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RESEARCH

Immunolocalization of SP22 (Park7) on the human sperm membrane is indicative of live birth

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Abstract

With the decline in sperm quality over the recent decades, there is little doubt that the decreased efficiency of spermatogenesis in men reflects their increasing susceptibility to environmental and pharmaceutical exposures that can compromise male fecundity. More than twenty years have passed since we discovered a novel sperm membrane protein (SP22) derived from the Park7 gene that is highly correlated with the fertility of rat sperm exposed to epididymal toxicants. We subsequently discovered that SP22 originates in the testis and is also compromised during exposure to testicular toxicants. Recently, we were given an opportunity to test the hypothesis that human sperm may also have reduced fertility when levels of SP22 are significantly decreased. Using a recombinant antibody to the functional epitope of SP22, we compared sperm from donors who achieved a live birth to donors who were not able to achieve a pregnancy. Sperm SP22 was significantly reduced in a group of donors not able to achieve a pregnancy. A similar reduction in birth was also observed among specific Park7 and SP22 exons when a group of donors unable to achieve a pregnancy was compared to the live birth donors. To our knowledge, this is the first demonstration that SP22 expression can be compromised in men with reduced fertility. This opens the door to incorporating SP22 analyses in future epidemiology studies and developing over-the-counter testing for men with suspected male infertility.

Lay summary

Epidemiological studies suggest that sperm quality in men has been declining; however, no biomarkers of fertility were measured in these studies. We discovered a protein (SP22) that was highly correlated with fertility of sperm following toxicant exposures. We sought to evaluate the expression of this protein in men with differing fertility status. Using an antibody to SP22, we compared sperm from donors who achieved a live birth to those who were not able to achieve a pregnancy. This is the first demonstration that SP22 expression, or any sperm membrane protein, is compromised in a random group of men with reduced fertility and provides an opportunity to quantify the expression of SP22 in future epidemiology studies. We also believe that smart phone technology can now be expanded to quantify SP22 in a man's sperm. This will enable a couple to make more informed decisions about appropriate assisted reproductive procedures.

Keywords: SP22 (Park7); immunolocalization; recombinant antibody; human donors; exon usage; fertility biomarker; novel applications

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Introduction

For several decades there has been much attention given to alterations in sperm quality, possibly induced by environmental or pharmaceutical exposures. The study by Jensen *et al.* 2007 noted that 20% of young men had poor semen quality, and there was a corresponding increase in assisted reproduction. Between 1930 and 2000, sperm counts declined from 120 to 60 million sperm per mL, while the percentage of subfertile men (<40 million/mL) increased from 18 to 40 million sperm per mL (Sharpe 2012). Another study reported a decrease in the sperm count in southern Europe between 2001 and 2011 (Mendiola *et al.* 2013).

A study by Levine *et al.* (2017) reported significant decreases in both sperm concentration and total sperm count in western countries between 1973 and 2011. It was soon obvious that the decrease in the sperm number was associated with sperm quality factors such as morphology (Rolland et al. 2013). However, some studies noted significant decreases in the sperm morphology and motility when there was no decrease in the sperm number (Garcia-Grau *et al.* 2023, Levine *et al.* 2023). The most notable change in the sperm quality over 20 years was a decrease in progressive motility to 12% and an increase in non-progressive motility to 21%. These alterations likely reflected changes in other qualitative endpoints. The prevalent thought is that the decline in the sperm quality results from exposure to environmental toxicants such as endocrine disruptors and pharmaceuticals (Lv *et al.* 2021, Stukenborg *et al.* 2021).

Sperm DNA fragmentation (Lewis & Aitken 2005, Ribas-Maynou *et al.* 2021), sperm miRNAs and RNAs (Platts *et al.* 2007, Jodar *et al.* 2013, Yuan *et al.* 2016, Burl *et al.* 2018, Salas-Huetos *et al.* 2020) and seminal plasma extracellular vesicles (Abu-Halima *et al.* 2016, Kumar & Singh 2020, Hong *et al.* 2021, Barranco *et al.* 2022) also impact sperm. Together this provides a more comprehensive picture of sperm quality and mechanism(s) of perturbation. However, up until now, no human study has directly explored changes in sperm quality, reflected in the sperm membrane proteome. Tasked with the need to identify a sensitive biomarker of sperm fertility (Klinefelter *et al.* 1997), a study using known epididymal toxicants cast a wide net to determine how sperm motility, sperm morphology, cauda epididymal sperm reserves, epididymal testosterone and two-dimensional gel evaluation of proximal cauda sperm impacted fertility following *in utero* insemination. Fertility was reduced significantly by each toxicant at the higher dose tested, and by epichlorohydrin and hydroxyflutamide at the lower dose as well. One 22 kDa protein (SP22) was diminished in 2D gels in the same fashion. This protein was highly correlated with and predictive of fertility.

SP22 is a member of a recently described highly conserved reproductive system gene family (Welch *et al.* 1998). In rats, the 189 amino acid protein is also referred to as contraceptive-associated protein or CAP1 (Wagenfeld et al. 1998). The protein was thought to be shed from the sperm membrane as it was increased in the epididymal luminal fluid of rats exposed to the antifertility drug ornidazole. In humans, the protein has been referred to as DJ1 (Nagakubo *et al.* 1997), RS (Hod *et al.* 1999) and more recently intensively studied as Park7, for its role in familial Parkinson's disease (Bonifati *et al.* 2003).

While SP22 is expressed ubiquitously, a unique isoform with an extended 5′ untranslated region (UTR) is present in the testis. This was confirmed by a Northern blot analysis after screening a rat testis cDNA library (Welch *et al.* 1998). Other transcripts DJ1, RS and Park7 expressed in the testis possess a shorter 5′ UTR. SP22 exon usage was evaluated focusing on exons 5 and 6 that encompass the epitope of the antibody as a function of both live birth donors and donors that failed to achieve a pregnancy. Herein, we describe the localization of SP22 in human sperm derived from live birth donors and from donors that failed to achieve a pregnancy, using a highly specific antibody to the presumptive functional epitope present on the equatorial segment of sperm. To our knowledge, this is the first study on the localization of SP22 on human sperm indicative of differing functional live birth status. In addition to determining the differential exon occupancy between live birth donors and donors that failed to achieve pregnancy, we also observed a strong correlation between SOD1, known to play a role in maintaining the oxidative status of sperm, and Park7 exons 5 and 6.

Materials and methods

Donor sperm

After a human studies review and approval, a Materials Transfer Agreement between the U.S. Environmental Protection Agency and Biorepository at Augusta University was awarded for the use of the well-characterized samples from the Assessment of Multiple Intrauterine Gestations from Ovarian Stimulation (AMIGOS) trial (Diamond *et al.* 2011, 2015). Frozen semen from the Biorepository was shipped to the agency's Reproductive and Developmental Biology branch and immediately stored at −70°. The shipment consisted of ten sperm samples from donors achieving a live birth and ten samples from donors who failed to achieve a pregnancy.

Sperm isolation

Cryovials from four donors were separately allowed to stand for 15 min at room temperature and then 15 min at 34 °C. The 0.5–1 mL of neat, thawed semen was resuspended slowly in 2 mL of sperm washing medium (#9983, Irvine Scientific, USA). After verifying motility,

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the sperm were pelleted for 10 min in Beckman JA-14 rotor at 1,500 r.p.m. (300 ***g***) without the brake on. The supernatant was removed slowly with a 1 mL pipet, and the pellet was resuspended again in 2 mL of sperm washing medium centrifuged as above. This pellet was resuspended in a sperm isolation buffer (SIB) used to develop the methodology using rabbit sperm. This buffer consists of Hanks' balanced salt solution containing no sodium bicarbonate or phenol red, to which sodium bicarbonate, HEPES, glucose and sodium pyruvate were added.

Immunostaining

SP22 antibody

The recombinant antibody was generated by Creative Biolabs (USA) to amino acids 80–105 of the human SP22 protein. Sperm (10×10^6) were incubated overnight at 4 °C in 0.5 mL of SIB containing 5 μL (0.01 mg) of mouse recombinant antibody. The sperm were then pelleted briefly in a microfuge tube and washed once in 1 mL of SIB. The pellet was resuspended in SIB with 10 μL of anti-mouse Alexa 488. After 1 h at 34 °C, the sperm were washed with 1 mL of SIB and resuspended in 0.25 mL of SIB and 0.25 mL of fixative (4% paraformaldehyde in Sorenson's phosphate buffer). Slides were prepared by adding Vectashield, USA (10 μL), 20 μL of sample, cover slipping and sealing with nail polish.

Photomicroscopy

A Leica DM600B and LAS AF fluorescence software were used to capture images of donor sperm stained with the recombinant SP22 antibody. Images of four donors (two live birth and two non-live birth) were analyzed at a time. After acquisition of the differential interference contrast image in black and white, the fluorescent image was acquired and converted to pseudocolor (red). These two images, differential interference and fluorescent, were merged. We determined that the distinction between the red antibody image for the equatorial segment and the unstained image was clearer with this exposure method. Importantly, the exposure time for each image was kept constant across donors. Six to twelve merged images were evaluated for each donor. The percentage of stained sperm was simply the number of stained sperm divided by the total sperm counted.

RNA isolation and analysis

A separate aliquot of each sample was subjected to RNA-seq analysis (Swanson *et al.* 2020). In brief, sperm RNA was isolated (Krawetz *et al.* 2011, Sendler *et al.* 2011, Jodar *et al.* 2013, Sendler *et al.* 2013), (1–3) sequenced Jodar *et al.* (2015) and analyzed as sperm RNA elements (SREs) and short exon sequences (Estill *et al.* 2019*a*,*b*). Each SP22/Park7 RE (exon) was assessed as a function of live birth and compared using a pairwise *T*-test.

Statistical analysis

Data were analyzed with GraphPad Prism version 10.1.2 (https://www.graphpad.com) using analyses such as mixed effects analysis, unpaired *T*-tests and correlation analysis. Significance of $P < 0.0001$ and <0.05 was achieved for the unpaired *T*-test and correlation analysis, respectively. Standard error is provided for exon analysis.

Results

Samples were selected from the 900 couples participating in the AMIGOS trial (Diamond *et al.* 2015).

The AMIGOS study is characterized by males with predominately 'normal semen parameters' among the couples presenting with unexplained infertility. The uniqueness of the samples limited the number of subjects that could participate in this analysis.

Expression of the 1.5 kb SP22 transcript begins in meiosis on postnatal day 33 with the formation of pachytene spermatocytes (Fig. 1A) and increases throughout

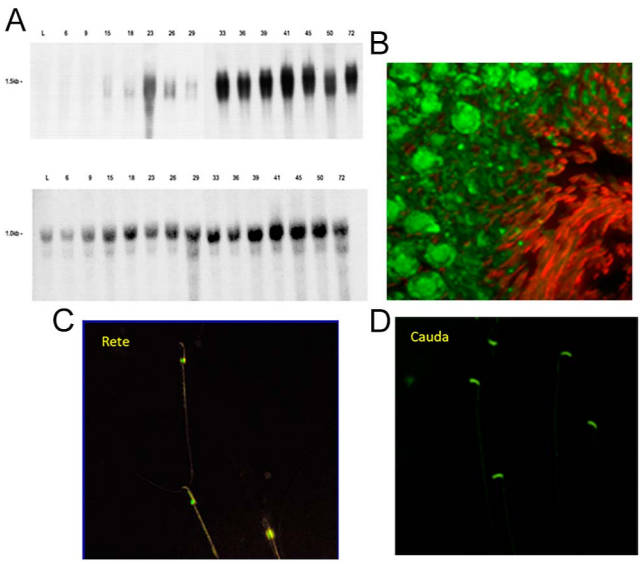

Figure 1

SP22 transcripts and localization in the testis and epididymis. (A) Northern blot depicting the 1.5 and 1.0 kb transcripts during postnatal day 6 through postnatal day 72 of spermatogenesis in the rat. Note that the 1.0 kb transcript is expressed in the liver (L) and throughout spermatogenesis, whereas the 1.5 kb transcript is testis-specific, begins expression on postnatal 33 during meiosis and increases expression until the spermatids are released. (B) A fluorescent micrograph showing the green nuclear, cytoplasmic and cytoplasmic droplet localization of SP22 in the rat testis before spermatid elongation. The red localization in the nucleus and cytoplasmic droplet of elongating spermatids is evident. (C) A fluorescent micrograph with green localization is the cytoplasmic droplet of rete testis sperm. (D) A fluorescent micrograph showing the translocation of SP22 staining to the equatorial segment of cauda epididymal sperm.

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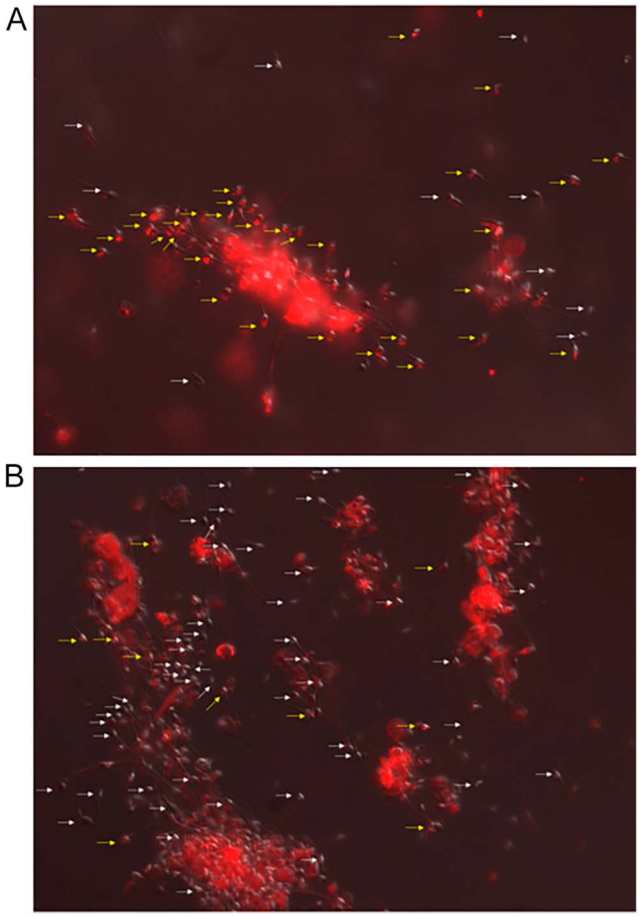

Figure 2

Examples of live birth and non-live birth immunostaining for SP22. (A) Punctate equatorial staining is evident on the red sperm heads (yellow arrows) extending out of the clustered sperm from a live birth donor. Non-staining sperm are also indicated (white arrows). (B) The non-live birth donor has very few sperm stained for SP22, but many unstained sperm.

spermatogenesis in the rat, where it is ultimately expressed in the nucleus and cytoplasm of round and elongating spermatids (Fig. 1B). Once sperm leave the testis, it is translocated from the cytoplasmic droplet on the rete sperm to the equatorial segment of the epididymal sperm (Fig. 1C and D).

To determine the percentage of sperm stained for SP22, the total number of sperm counted per donor ranged from 100 to 500. The percentage stained for the live birth donors ranged from 67 to 84, and the percentage stained for those donors that failed ranged from 15 to 51. Sperm in stained clumps were avoided, as were sperm with non-specific staining over the tail. An image from the live birth group with 76% staining is shown in Fig. 2A, whereas an image from the failed group with 18% non-live birth staining is shown in Fig. 2B. The average percentage of live birth donor sperm stained for SP22 was 71%, and that of the failed donor sperm was 33%,

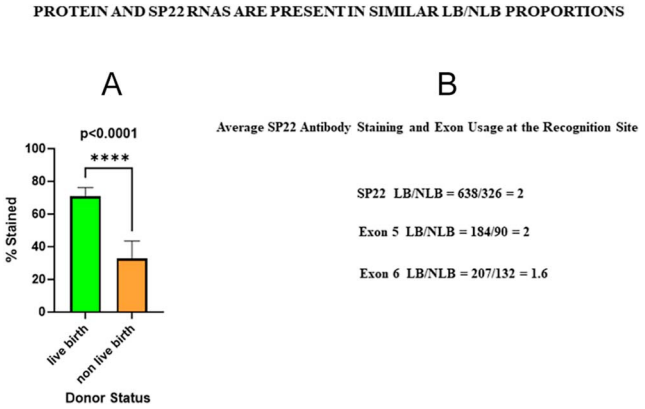

Figure 3

SP22 and SP22 RNAs exist in similar proportions in exons 5 and 6. (A) A graph showing highly significant decrease in SP22 staining in the non-live birth donors compared to the live birth donors. (B) A depiction of the SP22 antibody staining vs average exon 5 and 6 usage. The ratio of SP22 staining in live birth to non-live birth donors was 2.0. The ratio of exon 5 usage in live birth to non-live birth donors was 2.0, and the ratio of exon 6 usage in live birth to non-live birth donors was 1.6.

$P < 0.0001$ (Fig. 3). Interestingly, the ratios of live birth to non-live birth data were reasonably similar whether antibody staining to SP22 (638/326 = 2.0), the usage of exon 5 (184/90 = 2.0) and the usage of exon 6 (207/132 = 1.6) were compared. Figure 4 summarizes the exon usage of the 8 Park7 exons, including exons 5 and 6 that correspond to the epitope. The levels of sperm RNA elements (SREs) in both live birth and non-live birth donors in exons 1 through 8 are shown. The levels of the functional transcripts encoding the SP22 epitope are underlined for exons 5 and 6. Interestingly,

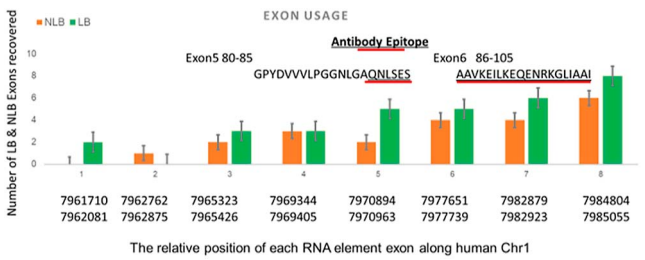

Figure 4

Comparison of RNA elements across the Park7 locus as a function of live birth and non-live birth. A graph showing the 8 Park7 RNA elements. The position of these exons on CRCh38/hg38p14 begins with the one which corresponds to an enhancer region, followed by the amino acid sequence of the highlighted recombinant SP22 antibody epitope in exons 5 and 6. Exon 7 corresponds to a transcribed YRNA, and 8 is the terminating exon. The antibody epitope contained in exons 5 and 6 is underlined. The success (green) of couples who achieved live birth was compared (orange) with who were unable to conceive. Exon 5 shows a marked decrease for those couples that were unable to conceive. The standard error for each group is indicated.

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the short epitope sequence corresponding to *QNLSES* in exon 5 is expressed to a greater extent in the live birth donors than in the failed non-live birth donors. The epitope sequence corresponding to *AAVKEILKEQENRKGLIAAI* in exon 6 was also expressed to a greater extent in the live birth vs the failed non-live birth donors. The differences in the number of fertile donors expressing exon 5 were 2.5 times higher than the donors that failed to achieve pregnancy, whereas the remaining exons generally showed no difference. This is indicative of the potential diagnostic capacity of exon 5 isolated from mature sperm where individuals display differential states of integrity upon comparison (Sendler *et al.* 2013).

As shown in Fig. 4, the functional epitope begins at amino acid 80 in exon 5 and terminates at amino acid 105 in exon 6. Thus, only five amino acids reside in exon 5, while 20 amino acids reside in exon 6. Interestingly, both amino acid sequences are predicted to be a functional helix as the antibody to the full-length recombinant recognized both linear and looped peptides using Pepscan's CLIPS technology (Pepscan, the Netherlands). Indeed, it was this finding that leads us to generate a recombinant human antibody specific to the amino acids 80 through 105. To test the specificity of the antibody, we used an overlapping PepSet of 35, 20 mer peptides, each overlapping the next by 15 C-terminal amino acids. The absorbance of each peptide was measured using peroxidase, following the addition of the anti-mouse second antibody. Consistently, the only peptide of the set was peptide 17, which solely contained the entire epitope.

A correlation analysis (Fig. 5A) was performed on those donors with detectable transcripts in exons 5 and 6. This analysis revealed a significant correlation between the expression of exons 5 and 6, $P < 0.003$, $r = 0.86$, and the epitope when a simple linear regression was tested when both live birth and failed non-live birth donors were considered together. Figure 5B illustrates the correlation between exon 6 and SOD1, $P < 0.05$, $r = 0.67$. Figure 5C illustrates the correlation between exon 5 and SOD, $P < 0.05$, $r = 0.74$. This suggests that these three proteins or RNAs may be of predictive value, opening the door to developing a sperm quality measure.

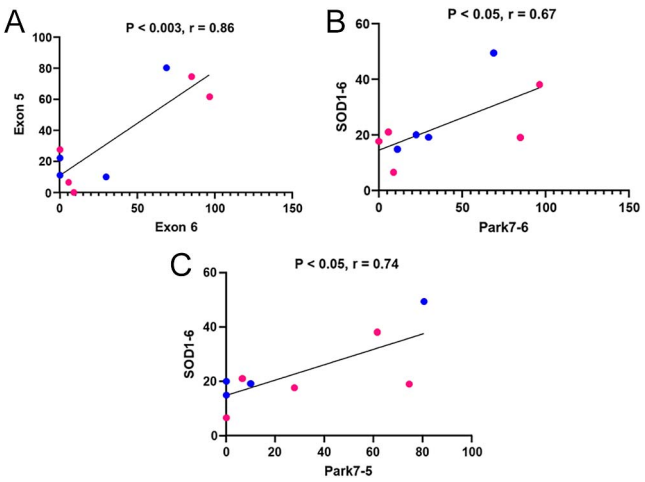

Figure 5

Correlation between transcripts in live birth and non-live birth donors. (A) Graph showing the significant, positive correlation observed between exons 5 and 6. In these graphs, the live birth values are displayed in red, and the non-live birth values are displayed in blue. (B) Graph showing the significant, positive correlation observed between SOD1 and exon 6 of Park7. (C) Graph showing the significant, positive correlation observed between SOD1 and exon 5 of Park7.

Discussion

It is important to note that while SP22 was discovered studying epididymal toxicants (Klinefelter *et al.* 1997), the initial molecular biology (Welch *et al.* 1998) established the origin of SP22 in the meiotic germ cells of spermatogenesis. We reasoned that testicular toxicants would also compromise SP22 expression and fertility. This proved to be the case as we found exposure to be prevalent by products of drinking water disinfection such as dibromoacetic acid (Klinefelter *et al.* 2004) and bromochloroacetic acid (Klinefelter *et al.* 2002) produced significant decreases in the SP22 isolated from cauda sperm membrane extracts and the fertility of these sperm observed by *in utero* insemination (Klinefelter 2008). These results were subsequently extended to a similar study in the rabbit (Veeramachaneni *et al.* 2007). This laid the groundwork for years of research to identify the functional epitope in human sperm, generation of a recombinant antibody to the epitope and our search for human sperm from donors that were fertile and couples that could not achieve a pregnancy.

While numerous proteins have now been implicated with the fertility of sperm, SP22 is the only sperm protein that has been demonstrated in multiple studies to be a biomarker of fertility. Despite the novel data provided herein, a diagnostic platform that can identify the fertility status of live, motile sperm based on the expression of SP22 is not under development at the moment. However, a recent review by Gonzalez et al. (2021) describes a variety of over-the-counter products that evaluate the number and motility of sperm in a semen sample. The most recent devices rely on smartphone technology to capture images of sperm. Looking forward, it should be possible to capture a number of motile sperm that express a requisite level of SP22 to guide treatment (continued intercourse, *in utero* insemination, *in vitro* fertilization or intracytoplasmic sperm injection). In future epidemiology studies, SP22 expression analysis could prove useful when assessing the impact of chemical and pharmaceutical exposures. Often exposure and time to pregnancy data are not available, but with the ability to evaluate SP22 expression, men enrolled in a

study may be able to make more informed decisions about their fertility status. Given the spectrum of diseases in which SP22 participate, expression analysis could prove useful when assessing the impact of chemical and pharmaceutical exposures. With the ability to evaluate SP22 expression, we are being provided with the opportunity to inform decisions about their fertility and exposure(s) status.

While this is the first demonstration of an expressed sperm protein that is compromised in men with reduced fertility, SP22, Park7 and DJ1 are aliases that describe this multifunctional protein in many systems. Its extensive influence reflects its wide distribution throughout the body from the testis to the brain, as summarized in the human protein atlas (Uhlen *et al.* 2005). Alterations can present in a spectrum of diseases, including male infertility, Parkinson's disease and various cancers (Sun & Zheng 2023). Uniprot references over 234 single nucleotide polymorphisms, 189 of which correspond to changes in the protein sequence. It should now be possible to identify protein sequence alterations using the antibody described above. In various autosomal recessive models, mutations can present as reduced male fertility or early onset of Parkinson's disease (Seyedtaghia *et al.* 2023). Such mutations could also be addressed using the antibody described above.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the work.

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Author contribution statement

GK was responsible for study design, experimental methods and data collection. SK was responsible for genetic data collection and analysis. MD was involved with donor sample inventory and design.

Human study approval

This work received a human studies review and approval. The approval was provided by the EPA Human Subjects Review Official (HSRRO).

Disclaimer

The research described in this article has been reviewed by the Center for Public Health and Environmental Assessment, US, EPA, and approved for publication. The contents of this article should neither be construed to represent agency policy nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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