The association between mutations in ubiquitin-specific protease 26 (USP26) and male infertility: a systematic review and meta-analysis

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During recent decades, the association between mutations in ubiquitin-specific protease 26 (USP26) and male infertility remains doubtful. We conducted this meta-analysis to evaluate the association between mutations in USP26 and male infertility according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines. It was registered in the International Prospective Register of Systematic Reviews (PROSPERO; CRD42021225251). PubMed, Web of Science, and Scopus were systematically searched for comparative clinical studies, which were written in English and provided eligible data. Studies were included when they compared USP26 mutations in azoospermic, oligozoospermic, and asthenozoospermic patients with controls with normal sperm parameter values or whose partners had experienced spontaneous pregnancy. Pooled odds ratio (OR) with 95% confidence interval (CI) was calculated with random effect models. Overall, twelve studies with 3927 infertility patients and 4648 healthy controls were included. The association between overall USP26 mutations and infertility was not significant (OR = 1.60, 95% CI: 0.51–5.01). For specific mutations, the pooled ORs were 1.65 (95% CI: 1.02–2.69) for cluster mutation (including 370–371insACA, 494T>C, and 1423C>T), 1.80 (95% CI: 0.35–9.15) for c.576G>A, 1.43 (95% CI: 0.79–2.56) for c.1090C>T, and 3.59 (95% CI: 2.30–5.59) for c.1737G>A. Our results suggest that several mutations (cluster mutation, c.1737G>A) may play roles in male infertility, while others (c.576G>A and c.1090C>T) do not show notable associations with male infertility. More high-quality clinical researches are needed for validation.

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INTRODUCTION

Infertility is characterized as the failure to obtain a pregnancy after undergoing regular and unprotected sexual behavior for 1 year. About 8%–12% of reproductive-aged couples are affected all over the world.1 Furthermore, the existing body of researches suggests that 20%–30% of infertility cases result solely from males, and 50% of infertility cases are associated with male infertility.2 In the last few years, a high prevalence of genetic causes for spermatogenic impairment has been reported.3 Therefore, there has been increasing attention to the genetic landscape of the patients. This landscape consists of a small number of altered genes (gene mutations) in a high percentage of cases and a much larger number of genes which are altered infrequently.4 Owing to the complexity of spermatogenesis, semen composition, and testicular histological diversity, the gene landscape of male infertility is very complicated. Actually, more than 2000 genes are involved in spermatogenesis.5 Genetic abnormalities are responsible for about 25% of azoospermic cases, and the number of genetic abnormalities that are identified in the semen composition and etiological categories is steadily growing.6 Genetic screening is associated with diagnosis, genetic counseling, and clinical decision-making.7 However, even with a huge development of detecting techniques, the etiology of infertility in numerous patients remains unknown. The identification of novel genetic factors in idiopathic infertility, which refers to infertility without a clear-cut cause identified,8 is crucial for the treatment of these patients.8

Male infertility mainly results from spermatogenic defects, to which X chromosomal dosage is closely connected.7 Since only one X chromosome exists in males, there is no normal allele that can compensate for loss-of-function mutations in single-copy X chromosomal genes. Therefore, X-linked genes are thought to play vital roles in male spermatogenic failure. Furthermore, with the development of next-generation sequencing (NGS), increasingly, novel genes have been discovered to be associated with male infertility, such as bromodomain and WD repeat domain containing 1 (BRWD1), DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3 beta (DNMT3B), ring finger protein 17 (RNF17), ubiquitin protein ligase E3 component n-recognition 2 (UBR2), ubiquitin-specific peptidase 1 (USP1), and ubiquitin-specific peptidase 26 (USP26).8 However,
definite evidence is still lacking for a gene–disease relationship of these genes.⁹ USP26 is one of the genes, which is assumed to be relevant to male infertility. The USP26 gene, located at Xq26.2, presents as a single exon on the X chromosome and encodes a protein containing 913 amino acids. It is a member of a large family of de-ubiquitinating enzymes and is exclusively expressed in the testis.¹⁰ Ubiquitination is an important biological process that is involved in the stability and degradation of cellular proteins.¹¹ The addition of ubiquitin to substrate proteins can enhance the degradation of target proteins.¹² Conversely, the removal of ubiquitin from substrate proteins (also called de-ubiquitination) can prevent these proteins from being degraded.¹³ The balance between ubiquitination and de-ubiquitination is crucial for biological activity, including the completion of spermatogenesis.¹⁴,¹⁵ The USP26 gene was first reported in 2001.¹⁶ Owing to the importance of the de-ubiquitinating enzymes in male infertility, more and more attention has been paid to the association between USP26 and male infertility.

In recent years, over 20 mutations in the USP26 have been reported to be associated with male infertility.¹⁷ Several mutations are mentioned frequently, such as the cluster mutation (370–371insACA, 494T>G, and 1423C>T) in the same allele, causing the amino acid changes including T123–124ins, L165S, and H475Y), c.576G>A,¹⁸ c.1090C>T,²⁰ and c.1737G>A.²¹ The cluster mutation is presumed to be concerned with Sertoli cell-only syndrome.²² Several research papers describe a remarkable relationship between USP26 mutations and male infertility,²³,²⁴,²⁵ while others do not identify a significant association between them.²⁶,²⁷–²⁹ Whether specific single-nucleotide polymorphisms, such as c.576G>A, play a crucial role in male infertility remains unknown.

Although two meta-analytic studies on this issue have been performed, the conclusions from them are different. Xia et al.²⁷ reported an association of USP26 mutations with male infertility, while the result reported by Zhang et al.²⁸ did not support a significant association between USP26 mutations and male infertility. Moreover, an increasing number of single-nucleotide polymorphisms of USP26 have recently been found. Therefore, our study included more records and investigated some of the new single-nucleotide polymorphisms (SNPs) of USP26 to clarify the association between USP26 mutations and male infertility.

MATERIALS AND METHODS

This work was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines. According to PRISMA 2020 guidelines, as shown in Supplementary Table 1, the populations were adult males, the exposures were patients with azoospermia, oligozoospermia, or asthenozoospermia, the comparators were individuals with normal fertility or normal spermatogenic function, and the outcomes were the results of the assessment of mutations of USP26. It was also registered in the International Prospective Register of Systematic Reviews (PROSPERO; CRD42021225251).

Search strategy

Studies published before March 2021 were identified from PubMed, Scopus, and Web of Science. No retrieval limitation was applied. The searching strategy was (“infertility” OR “azoospermia” OR “oligozoospermia” OR “oligoasthenozoospermia” OR “asthenozoospermia”) AND (“USP26” OR “ubiquitin-specific protease 26”). Furthermore, the reference lists of the included studies and related reviews and reports were also screened.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) clinical studies reported the association between USP26 mutations and male infertility; (2) both the number of patients and controls, and the number of patients and controls with USP26 polymorphisms can be extracted according to reported data; (3) the studies were published in English; (4) the studies included patients which were azoospermia, oligozoospermia, and/or asthenozoospermia; and (5) the studies included controls with normal sperm parameter values or had experienced a partner’s spontaneous pregnancy.

The exclusion criteria were as follows: (1) the article was a review, comment or abstract; or (2) when there were multiple publications from the same study group, the study that reported most complete and recent results was included.

Data extraction and quality assessment

Two authors (QYL and YCZ) extracted the following information from each study independently: name of first author, publication year, country, age, description of cases (infertility and subgroups), description of control, the sample size, the number of patients and controls, the number of patients and controls with USP26 polymorphisms, genotyping method, and the result of genotyping method (the mutations of USP26). In the end, the accuracy of the data extraction was checked again.

Quality assessment was independently conducted by two investigators (XML and LCW) according to the National Institutes of Health (NIH) Quality Assessment tool for case-control studies (https://www.ncbi.nlm.nih.gov/health-topics/study-quality-assessment-tools). The NIH’s scales consist of twelve parameters of quality (Supplementary Table 2). We scored these items and classified the included studies as “good”, “no”, or “others (CD: cannot determine, NA: not applicable, and NR: not reported)” according to the scales.

Statistical analyses

For each USP26 mutation reported in the included study, we estimated the odds ratio (OR) and the 95% confidence interval (CI), by retrieving the number of cases and controls. To obtain conservative results, the random-effects model was employed for pooled analysis. The Chi-square test and inconsistency index (I²) were applied to evaluate the heterogeneity. Subgroup analyses were performed according to different case types (azoospermia, oligozoospermia, and unselected cases). Unselected cases are those in which the detailed numbers of patients with azoospermia or oligozoospermia were not reported in the included article, while the study reported the total number of patients with azoospermia or oligozoospermia instead. All statistical analyses were executed by using Review Manager 5.3 (Cochrane Collaboration, Oxford, UK), and P < 0.05 was considered to be statistically significant. Funnel plots were applied for the assessment of publication bias. In addition, a sensitivity analysis was performed by excluding the lowest-weighted or lowest-scored studies.

RESULTS

Characteristics of the included studies

The search and screening process is shown in Figure 1. Studies included in our analysis were published between March 2005 and December 2016. A total of 12 studies⁸,¹⁷–²⁶,²⁷ with 3927 infertility patients and 4648 healthy controls were included in this meta-
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The association between overall mutations of USP26 and male infertility

Six studies containing 2943 patients and 2478 controls were involved in the analysis of the association between the overall mutations of USP26 and male infertility. The pooled OR was 1.60 (95% CI: 0.51–5.01; P = 0.42; Figure 2). However, heterogeneity among these studies was significant (P < 0.01; Supplementary Figure 1).

The associations between cluster mutation of USP26 and male infertility

Ten studies containing 1637 patients and 4052 controls were involved in the analysis of the cluster mutation of USP26. The cluster mutation of USP26 included the mutations of 370–371insACA, 494T>C, and 1423C>T. The pooled OR for overall results was 1.65 (95% CI: 1.02–2.69, P = 0.04), which indicated that the cluster mutation of USP26 was associated with overall male infertility (Figure 3a). In subgroup analysis, the pooled ORs were 1.97 (95% CI: 0.96–4.04, P = 0.06) for azoospermia, 0.88 (95% CI: 0.24–3.25, P = 0.85) for oligozoospermia, and 3.00 (95% CI: 0.35–25.61, P = 0.32) for unselected cases (Figure 3a). Although the results of the subgroup analysis did not show a significant association between cluster mutation of USP26 and male infertility, the overall results showed a statistical significance which revealed a potential association between them (Figure 3a). Moreover, the funnel plot suggested that the publication bias was limited (P = 0.45; Supplementary Figure 2).

The associations between c.576G>A and male infertility

Five studies containing 781 patients and 772 controls were involved in the analysis of the c.576G>A mutation of USP26. The pooled OR was 1.01 (95% CI: 0.45–2.24, P = 0.98; Figure 3b). For the subgroup analysis, the pooled ORs were 1.21 (95% CI: 0.40–3.63, P = 0.73) for azoospermia, 0.40 (95% CI: 0.11–1.46, P = 0.17) for oligozoospermia, 1.80 (95% CI: 0.35–9.15, P = 0.48) for unselected cases (Figure 3b). Neither overall nor subgroup analysis showed an obvious association between c.576G>A and male infertility. The funnel plot suggested that the publication bias may exist (P < 0.01; Supplementary Figure 3).

The association between c.1090C>T and male infertility

Five studies containing 828 patients and 532 controls were involved in the analysis of the c.1090C>T mutation of USP26. The pooled OR was 1.43 (95% CI: 0.79–2.56, P = 0.24; Figure 4a). For the subgroup analysis, the pooled ORs were 2.61 (95% CI: 0.73–9.36, P = 0.14) for azoospermia, and 1.21 (95% CI: 0.63–2.34, P = 0.57) for unselected cases (Figure 4a). The results indicated that the c.1090C>T was not significantly associated with male infertility. The funnel plot suggested that the publication bias was limited (P = 0.81; Supplementary Figure 4).

Figure 1: Flow diagram of the included study in this meta-analysis.

Figure 2: Forest plot of the association between overall mutations of ubiquitin-specific protease 26 (USP26) and male infertility. CI: confidence interval; df: degrees of freedom; M–H: Mantel–Haenszel.
Table 1: Main characteristics of studies in meta-analysis

| Reference          | Country | Ethnic group | Number of cases (age [year], range) | Number of controls (age [year], range) | Genotyping method | Diagnostic examination                                                                 | Patient description                                                                 | Control description                  |
|--------------------|---------|--------------|-------------------------------------|-----------------------------------------|-------------------|---------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------|
| Paduch et al.22, 2009 | USA     | Caucasian    | 188 (NM) 17 (NM)                    |                                          | PCR, HPLC         | Semen analysis, serum hormones, karyotype and physical examinations                    | Patients with azoospermia or severe oligozoospermia                                | Fertile men                           |
| Stouffs et al.17, 2005 | Belgium | Caucasian    | 143 (NM) 152 (NM)                   |                                          | PCR, TaqMan       | Histological examination of testicular tissues                                         | Diagnosed with Sertoli cell-only syndrome                                          | Normal sperm parameters               |
| Ravel et al.24, 2006 | France  | Mixed        | 99 (NM) 1334 (NM)                   |                                          | PCR, TaqMan       | Semen analysis                                                                         | Azoospermia, extreme oligozoospermia (<1×10⁶ sperm per ml), severe oligozoospermia (1×10⁵–5×10⁵ sperm per ml), moderate oligozoospermia (5×10⁴–10×10⁴ sperm per ml), mild oligozoospermia (10×10³–20×10³ sperm per ml) | Fertility status or sperm count of the individual was known |
| Stouffs et al.25, 2006 | Belgium | Caucasian    | 146 (NM) 202 (NM)                   |                                          | PCR, TaqMan       | Semen analysis, karyotype examination, testicular biopsy                               | Cryptozoosperm (sperm concentration <0.1×10⁶ ml⁻¹), severe oligozoospermia (sperm concentration of 0.1×10⁵–5×10⁵ ml⁻¹) | Normal spermatogenesis on testicular biopsy, normal sperm parameters or proven fertility |
| Lee et al.13, 2008  | China   | Asian        | 200 (NM) 200 (NM)                   |                                          | PCR, TaqMan       | Detailed history, physical examination, semen analyses, hormone assays, karyotyping test, molecular test, testicular biopsies | Severe oligozoospermia (spermatozoa count <5×10⁶ ml⁻¹) or nonobstructive azoospermia | Proven fertility                       |
| Christensen et al.18, 2008 | USA     | Caucasian    | 96 (NM) 96 (NM)                     |                                          | PCR               | Semen analysis                                                                         | Severely oligozoospermic (<5 million ml⁻¹) or azoospermic patients                  | Known paternity                       |
| Ribański et al.26, 2009 | Israel  | Caucasian    | 300 (NM) 287 (NM)                   |                                          | PCR               | Semen analysis, karyotype analysis                                                     | Azoospermic, oligozoospermic, infertile with unknown semen parameters               | Sperm bank donors, known fertile men with at least 2 children                       |
| Shi et al.20, 2011  | China   | Asian        | 221 (24–42) 101 (25–40)            |                                          | PCR               | Detailed history, physical examination, semen analyses, hormone assays, karyotyping test, plasma biochemical analysis | Azoospermia, oligoasthenozoospermia, asthenozoospermia, oligozoospermia | Normozoospermia                       |
| Asadpor et al.23, 2013 | Iran    | Asian        | 166 (25–46) 60 (24–46)             |                                          | PCR-SSCP, TaqMan  | Detailed history, physical examination, semen analyses, hormone assays, karyotyping test, molecular test, testicular biopsies | Nonobstructive azoospermia                                                       | Normal semen analysis, at least one child within 3 years without assisted reproductive technologies and no history of miscarriages |
| Li et al.8, 2015    | NM      | NM           | 1514 (NM) 1418 (NM)                 |                                          | PCR, Custom-designed capture array          | Semen analyses, karyotyping test                                                        | Nonobstructive azoospermia                                             | Proven fertile                         |
| Luddi et al.21, 2016 | NM      | Caucasian    | 78 (25–44) 72 (28–42)              |                                          | HRM analysis, direct sequencing              | Semen analysis                                                                         | Azoospermic patients                                                                 | Normal sperm parameters                 |
| Ma et al.27, 2016   | China   | Asian        | 776 (24–46) 709 (29–51)            |                                          | AllPrep DNA/RNA Mini Kit, Illumina HiSeq 2000 platform | Semen analyses, karyotyping test                                                        | Nonobstructive azoospermia                                                       | Had fathered at least 1 child without assisted reproductive techniques               |

NM: not mentioned; PCR: polymerase chain reaction; HPLC: high-performance liquid chromatography; SSCP: single-strand conformation polymorphism; HRM analysis: high-resolution melting analysis
The association between c.1737G>A and male infertility

Five studies containing 932 patients and 636 controls were involved in the analysis of the c.1737G>A mutation of USP26. The pooled OR was 3.59 (95% CI: 2.30–5.59, P < 0.01; Figure 4b). For the subgroup, the pooled ORs were 2.31 (95% CI: 0.64–8.34, P = 0.20) for azoospermia and 3.52 (95% CI: 1.71–7.24, P < 0.01) for unselected cases (Figure 4b). Although the results of azoospermia did not indicate the association between c.1737G>A and male infertility, the unselected case and overall cases showed a statistical significance, which revealed a potential relationship between them. The funnel plot suggested that the publication bias was limited (P = 0.56; Supplementary Figure 5).

Publication bias and sensitivity analysis

Publication bias was assessed by the funnel plot. As shown in Supplementary Figure 2–5, the publication bias was limited in our study. In sensitivity analyses, all the outcomes remained consistent with the previous outcomes, suggesting that no individual study significantly affected the pooled OR of the association between mutations in USP26 and male infertility (Supplementary Figure 6 and 7).

DISCUSSION

In recent years, the outcomes of the analysis of the relationship between USP26 variants and male infertility have been controversial. Therefore, a meta-analysis including larger samples and updated
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Figure 4: Forest plots of the association between variants of ubiquitin-specific protease 26 (USP26) and male infertility: (a) c.1090C>T, (b) c.1737G>A.

CI: confidence interval; df: degrees of freedom; M–H: Mantel–Haenszel.

results is needed. Our analysis involved 3927 patients and 4648 controls from 12 studies. From the result of our analysis, the mutations of USP26 did not lead to the occurrence of male infertility inevitably. Only some specific mutations play important roles in the male infertility, such as cluster mutation and c.1737G>A. In fact, in the previous study, only one out of nineteen mutations results in the disappearance of the enzyme activity. As for the c.576G>A, it might play a crucial role in male infertility, including asthenozoospermia and oligoasthenozoospermia, though this polymorphism is a synonymous mutation which does not change the amino acid sequence. However, SNPs can influence the gene function through modifying the stability of mRNA. Nevertheless, our results did not show a significant association between mutation of c.576G>A and male infertility, which may be due to we did not conduct the analysis on cases of asthenozoospermia or oligoasthenozoospermia. For the c.1090C>T, our results did not support the association between male infertility and this mutation. This is also consistent with the previous meta-analysis by Zhang et al.

Spermatogenesis is an extremely complicated process. Several cell types, hormones, paracrine factors, genes, and epigenetic regulators are involved in the differentiation of spermatogonia into spermatozoa. Such an intricate process requires the precise expression of functional enzymes. Therefore, the balance of ubiquitination and de-ubiquitination plays a critical role in spermatogonia. De-ubiquitinating enzymes are responsible for regulating ubiquitin-dependent processes, which is important for protein stability and activity. USP26 belongs to a de-ubiquitinating enzyme family. In a previous study, mutations of USP26 were found to affect spermatogenesis and hormone secretion and cause male subfertility. As for the mechanism, androgen receptor (AR) pathway is considered to be involved in the association between USP26 and male infertility. Androgens such as testosterone are steroid hormones that are essential for normal male reproductive development and function. The AR signaling in the testis is essential for spermatogenesis. Substrates of USP26 include AR, MDM2 proto-oncogene (MDM2), SMAD family member 7 (SMAD7), and protein regulator of cytokinesis 1 (PRC1). Because USP26 can prevent the degradation of AR, the downregulation of USP26 may affect spermatogenesis.

However, the role of USP26 in male infertility remains doubtful because USP26-knockout mice are reported to be fertile. Nevertheless, the function of USP26 might be different between humans and mice. A frameshift mutation in the USP26 gene in a patient is reported to result in severe oligozoospermia, which indicates a crucial role of USP26 in humans.
Interestingly, the defects in fertility caused by USP26 mutations in an animal study were found to be dependent on the genetic background. A mutation was introduced into the USP26 gene in mice, and the USP26-mutant males backcrossed to a DBA/2 background are found to be sterile with atrophic testes. Xia et al. also find that the association between USP26 mutation and male infertility is significant only in Asian, but not in Euramerican populations. Therefore, further studies should pay more attention to the genetic background of patients and enroll individuals with similar as possible genetic backgrounds. In addition, studies on the relationships between different mutation variants of USP26 are also needed for a better understanding of male infertility. Although the expression level of USP26 in different tissues has been explored, comparison of the expression levels of USP26 in azoospermia patients and healthy individuals is also needed to be further investigated.

The main limitation of our study is that the definitions of case and control were different in the included study. Patients in some studies were azoospermic while others were oligozoospermic, and the definitions of control also varied from different studies. Only one study reported the data on oligoasthenozoospermic and asthenozoospermic patients, so we did not analyze these patients. Furthermore, in some studies such as those reported by Stouffs et al. and Shi et al. individuals with normal sperm parameter values were defined as controls. However, known fertile men with at least 2 children were enrolled as control group in the study by Ribarski et al. As mentioned in the introduction, several studies described a significant association between the mutations and male infertility, while others reported negative outcome. Since researchers are willing to report the positive outcome, more negative outcome may not be reported, suggesting potential publication bias. In addition, the sample sizes of some included studies were limited. We only searched PubMed, Scopus, and Web of Science; some articles in other databases satisfied our criteria may not be included. Although we tried to contact with study authors to identify additional studies, some authors did not reply. In addition, some later studies after March 2021 may not be included. Furthermore, the factors that could influence the male infertility, such as age, were different in cases of each included study. Therefore, more high-quality clinical researches are needed for further validation.

CONCLUSION

Our results suggest that several variants (cluster mutation and c.1737G>A) of USP26 may play roles in male infertility. More high-quality clinical research is needed for further validation.

AUTHOR CONTRIBUTIONS

QYL and YCZ proposed the conception and design. XML, LCW and JHL provided the administrative support. CW and ZL supplied the study materials. XML and LCW collected and assessed the data. GDS, BLC and ML analyzed the data. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary Information is linked to the online version of the paper on the Asian Journal of Andrology website.

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**Supplementary Figure 1:** Tunnel plot of the overall mutations of USP26. USP26: ubiquitin-specific protease 26.

**Supplementary Figure 2:** Tunnel plot of the cluster mutation of USP26. USP26: ubiquitin-specific protease 26.

**Supplementary Figure 3:** Tunnel plot of the c.576G>A.

**Supplementary Figure 4:** Tunnel plot of the c.1090C>T.
Supplementary Figure 5: Tunnel plot of the c.1737G>A.

Supplementary Figure 6: Sensitivity analysis of the association between overall mutations of USP26 and male infertility. USP26: ubiquitin-specific protease 26.

Supplementary Figure 7: Sensitivity analysis of the association between variants of USP26 and male infertility. (a) Cluster mutation of USP26, (b) c.576G>A, (c) c.1090C>T, (d) c.1737G>A. USP26: ubiquitin-specific protease 26.
Supplementary Table 1: PECOS table

| Participants | Adult male |
|--------------|------------|
| Exposure     | Individuals which were azoospermia or oligozoospermia |
| Comparisons  | Individuals which are tested of normal sperm parameters or spontaneous pregnancy occurred |
| Outcomes     | Results of the assessment of mutations of usp26 |
| Study design | Case-control study |

PECOS: P: Participants, E: Exposure, C: Comparisons, O: Outcome, S: Study design

Supplementary Table 2: The parameters in National Institutes of Health's scales

1. Research question
2. Study population
3. Sample size justification
4. Groups recruited from the same population
5. Inclusion and exclusion criteria
6. Case and control definitions
7. Random selection of study participants
8. Concurrent controls
9. Exposure assessed prior to outcome measurement
10. Exposure measures and assessment
11. Blinding of exposure assessors
12. Statistical analysis.