Clinical implications of sperm DNA damage in IVF and ICSI: updated systematic review and meta-analysis

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Clinical implications of sperm DNA damage in IVF and ICSI: updated systematic review and meta-analysis

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I noticed a mistake on Fig. 1 (apologies for missing this last time). The total in the 'Records excluded based on full text, with reasons' should be **210**, not 206. This is the correct total from all the numbers given in this box (46+11+1+69+3+73+7), and also corresponds with the difference between 302 full-text studies assessed, and 78 used in your analysis (210+10+4 = 224; 302–224 = 78) I have corrected this to 210 in your results, on p. 14. Please change 206 to 210 on Fig. 1 and upload the corrected version of this figure with your final submission.

There is a change still needed to two of your Supporting information figures: In Fig. S3 and Fig. S8, please change Yang *et al.* (2009) to Yang *et al.* (2019).

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Running title: DNA damage in IVF and ICSI

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ABSTRACT

The clinical effect of sperm DNA damage in assisted reproduction has been a controversial topic during recent decades, leading to a variety of clinical practice recommendations. While the latest European Society of Human Reproduction and Embryology (ESHRE) position report concluded that DNA damage negatively affects assisted reproduction outcomes, the Practice Committee of the American Society for Reproductive Medicine (ASRM) does not recommend the routine testing of DNA damage for *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Herein, our aim was to perform a systematic review and meta-analysis of studies investigating whether sperm DNA damage affects clinical outcomes in IVF and ICSI, in order to contribute objectively to a consistent clinical recommendation. A comprehensive systematic search was conducted according to PRISMA guidelines from the earliest available online indexing year until March 2020, using the MEDLINE-PubMed and EMBASE databases. We included studies analysing IVF and/or ICSI treatments performed in infertile couples in which sperm DNA damage was well defined and assessed. Studies also had to include information about pregnancy, implantation or live birth rates as primary outcomes. The NHLBI-NIH quality

assessment tool was used to assess the quality of each study. Meta-analyses were conducted using the Mantel–Haenszel method with random-effects models to evaluate the Risk Ratio (RR) between high-DNA-damage and control groups, taking into account the 95% confidence intervals. Heterogeneity among studies was evaluated using the I^2 statistic. We also conducted sensitivity analyses and *post-hoc* subgroup analyses according to different DNA fragmentation assessment techniques. We identified 78 articles that met our inclusion and quality criteria and were included in the qualitative analysis, representing a total of 25,639 IVF/ICSI cycles. Of these, 32 articles had sufficient data to be included in the meta-analysis, comprising 12,380 IVF/ICSI cycles. Meta-analysis revealed that, considering IVF and ICSI results together, implantation rate (RR = 0.74; 95% CI = 0.61–0.91; I^2 = 69) and pregnancy rate (RR = 0.83; 0.73–0.94; I^2 = 58) are negatively influenced by sperm DNA damage, although after adjustment for publication bias the relationship for pregnancy rate was no longer significant. The results showed a non-significant but detrimental tendency (RR = 0.78; 0.58–1.06; I^2 = 72) on live birth rate. Meta-analysis also showed that IVF outcomes are negatively influenced by sperm DNA damage, with a statistically significant impact on implantation (RR = 0.68; 0.52–0.89; I^2 = 50) and pregnancy rates (RR = 0.72; 0.55–0.95; I^2 = 72), although the latter was no longer significant after correction for publication bias. While it did not quite meet our threshold for significance, a negative trend was also observed for live birth rate (RR = 0.48; 0.22–1.02; I^2 = 79). In the case of ICSI, non-significant trends were observed for implantation (RR = 0.79; 0.60–1.04; I^2 = 72) or pregnancy rates (RR = 0.89; 0.78–1.02; I^2 = 44), and live birth rate (RR = 0.92; 0.67–1.27; I^2 = 70). The current review provides the largest evidence to date supporting a negative association between sperm DNA damage and conventional IVF treatments, significantly reducing implantation and pregnancy rates. The routine use of sperm DNA

testing is therefore justified, since it may help improve the outcomes of IVF treatments and/or allow a given couple to be advised on the most suitable treatment. Further well-designed controlled studies on a larger number of patients are required to allow us to reach more precise conclusions, especially in the case of ICSI treatments.

Key words: infertility, sperm, DNA damage, DNA fragmentation, IVF, ICSI, meta-analysis, systematic review.

CONTENTS

I. Introduction
II. Aims
III. Methods
(1) Systematic review and meta-analysis registration
(2) Data sources and search strategy
(3) Study eligibility
(4) Study selection procedure and quality analysis
(5) Data extraction for systematic review and statistics
(6) Data extraction for meta-analysis and statistics
IV. Results
(1) Identification and selection of articles for qualitative analysis
(2) Systematic review: qualitative analysis
(*a*) Qualitative analysis in relation to fertilization method
(*b*) Qualitative analysis in relation to DNA damage detection method
(3) Inclusion of studies in the meta-analysis
(4) Meta-analysis: quantitative analysis

(*a*) IVF and ICSI
(*b*) IVF results
(*c*) ICSI results
(5) Sensitivity analyses
(*a*) HKSJ method
(*b*) Systematic study exclusion
(6) *Post-hoc* analyses
V. Discussion
(1) IVF outcomes
(2) ICSI outcomes
(3) Differences between IVF and ICSI
(4) DNA fragmentation assessment methods
(5) Strengths and limitations
VI. Conclusions
VII. Acknowledgements and author contributions
VIII. References
IX. Supporting information

I. INTRODUCTION

Infertility affects millions of couples worldwide, with an incidence of between 7% and 15% (Datta *et al.*, 2016). Assisted Reproduction Techniques (ART), including intrauterine insemination (IUI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), are the most common therapeutic options available to infertile couples. Although these methods have improved progressively over recent decades, ART data generated by national registries and collected by the European IVF-Monitoring

Consortium (European Society of Human Reproduction and Embryology; ESHRE), which include more than 500,000 IVF and ICSI cycles, indicate that there are still important limitations to achieving reasonable pregnancy rates, reported to be only 28.5% and 26.2% for IVF and ICSI, respectively (De Geyter *et al.*, 2020).

It is well known that male factors contribute to infertility at approximately the same rate as female factors. However, accumulating data analysing the relationship between conventional sperm analysis (sperm concentration, motility and morphology) and clinical results show that these male factors are not always predictive of the performance of ART (Lewis, 2007). This fact, coupled with the low pregnancy rates for IVF and ICSI, has led to an increasing need to develop precise methods for sperm-quality diagnosis with a prognostic value in ART (Simon & Lewis, 2011). Given the role of diminished DNA integrity in a wide range of diseases, sperm DNA fragmentation assessment has become a focus of studies in human sperm since the 1990s (Bianchi *et al.*, 1993; Sun, Jurisicova & Casper, 1997). Different testicular and post-testicular origins have been identified to explain the presence of both single- and double-stranded DNA breaks in mature sperm cells (Ribas-Maynou & Benet, 2019). First, apoptosis may take place during spermatogenesis leading to the expression of apoptotic markers in defective cells, which are either eliminated by Sertoli cells or enter an apoptotic-like status leading to ejaculated sperm with fragmented DNA (Sakkas & Alvarez, 2010). Second, during meiosis, unrepaired DNA breaks produced during the DNA recombination process may remain in sperm cells leading to DNA fragmentation (Lange *et al.*, 2011; Gunes, Al-Sadaan & Agarwal, 2015). Third, sperm chromatin remodelling to replace histones with protamines may also be a source of DNA damage. This process, facilitated by endogenous topoisomerase II, generates and repairs DNA breaks with the aim of reducing DNA torsional stresses (McPherson & Longo, 1993;

150 Muratori *et al.*, 2019). Finally, oxidative stress is also a major cause of DNA breaks
151 through free radical generation in endogenous or exogenous processes (Aitken *et al.*,
152 2016). Endogenous oxidative stress may be a by-product of physiological sperm
153 activity, such as mitochondrial ATP generation through oxidative phosphorylation, or it
154 could be increased as a result of abnormal sperm–seminal fluid redox balance, leading
155 to the ejaculation of immature sperm or a reduction in antioxidant protection of the
156 ejaculate. Exogenous sources of oxidative stress include varicocele, infections, drug
157 consumption, heat stress, radiation and pollution, all of which are associated with
158 reduced sperm DNA integrity (Ribas-Maynou & Yeste, 2020).
159 One objective of clinical studies carried out to date has been to determine the
160 relationship between DNA damage in sperm and clinical outcomes after IVF and ICSI.
161 Conflicting results across studies have led to controversy about the utility of DNA
162 damage in ART management. Previous systematic reviews and meta-analyses that have
163 tested the contribution of DNA integrity to IVF/ICSI outcomes have not resolved this
164 controversy: some indicate that DNA fragmentation has a detrimental association with
165 pregnancy rates, while others report no such association (A.R. Collins *et al.*, 2008;
166 Deng *et al.*, 2019; Evenson & Wixon, 2006; Simon *et al.*, 2017; Zhang *et al.*, 2015).
167 These conflicting views have resulted in the major andrology and fertility societies
168 reporting different opinions and directions in their clinical practice guidelines. On the
169 one hand, the ESHRE, in a position report published by the Andrology Interest Group in
170 2010 (Barratt *et al.*, 2010), stated that the evidence supports a negative impact of sperm
171 DNA damage on embryo development and that infertile patients show increased levels
172 of DNA fragmentation compared to controls, thus supporting analysis of DNA damage
173 in infertile patients. However, this report also identified the need for well-designed
174 clinical studies involving large sets of patients and efforts to standardize clinical assays

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175 and their cut-off values. Similarly, the European Academy of Andrology, in 2018,
176 stated that DNA damage may impair ICSI and IVF cycles by reducing pregnancy rate,
177 and suggested the inclusion of DNA damage testing, identifying sperm chromatin
178 structure assay (SCSA) and Alkaline Comet as the best methods (Colpi *et al.*, 2018).
179 Similarly, the European Association of Urology recognizes that reduced DNA integrity
180 negatively impacts conception rates (Jungwirth *et al.*, 2018). On the other hand, the
181 Practice Committee of the American Society for Reproductive Medicine (ASRM),
182 despite recognizing that increased DNA damage is associated with reduced fertility and
183 might be informative in IVF and ICSI, stated that there is insufficient evidence to apply
184 routine testing in ART. This conclusion was related to three specific deficiencies in the
185 available evidence: (*i*) the application of uncertain and non-standardized cut-off values;
186 (*ii*) the limited data available showing an association of DNA damage with both IVF
187 and ICSI clinical outcomes; and (*iii*) the need for randomized controlled trials (Practice
188 Committee of the American Society for Reproductive Medicine, 2013, 2015). Similar
189 conclusions were reached by the American Urological Association (AUA), who
190 indicated that there was insufficient evidence supporting the routine analysis of DNA
191 damage, due to the small effect on IVF/ICSI pregnancy rates identified by some meta-
192 analyses (Jarow *et al.*, 2010). A recent AUA/ASRM guideline that is more supportive of
193 DNA fragmentation testing has been published recently. In this guideline, DNA damage
194 is identified as possibly leading to infertility and miscarriage, but it does not
195 recommend routine testing of DNA fragmentation in the initial evaluation of male
196 infertility due to the lack of prospective studies (Schlegel *et al.*, 2020).
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198 **II. AIMS**

199 While most of the opinions and guidelines described above were published more than

six years ago, new data have become available in scientific databases since then. The analysis of these data in a comprehensive way is therefore timely. The present systematic review and meta-analysis aims to be the most up to date and comprehensive assessment of the association between DNA damage and IVF and ICSI outcomes. In addition, testing for differential effects observed using different DNA damage assessment methods is a secondary objective of this review. We focus herein on qualitative and quantitative data obtained from high-quality studies related to the clinical and laboratory outcomes of IVF and ICSI treatments.

III. METHODS

(1) Systematic review and meta-analysis registration

This systematic review and meta-analysis were performed following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Liberati *et al.*, 2009). The protocol used was registered in the international prospective register of systematic reviews (PROSPERO 2020: www.crd.york.ac.uk/prospero) under the code CRD42020176683.

(2) Data sources and search strategy

A systematic search of the literature was conducted using the MEDLINE-PubMed (www.ncbi.nlm.nih.gov/pubmed) and EMBASE (https://www.embase.com/#search) databases, encompassing articles from the earliest record until 27 March 2020. The search was conducted according to the inclusion and exclusion criteria defined in a PICOS (Population, Intervention, Comparison, Outcome, Study) design structure (see online supporting information, Table S1). The defined criteria used different key words and medical subject headings terms that were combined with key words relating to

infertility, DNA damage and oxidative stress to define the search strategy for both databases (Appendix S1).

(3) Study eligibility

Eligibility of a study for inclusion in the systematic review and meta-analysis was determined following the criteria defined in the PICOS design (Table S1), and also according to the study-quality analysis defined in Section III.4. Studies that satisfied the following criteria were included: (*i*) performed in human infertile couples where a male factor was assessed; (*ii*) treatments with conventional IVF or ICSI, in combination with screening for sperm DNA damage; (*iii*) comparisons performed between couples with low and high exposure to oxidative damage or between pregnant and non-pregnant couples; (*iv*) primary outcomes defined were pregnancy rate, implantation rate and live-birth rate, and secondary outcomes were fertilization rate, blastocyst rate, embryo quality and sperm DNA damage.

Studies performing intrauterine insemination or performing IVF or ICSI with an additional treatment either to the male partner, female partner, sperm cell or oocyte were excluded from this review. Examples of these additional treatments include oral antioxidant intake by the male partner, non-conventional sperm selection different from swim-up or density gradients, such as Annexin-V magnetic-activated cell sorting (MACS) or motile sperm organelle morphology examination (MSOME). Review articles, case reports, letters, commentary articles or previous systematic reviews and meta-analyses were considered non-eligible for inclusion.

(4) Study selection procedure and quality analysis

Study selection for the systematic review and for the subsequent meta-analysis was

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250 performed in different stages, as depicted in the flowchart shown in Fig. 1. Initially, all
251 records from the searches in PubMed and EMBASE were collected in a database
252 including reference, DOI, title, abstract, authors and article type. Duplicated articles and
253 review articles were excluded. The second step comprised title and abstract screening
254 performed by two specialists in male (in)fertility (J.R.-M. and A.S.-H.), excluding
255 articles that did not meet the eligibility criteria or that did not include a relevant
256 outcome. Following this stage, full texts of all eligible articles were downloaded and
257 used for a third round of screening by the same authors, performed using the eligibility
258 criteria defined by the PICOS design (Table S1). Any discrepancies between J.R.-M.
259 and A.S.-H. during the second and third exclusion stages were discussed to reach a
260 consensus.
261 An additional quality analysis for each included study was performed following the
262 NHLBI-NIH guidelines using the Quality Assessment Tool for Observational Cohort
263 and Cross-Sectional Studies (www.nhlbi.nih.gov/health-topics/study-quality-
264 assessment-tools). This tool was designed to assist reviewers in assessing published
265 work for potential flaws that may compromise the validity of a study; it provides 14
266 'Yes'/'No' questions that have to be answered for each study. The sum of positive
267 answers results in a value on a 0–14 scale and represents an indicator of study quality.
268 For the present systematic review and meta-analysis, we excluded studies for which this
269 score was less than 5, which are classified as 'poor' or 'very poor' in quality. As an
270 additional quality assessment, we excluded studies with a sample size of less than 25
271 couples.
272
273 **(5) Data extraction for systematic review and statistics**
274 From the final list of articles included in the qualitative analysis, the following data

were extracted from each study: author/s, publication date, journal, title of article, study design, parameter studied, methodology, cut-off value used to discriminate high or low values of the parameter studied, sample size, fertilization method (IVF, ICSI, IVF and ICSI), and aim and principal conclusion (Table S2). The studies included in the systematic review were classified according to their findings regarding the presence or absence of an association between DNA damage and implantation rate, pregnancy rate, live-birth rate, fertilization rate, embryo quality, and blastocyst formation. These data were also separated by fertilization method, and by DNA damage assessment method, in order to investigate possible differences among these methods. Comparison of the frequencies of studies that found or failed to find an association between DNA damage and these parameters was performed using Chi-squared tests.

(6) Data extraction for meta-analysis and statistics

Out of the studies that were included in the qualitative analysis, only those that reported sufficient data to construct a two-by-two table (e.g. normal/increased DNA damage *versus* pregnant/non-pregnant) were included in the meta-analysis. Data relating to implantation rate, pregnancy rate and live birth rate in the normal or increased DNA damage groups were extracted and used in the statistical analyses described below. Meta-analyses were conducted using R software v. 4.0.0 (R Core Team, 2020), according to the guide provided by Harrer *et al.* (2019*a*) including the R packages '*meta*' (Balduzzi, Rücker & Schwarzer, 2019) and '*dmetar*' (Harrer *et al.*, 2019*b*) in accordance with the Cochrane guidelines. Study-by-study comparisons were synthesized using the Mantel–Haenszel method with random-effects models comparing risk ratios (RRs) and 95% confidence intervals (95% CI). RRs were preferred to odds ratios (ORs) following the Cochrane Handbook because they are easier to interpret.

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300 Summary estimates describe the outcome results when comparing normal and increased
301 DNA damage groups with respect to three clinical ART data indicators (implantation
302 rate, pregnancy rate and live birth rate) and treatment type (IVF and/or ICSI). A RR
303 below 1 suggests a higher risk of an unsuccessful outcome in the DNA damage group
304 compared to the normal DNA group. A RR above 1 suggests a lower risk of an
305 unsuccessful outcome in the DNA damage group compared to the normal DNA group.
306 The DNA damage cut-off selected was based on that used in each included study
307 individually. Statistical significance was set at $P<0.05$ (two-way).
308 Heterogeneity among studies was evaluated *via* a Chi-squared test and the I^2 index with
309 the significance level set at $P<0.10$. We assume that I^2 values indicate moderate
310 heterogeneity for values <50%, substantial heterogeneity if the values are between
311 ≥50% and <75%, and considerable heterogeneity for ≥75%. Tests for funnel plot
312 asymmetry were performed when at least 10 studies were included in the meta-analysis.
313 When publication bias was detected we performed a sensitivity analysis adjusting the
314 pooled RR estimate through the Copas selection model (Carpenter *et al.*, 2009). Copas
315 analyses were performed with the '*Copas*' function from the R package '*metasens*'
316 (Schwarzer, Carpenter & Rücker, 2019).
317 Sensitivity analyses were conducted by systematic exclusion of one study at a time and
318 recalculating summary RRs. We considered a study as influential if removing it: (1)
319 changed the magnitude of the association by more than 20%; (2) changed the RR to
320 significant or non-significant; (3) changed the direction of the association; or (4)
321 explained the heterogeneity. Primary analyses were also repeated using the Hartung,
322 Knapp, Sidik and Jonkman (HKSJ) method to adjust the random-effects model. The
323 DerSimonian–Lair method is prone to producing statistically significant results, mainly
324 when there is substantial inter-study heterogeneity and the number of studies included

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325 in the analyses is small (IntHout, Ioannidis & Borm, 2014). Because in some of our
326 analyses there was evidence of significant heterogeneity, and the HKSJ method
327 performs better in this situation, we repeated the analyses using this method to test the
328 robustness of our results.
329 *Post-hoc* subgroup analyses were performed when 10 or more studies were available,
330 considering the evaluation technique [SCSA, terminal deoxynucleotidyl transferase-
331 mediated nick end labeling (TUNEL), sperm chromatin dispersion (SCD), Alkaline
332 Comet and Acridine Orange] as a subgroup variable.
333

334 **IV. RESULTS**

335 **(1) Identification and selection of articles for qualitative analysis**

336 A flowchart showing the article identification and selection process is depicted in Fig. 1.
337 After the initial search, a total of 3,642 records were identified, of which 267 were
338 excluded as duplicates or review articles. Out of the 3,375 articles screened, 3,073 were
339 excluded based on the title and abstract as not meeting the scope of this study, not being
340 performed in humans, or presenting outcomes not relevant to this review. This resulted
341 in 302 articles that were downloaded and subjected to full-text assessment according to
342 the inclusion/exclusion criteria summarized in Section III.3. A total of 210 of these were
343 excluded (Fig. 1), with a further 10 excluded that failed to reach a quality score of 5 and
344 4 excluded due to small sample size. These exclusion steps led to the inclusion of 78
345 articles for qualitative analysis. Quality scores for each of these articles are provided in
346 Table S3: mean quality score was 7.35 with a range of 5–11.
347

348 **(2) Systematic review: qualitative analysis**

349 The 78 studies included in our qualitative review comprise data from 25,639 ART

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350 cycles. Among these, twelve studies (15%) involving 1,748 patients used IVF, 23
351 studies (29%) involving 4,664 patients used ICSI, 34 studies (44%) involving 16,336
352 patients used both IVF and ICSI but presented separate data for each method, and nine
353 studies (12%) involving 2,891 patients presented combined data from IVF and ICSI
354 cycles. Full information about each study is provided in Table S2.
355 Table 1 shows the percentage of studies that identified a negative influence of sperm
356 DNA damage on clinical parameters (implantation rate, pregnancy rate, live-birth rate)
357 and on laboratory parameters (fertilization rate, embryo quality and blastocyst
358 formation), classified according to the fertilization method (IVF and ICSI) and the most
359 common methods used to assess DNA damage (SCSA, TUNEL, SCD and Alkaline
360 Comet).
361
362 (*a*) *Qualitative analysis in relation to fertilization method*
363 For three clinical parameters (implantation rate, pregnancy rate and live-birth rate),
364 there were no statistically significant differences between IVF cycles and ICSI cycles in
365 the total percentage of studies reporting a negative association with DNA damage.
366 However, it is worth noting that these percentages were slightly higher for studies
367 involving IVF (74%, 61% and 60% for implantation rate, pregnancy rate and live-birth
368 rate, respectively) than for those involving ICSI (60%, 45% and 54%, respectively),
369 implying that there may be a greater influence of DNA damage on these parameters in
370 IVF than in ICSI (Table 1).
371 For the laboratory parameters, there was a significant difference between IVF and ICSI
372 cycles in the percentage of studies reporting a negative association with sperm DNA
373 damage only for fertilization rate (76% for IVF and 52% for ICSI) ($P = 0.039$). For
374 embryo quality, the percentage of studies finding this negative association was slightly

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375 higher in IVF than in ICSI (68% *versus* 58%, respectively), although the difference did
376 not reach statistical significance. An influence of DNA damage on blastocyst formation
377 rates was found in 67% of studies in both cases (Table 1).
378

379 (*b*) *Qualitative analysis in relation to DNA damage detection method*

380 We identified differences between methods assessing DNA damage in the percentage of
381 studies supporting a negative association between DNA damage and both pregnancy
382 rate and live-birth rate in IVF (Table 1A). For pregnancy rate, these differences were
383 observed between SCD and Comet (31% *versus* 100%; $P = 0.005$) and SCD and
384 TUNEL (31% *versus* 73%; $P = 0.024$). For live-birth rate, significant differences were
385 found between SCD and Comet (0% *versus* 100%; $P = 0.025$) and between SCD and
386 SCSA (0% *versus* 80%, $P = 0.028$); however, one should note that the number of
387 studies reporting live-birth rates was small. Regarding IVF laboratory parameters, most
388 studies supported a negative association between DNA damage and fertilization rate,
389 embryo quality and blastocyst formation for all techniques.

390 For the ICSI studies (Table 1B), no significant differences were found between the
391 different DNA damage assessment techniques in the percentage of studies reporting a
392 negative association of DNA damage with clinical or laboratory parameters. The high
393 heterogeneity in the results precludes firm conclusions regarding the different DNA
394 damage assessment methods, but around half of all studies showed a negative
395 association with clinical and laboratory parameters (Table 1B). There were too few
396 studies reporting blastocyst formation to establish reliable conclusions.
397

398 **(3) Inclusion of studies in the meta-analysis**

399 Out of the 78 articles included in the qualitative analysis, 46 did not provide sufficient

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400 information to construct a two-by-two table for any of the three parameters used in the
401 meta-analysis: implantation rate, pregnancy rate and live birth rate (Fig. 1). Of the 32
402 articles selected for data analysis, five involved IVF, 14 ICSI, and 13 both IVF and
403 ICSI, together representing 12,380 ART cycles. From the studies including IVF data,
404 eight studies (4,055 cycles) had sufficient data on implantation rate, 15 studies (3,711
405 cycles) had data on pregnancy rate, and six studies (1,634 cycles) had data on live-birth
406 rate. From those involving ICSI, 11 studies (3,405 cycles) provided implantation rate
407 data, 25 studies (5,467 cycles) reported pregnancy rate, and nine studies (3,017 cycles)
408 presented data on live-birth rate.
409

410 **(4) Meta-analysis: quantitative analysis**

411 Fig. 2 provides a summary of the pooled RR values from the meta-analysis assessing
412 the association between sperm DNA fragmentation index and different ART outcomes
413 (implantation rate, pregnancy rate and live-birth rate) for the combined IVF and ICSI
414 data (Fig. 2A), and for IVF or ICSI considered separately (Fig. 2B).
415

416 (*a*) *IVF and ICSI*

417 The combined IVF and ICSI results showed a significant detrimental association
418 between sperm DNA damage and implantation rate (RR; 95% confidence interval)
419 (0.74; 0.61–0.91; P<0.01), with substantial heterogeneity among studies (I^2 = 69%,
420 P<0.01) (Fig. S1). A funnel plot showed no evidence of asymmetry upon visual
421 inspection (Fig. S2), although evidence for publication bias was detected *via* an Egger's
422 test (P=0.022). The adjusted estimate from the Copas selection model remained
423 significant (0.82; 0.70–0.96; P = 0.01) after accounting for publication bias.

For pregnancy rate, a similar negative association with sperm DNA damage was observed (0.83; 0.73–0.94; $P<0.01$) (Fig. S3), and heterogeneity was substantial (I^2 = 58%, $P<0.01$). A funnel plot showed little evidence of asymmetry upon visual inspection (Fig. S4), although an Egger's test found evidence for publication bias ($P<0.01$). The adjusted estimate from the Copas selection model suggested that, after accounting for publication bias, there was no longer a significant association between sperm DNA damage and pregnancy rate (1.05; 0.93–1.19; $P = 0.43$).

For live-birth rate, a non-significant negative association with sperm DNA damage was observed (0.78; 0.58–1.06; $P = 0.12$). The heterogeneity was again substantial (I^2 = 72%, $P<0.01$) (Fig. S5). While a funnel plot showed no evidence of asymmetry upon visual inspection (Fig. S6), evidence for publication bias was detected *via* an Egger's test ($P<0.01$). The adjusted estimate from the Copas selection model indicated, after accounting for publication bias, that there was no evidence of an association between sperm DNA damage and live birth rate (0.90; 0.67–1.22; $P = 0.52$).

(*b*) *IVF results*

The results for IVF studies revealed a significant detrimental association between sperm DNA damage and implantation rate (0.68; 0.52 to 0.89; $P<0.01$) (Fig. 2B), with moderate heterogeneity among studies (I^2 = 50%, $P = 0.05$) (Fig. S7). Due to the small number of studies (<10) no funnel plot was constructed. Similarly, we found a significant negative association between sperm DNA damage and pregnancy rate (0.72; 0.55–0.95; $P = 0.02$) (Fig. S8), but in this case the heterogeneity was deemed substantial (I^2 = 72%, $P<0.01$). A funnel plot indicated no evidence of asymmetry upon visual inspection (Fig. S9), although an Egger's test ($P = 0.046$) detected evidence for publication bias. The adjusted estimate from the Copas selection model suggested that,

after accounting for publication bias, there was no evidence for an association between sperm DNA damage and pregnancy rate (0.82; 0.61–1.10; $P = 0.19$). For live-birth rate, a negative tendency was observed, but this was not statistically significant (0.48; 0.22–1.02; $P = 0.06$) and had considerable heterogeneity ($I^2 = 79\%$, $P<0.01$) (Fig. S10).

(*c*) *ICSI results*

The ICSI studies showed non-significant results for all parameters. There was a tendency for sperm DNA damage to affect implantation rate negatively (0.79; 0.60–1.04; $P = 0.09$), with substantial heterogeneity among studies ($I^2 = 72\%$, $P<0.01$) (Fig. S11). While visual inspection of the funnel plots revealed no apparent asymmetry (Fig. S12), evidence of publication bias was detected *via* the Egger's test ($P=0.016$), and the adjusted estimate from the Copas selection model analysis confirmed the absence of a significant association (1.05; 0.96–1.15; $P = 0.32$). The association between sperm DNA damage and pregnancy rate also was not significant but showed a tendency towards a negative effect (0.89; 0.78–1.02; $P=0.09$), with moderate inter-study heterogeneity ($I^2 = 44\%$, $P = 0.01$) (Fig. S13). While funnel plot asymmetry was not found by visual inspection (Fig. S14), the Egger's test showed significant publication bias ($P = 0.013$) and the Copas selection model confirmed the absence of a significant association (0.99; 0.89–1.12; $P = 0.99$). Finally, there was no significant relationship between DNA damage and live-birth rate (0.92; 0.67–1.27; $P = 0.62$), again with evidence for considerable heterogeneity ($I^2 = 70\%$, $P<0.01$) (Fig. S15).

(5) Sensitivity analyses

(*a*) *HKSJ method*

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473 We repeated our primary analyses with adjustments according to the HKSJ method. The
474 results were consistent with those obtained without adjustment (Appendix S2).
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476 (*b*) *Systematic study exclusion*
477 Systematic exclusion of one study at a time (Table S4) showed that the RR value for the
478 association of sperm DNA fragmentation with pregnancy rate in IVF studies became
479 non-significant after removing data from Bungum *et al.* (2007). For the live-birth rate
480 comparison, removing the Ni *et al.* (2014) study led to a significant negative
481 relationship, and removal of Simon *et al.* (2010) explained the heterogeneity. The
482 association between sperm DNA fragmentation and implantation rate after ICSI became
483 significantly negative after removing the study of Green *et al.* (2020). Removing the
484 study of Bungum *et al.* (2007) caused the RR for a negative association with pregnancy
485 rate in ICSI to become significant. Finally, in the live birth rate comparison, removal of
486 Sivanarayana *et al.* (2014) caused the direction of the trend to change, although this
487 remained non-significant.
488
489 **(6) *Post-hoc* analyses**
490 Due to the limited number of studies reporting certain outcomes, we could only perform
491 *post-hoc* analysis for the effects of sperm DNA damage on pregnancy rate after IVF and
492 ICSI and implantation rate after ICSI. These analyses were performed using the
493 technique for evaluating sperm DNA damage (SCSA, TUNEL, SCD, Alkaline Comet
494 and Acridine Orange) as a subgroup variable. Our results showed a significant RR for
495 only Alkaline Comet for pregnancy rate after IVF, for Acridine Orange and Alkaline
496 Comet for implantation rate after ICSI, and for Acridine Orange for pregnancy rate after
497 ICSI (Figs S16–S18). However, in all cases there was no statistically significant

subgroup effect, suggesting that the technique used does not modify the association. Moreover, in all three subgroup analyses, there was a high percentage (ranging from 46% to 68%) of residual variation due to heterogeneity. It is noticeable that there was a smaller number of trials and participants for the Alkaline Comet and Acridine Orange subgroups than for the other techniques, perhaps affecting the ability of this analysis to detect differences.

V. DISCUSSION

The use of DNA fragmentation testing in infertility treatments has been the subject of controversy in recent decades. The publication of studies and meta-analyses that arrived at contradictory conclusions has led to varying clinical recommendations related to its diagnostic and prognostic value by different clinical associations (Barratt *et al.*, 2010; Practice Committee of the American Society for Reproductive Medicine, 2013). In this context, and with the aim of moving towards a consensus in clinical practice, this systematic review and meta-analysis provides updated information regarding the relationship between sperm DNA damage and clinical outcomes in IVF and ICSI treatments. Our study design and search strategy, coupled with quality analysis, allowed us to analyse a large data set from high-quality studies related to this male factor in a systematic review that included data from 25,639 ART cycles, and in a meta-analysis that used data from 12,380 ART cycles. The analysis presented here is the most comprehensive to date, since we include implantation rate, pregnancy rate and live-birth rate in the same meta-analysis.

Our results from both the systematic review and meta-analysis suggest that sperm DNA damage has a negative influence on implantation and pregnancy rates after IVF treatment and a tendency towards reduction of live-birth rates (Fig. 2). In the systematic

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523 review, we observed that 60–74% of studies support these detrimental associations in
524 IVF treatments. A similar proportion (67–76%) of studies indicate that DNA damage
525 has a detrimental association with fertilization rate, embryo quality and blastocyst
526 formation (Table 1). These results demonstrate a general agreement in the literature, but
527 could not be extrapolated to ICSI procedures. In ICSI, no significant effects were
528 identified for any of the parameters (Fig. 2), and additional studies are needed to
529 understand the influence of sperm DNA integrity on ICSI outcomes. Our qualitative
530 analysis implies similar non-conclusive results, with a detrimental association of sperm
531 DNA damage with clinical parameters found by only 45–60% of studies and with
532 laboratory parameters for 52–67% of studies (Table 1).
533 The conclusions of previous systematic reviews and meta-analyses regarding an
534 association of DNA damage with ART outcomes are summarized in Table 2. Of these
535 studies, nine included pregnancy rate as main outcome (one assessing live birth rate as
536 additional outcome; Deng *et al.*, 2019), and one article analysed live birth rate (Osman
537 *et al.*, 2015). In summary: three of these studies did not find an association between
538 DNA damage and IVF/ICSI pregnancy rate (Evenson & Wixon, 2006; J.A. Collins,
539 Barnhart & Schlegel, 2008; Cissen *et al.*, 2016); one study obtained data supporting a
540 detrimental association of DNA fragmentation with IVF and ICSI pregnancy rate
541 (Simon *et al.*, 2017); and six studies found an association of sperm DNA integrity with
542 IVF outcomes, but not for ICSI cycles (Li *et al.*, 2006; Zini, 2011; Zhao *et al.*, 2014;
543 Osman *et al.*, 2015; Zhang *et al.*, 2015; Deng *et al.*, 2019).
544 Only four meta-analyses to date include data from more than 5,000 infertile couples,
545 three of which support a detrimental association of DNA damage with IVF outcome
546 (Zhang *et al.*, 2015; Simon *et al.*, 2017; Deng *et al.*, 2019), but only one found a
547 reduction of pregnancy rate in ICSI cycles (Simon *et al.*, 2017). Of the existing meta-

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548 analyses, only three used a quality filter for the inclusion of studies. The present study is
549 the first meta-analysis to include data exceeding 10,000 IVF/ICSI cycles, a quality
550 control filter, and an analysis of implantation, pregnancy and live-birth outcomes.
551

552 **(1) IVF outcomes**

553 To our knowledge, implantation rate has not been assessed in previous meta-analyses
554 investigating the effects of sperm DNA damage on IVF outcomes (Table 2). The
555 statistically significant RR of 0.68 (0.52–0.89) obtained here suggests that an increase
556 in sperm DNA damage to above the threshold value could lead to a 32% reduction in
557 embryo implantation compared to sperm with DNA damage below this threshold.
558 For pregnancy rate, we obtained a significant RR of 0.72 (0.55–0.95), implying that
559 patients with DNA damage above the cut-off would be expected to suffer a 5–45%
560 reduction in pregnancy achievement. The RR obtained here is in accordance with the
561 results from previous meta-analyses: Li *et al*. (2006) reported a RR of 0.68 (0.54–0.85);
562 Deng *et al*. (2019) reported a RR of 0.77 (0.59–1.00); Zini *et al*. (2011) calculated an
563 OR of 1.70 (1.30–2.23); Zhao *et al*. (2014) calculated an OR of 0.66 (0.48–0.90); and
564 Simon *et al*. (2017) reported an OR of 1.65 (1.34–2.04). [OR and RR obtained in meta-
565 analyses results rely upon how the outcome is analysed in the statistical study. If
566 authors assess whether an increase in DNA fragmentation is associated with a reduction
567 of the clinical condition, values lower than 1 are found when an association is present;
568 however, if authors analyse whether a reduction of DNA damage is associated with an
569 increase of the clinical condition, values higher than 1 indicate an association.]
570 For live-birth rate in IVF, our study found a RR of 0.48 (0.22–1.02), although it did not
571 quite meet statistical significance ($P = 0.06$). Our sensitivity analyses (Table S4)
572 indicated the RR became significant after removing a single study (Ni *et al*., 2014;

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recalculated RR = 0.37, 0.15–0.93). In addition, removal of a different study (Simon *et al*., 2010), significantly reduced heterogeneity. These results are in accordance with those of Osman *et al*. (2015), who obtained a significant RR of 1.27 (1.05–1.52) for live-birth rate, but contrast with Deng *et al*. (2019), who did not find a significant RR (0.84; 0.67–1.06). It is worth highlighting that meta-analysis results for live-birth rate should be considered with caution due to the lower number of studies and patients included.

(2) ICSI outcomes

Previous meta-analyses did not include an evaluation of implantation rate after ICSI cycles. Our results suggest a tendency for a negative effect of sperm DNA damage on implantation rate with a RR of 0.79 (0.60–1.04; $P = 0.09$). Sensitivity analysis showed that removal of a single study (Green *et al*., 2020) led to the result becoming statistically significant (recalculated RR = 0.75; 0.58–0.97), and this also reduced the heterogeneity to 56% (Table S4). Even with removal of this study, the confidence intervals are very close to 1, implying that any conclusion about the association of DNA damage with implantation rate is weak, and that additional high-quality studies are needed to provide more information on potential effects.

We obtained a similar result for pregnancy rate, with a non-significant RR of 0.89 (0.78–1.02, $P = 0.09$). The sensitivity analysis showed that this result would have become significant if the study of Bungum *et al*. (2007) had not been included (recalculated RR = 0.87; 0.76 to 0.99). Again, this significant association between DNA damage and implantation rate remains weak due to the proximity of the confidence interval to 1. Our result for pregnancy rate is in accordance with previous meta-analyses that failed to find a significant relationship between DNA damage and pregnancy rate in

ICSI cycles: Li *et al*. (2006) reported a RR of 0.76 (0.55–1.04); Deng *et al*. (2019) a RR of 0.75 (0.44–1.27); J.A. Collins *et al*. (2008) found an OR of 1.12 (0.59–2.15); Zini *et al*. (2014) reported an OR of 1.15 (0.90–1.55); and Zhao *et al*. (2014) an OR of 0.94 (0.70–1.25). Only one meta-analysis (Simon *et al.,* 2017) reported that pregnancy rate in ICSI is significantly affected by DNA fragmentation (RR=1.31; 1.08–1.59).

We did not find a significant effect of DNA damage on live-birth rate in ICSI, in agreement with a previous study (Deng *et al*., 2019), although Osman *et al*. (2015) did find a significant effect for this parameter (RR of 1.11; 1.0–1.23). The results for live-birth rate should be considered with caution due to the low number of studies and patients included (Fig. 2).

(3) Differences between IVF and ICSI

The results of the present meta-analysis show that sperm DNA damage is associated with clinical outcomes following IVF, but not in the case of ICSI. This difference could potentially be explained either by the presence of a bias between patient cohorts or by the inherent technical differences between these methods. First, we cannot exclude potential differences between IVF and ICSI patients: ICSI patients tend to be those with worse clinical conditions in parameters that are independent of DNA damage, which may impair ICSI outcomes of both the experimental and control groups in this meta-analysis. Second, ICSI involves single sperm selection by a technician and its direct injection into the oocyte. The ICSI technician selects a sperm cell with progressive motility and good morphology. Since these sperm-quality parameters are known to be negatively correlated with DNA damage (Aitken & De Iuliis, 2010; Belloc *et al.*, 2014), it is possible that ICSI-selected sperm cells have a lower incidence of DNA fragmentation than found in natural or processed semen. This hypothesis has been

proved for sperm selected using the MSOME (Gosálvez *et al.*, 2013; Pastuszek *et al.*, 2017), and may provide one explanation for the general lack of an effect of DNA damage on clinical outcomes in ICSI cycles.

Given that IVF and ICSI lead to similar fertilization (Practice Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2012) and pregnancy rates when DNA damage is not taken into account (Bhattacharya *et al.*, 2001; De Geyter *et al.*, 2020), Evers (2016) criticized the overuse of ICSI over IVF and suggested that ICSI should be reserved for couples with severe male factor infertility. Our systematic review and meta-analysis supports this view: IVF is more sensitive to sperm DNA damage than is ICSI, probably due to sperm micro-selection in ICSI. Therefore, in order to maximize the chances of success, patients with increased levels of sperm DNA damage should be directed to ICSI or the use of treatments like antioxidants that may help reduce DNA fragmentation (Salas-Huetos *et al.*, 2018; Smits *et al.*, 2019), although the benefits of such treatments are not yet well established. In this regard, measuring sperm DNA fragmentation levels may be highly relevant when determining the most appropriate ART for a given couple.

(4) DNA fragmentation assessment methods

In both our systematic review and meta-analysis we found heterogeneity related to the techniques used to assess DNA damage and their relation to the assessed outcomes. Considering IVF and ICSI together, our systematic review (Table 1) suggested that Alkaline Comet assay and TUNEL may be the most reliable methodologies in terms of predicting a negative effect of sperm DNA damage on pregnancy rate following IVF and ICSI, in agreement with the previously reported superior capacity of these assays in studies comparing different techniques in the same subset of patients (Ribas-Maynou *et*

al., 2013; Simon *et al.*, 2014*a*; Javed, Talkad & Ramaiah, 2019). The same studies also highlighted SCSA and SCD as useful measurement techniques, and showed that SCSA results were associated with some clinical outcomes, especially in IVF. While Comet assay and TUNEL are direct unequivocal measurements of DNA fragmentation, SCSA, SCD and Acridine Orange only provide an indirect assessment of DNA fragmentation, which may have implications on interpretation of any associations found. Indirect methods are based on higher susceptibility to chromatin decondensation of DNA that contains breaks, however, one should note that alterations in protamine disulphide bonds may give higher values in the SCSA, Acridine Orange and SCD methods without the presence of DNA fragmentation (Simon *et al.*, 2014*a*).

Since the available data from different methods are still limited, the conclusions obtained herein for each method analysed separately were weak, and further studies are needed to establish a clear understanding of their relative merits. Future research should perhaps focus on direct methods of DNA damage detection, as they provide more accurate detection of actual DNA fragmentation.

(5) Strengths and limitations

Our systematic review enabled us to assess additional parameters that could not be included in the meta-analysis, including fertilization rate, embryo quality and blastocyst formation. Our eligibility criteria, restricted to studies performing IVF or ICSI in couples with male factor infertility and without additional treatments, allowed inclusion of a larger number of studies compared to other systematic reviews or meta-analyses performed to date. The application of a quality-control filter ensured that the included studies met standardized quality criteria.

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672 However, our work has some limitations. Substantial to considerable heterogeneity was
673 identified among studies in most groups analysed in our meta-analysis. This
674 heterogeneity is typical for research targeting a multifactorial disease, but is particularly
675 high in infertility studies, as this condition can involve both the male and female partner
676 with multiple known and unknown factors. We cannot exclude that different patient
677 cohorts may exist between IVF and ICSI patients, and also note that female factors were
678 not controlled for in most of the studies included in our meta-analysis. This lack of
679 consideration of female factors might be due both to the difficulty in categorizing them
680 or due to the reduction that subdivisions cause in sample size. Another important
681 limitation of our study potentially increasing study heterogeneity is the inclusion of
682 different DNA fragmentation methods with different sensitivities for DNA damage
683 detection (see Section V.4). Additionally, we used the authors' stated cut-off values for
684 DNA damage, which varied among studies; in some cases, authors used cut-off values
685 established by other research groups. Finally, we detected significant publication bias
686 *via* the Egger's test in several cases. After accounting for publication bias, our
687 significant association of DNA damage with pregnancy rate in IVF studies became non-
688 significant indicating that, at least for this parameter, the results are sensitive to possible
689 publication bias. Finally, pregnancy loss is very likely to be associated with higher
690 sperm DNA damage indexes, although this reproductive parameter was not evaluated
691 here.

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693 **VI. CONCLUSIONS**

694 (1) This systematic review and meta-analysis identified a relationship between sperm
695 DNA fragmentation and clinical outcomes after IVF treatments, which was not
696 observed for ICSI treatments.

(2) The results suggest that it will be useful to test sperm DNA damage prior to conducting ART, to provide a well-founded basis to direct a couple to the most suitable method.

(3) We advise inclusion of sperm DNA damage testing in clinical recommendations and encourage further research in three directions: first, to assess the implications of DNA damage in ICSI; second, to standardize and compare DNA damage methodologies in IVF/ICSI; and third, to use direct methods of DNA damage assessment to measure the influence of DNA fragmentation on IVF/ICSI outcomes.

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Author contributions: J.R.-M. conceived the study, performed the search and eligibility selection, performed systematic review analysis, interpreted and discussed the results, wrote the manuscript, revised the manuscript and approved the final version. M.Y. conceived the study, contributed to eligibility selection, interpreted and discussed the results, revised the manuscript and approved the final version. N.B.-T. performed the meta-analysis and contributed to results interpretation. K.I.A. and E.R.J. revised the manuscript and approved the final version. A.S.-H. conceived the study, performed the search and eligibility selection, performed meta-analysis statistics, interpreted and discussed the results, wrote the manuscript, revised the manuscript and approved the final version. All authors provided substantial intellectual contributions.

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1176 **IX. SUPPORTING INFORMATION**

1177 Additional supporting information may be found online in the Supporting Information
1178 section at the end of the article.

1179 **Table S1.** PICOS design structure, including the inclusion and exclusion criteria and the
1180 key words used for the definition of the search strategy and the eligibility of the study.

1181 **Appendix S1.** Search strategy.

1182 **Table S2.** Studies included in the systematic review and meta-analysis, summarizing
1183 their main aim and principal conclusion.

1184 **Table S3.** Quality score determined for each study included in our qualitative analysis
1185 according to the criteria defined in the NHLBI-NIH guidelines for the quality
1186 assessment tool for observational cohort and cross-sectional studies.

1187 **Fig. S1.** Forest plot of risk ratio (RR) for each study investigating the association
1188 between sperm DNA damage and implantation rate in IVF and ICSI.

1189 **Fig. S2.** Funnel plot of risk ratio values for all IVF and ICSI studies reporting the
1190 effects of sperm DNA damage on implantation rate.

1191 **Fig. S3.** Forest plot of risk ratio (RR) for each study investigating the association
1192 between sperm DNA damage and pregnancy rate in IVF and ICSI.

1193 **Fig. S4.** Funnel plot of risk ratio values for all IVF and ICSI studies reporting the
1194 effects of sperm DNA damage on pregnancy rate.

1195 **Fig. S5.** Forest plot of risk ratio (RR) for each study investigating the association
1196 between sperm DNA damage and live-birth rate in IVF and ICSI.

1197 **Fig. S6.** Funnel plot of risk ratio values for all IVF and ICSI studies reporting the
1198 effects of sperm DNA damage on live-birth rate.
1199 **Fig. S7.** Forest plot of risk ratio (RR) for each study investigating the association
1200 between sperm DNA damage and implantation rate in IVF.
1201 **Fig. S8.** Forest plot of risk ratio (RR) for each study investigating the association
1202 between sperm DNA damage and pregnancy rate in IVF
1203 **Fig. S9.** Funnel plot of risk ratio (RR) values for all studies reporting the effects of
1204 sperm DNA damage on pregnancy rate in IVF.
1205 **Fig. S10.** Forest plot of risk ratio (RR) for each study investigating the association
1206 between sperm DNA damage and live-birth rate in IVF.
1207 **Fig. S11.** Forest plot of risk ratio (RR) for each study investigating the association
1208 between sperm DNA damage and implantation rate in ICSI.
1209 **Fig. S12.** Funnel plot of risk ratio (RR) values for all studies reporting the effects of
1210 sperm DNA damage on implantation rate in ICSI.
1211 **Fig. S13.** Forest plot of risk ratio (RR) for each study investigating the association
1212 between sperm DNA damage and pregnancy rate in ICSI.
1213 **Fig. S14.** Funnel plot of risk ratio (RR) values for all studies reporting the effects of
1214 sperm DNA damage on pregnancy rate in ICSI.
1215 **Fig. S15.** Forest plot of risk ratio (RR) for each study investigating the association
1216 between sperm DNA damage and live-birth rate in ICSI.
1217 **Appendix S2.** Sensitivity analysis.
1218 **Table S4.** Results of sensitivity analysis (no HKSJ adjustment) in which studies were
1219 excluded one at a time (sorted by I^2).

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1220 **Fig. S16.** Forest plot of risk ratio (RR) for each study investigating the association
1221 between sperm DNA damage and pregnancy rate in IVF, separated according to the
1222 technique used to assess DNA fragmentation
1223 **Fig. S17.** Forest plot of risk ratio (RR) for each study investigating the association
1224 between sperm DNA damage and implantation rate in ICSI, separated according to the
1225 technique used to assess DNA fragmentation
1226 **Fig. S18.** Forest plot of risk ratio (RR) for each study investigating the association
1227 between sperm DNA damage and pregnancy rate in ICSI, separated according to the
1228 technique used to assess DNA fragmentation
1229

FIGURE CAPTIONS

Fig. 1. Flowchart of the literature search and selection process. ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization.

Fig. 2. Forest plot of pooled risk ratios (RRs) for studies assessing the association between DNA damage and implantation rate, pregnancy rate and live birth rate for (A) all IVF and ICSI treatments, and (B) IVF and ICSI treatments separately. A RR value <1 indicates a greater risk of a negative outcome on the measured parameter in the DNA-damage group compared with the normal DNA group. ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization.

SUPPLEMENTARY FIGURE CAPTIONS

Fig. S1. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and implantation rate in IVF and ICSI. A RR value <1 indicates a greater risk of a negative outcome on implantation rate in the DNA-damage group compared with the normal DNA group. ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization.

Fig. S2. Funnel plot of risk ratio values for all IVF and ICSI studies reporting the effects of sperm DNA damage on implantation rate.

Fig. S3. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and pregnancy rate in IVF and ICSI. Other details are as in legend to Fig. S1.

Fig. S4. Funnel plot of risk ratio values for all IVF and ICSI studies reporting the effects of sperm DNA damage on pregnancy rate.

Fig. S5. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and live-birth rate in IVF and ICSI. Other details are as in legend to Fig. S1.

Fig. S6. Funnel plot of risk ratio values for all IVF and ICSI studies reporting the effects of sperm DNA damage on live-birth rate.

Fig. S7. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and implantation rate in IVF. Other details are as in legend to Fig. S1.

Fig. S8. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and pregnancy rate in IVF. Other details are as in legend to Fig. S1.

Fig. S9. Funnel plot of risk ratio (RR) values for all studies reporting the effects of sperm DNA damage on pregnancy rate in IVF.

Fig. S10. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and live-birth rate in IVF. Other details are as in legend to Fig. S1.

Fig. S11. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and implantation rate in ICSI. Other details are as in legend to Fig. S1.

Fig. S12. Funnel plot of risk ratio (RR) values for all studies reporting the effects of sperm DNA damage on implantation rate in ICSI.

Fig. S13. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and pregnancy rate in ICSI. Other details are as in legend to Fig. S1.

Fig. S14. Funnel plot of risk ratio (RR) values for all studies reporting the effects of sperm DNA damage on pregnancy rate in ICSI.

Fig. S15. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and live-birth rate in ICSI. Other details are as in legend to Fig. S1.

Fig. S16. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and pregnancy rate in IVF, separated according to the technique used to assess DNA fragmentation (SCSA, TUNEL, Alkaline Comet, Acridine Orange and SCD). SCD, sperm chromatin dispersion; SCSA, sperm chromatin structure assay; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labelling. Other details are as in legend to Fig. S1.

Fig. S17. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and implantation rate in ICSI, separated according to the technique used to assess DNA fragmentation (SCSA, Acridine Orange, SCD and Alkaline Comet). SCD, sperm chromatin dispersion; SCSA, sperm chromatin structure assay; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labelling. Other details are as in legend to Fig. S1.

Fig. S18. Forest plot of risk ratio (RR) for each study investigating the association between sperm DNA damage and pregnancy rate in ICSI, separated according to the technique used to assess DNA fragmentation (SCSA, TUNEL, Alkaline Comet,

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1316 Acridine Orange and SCD). SCD, sperm chromatin dispersion; SCSA, sperm chromatin
1317 structure assay; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end
1318 labelling. Other details are as in legend to Fig. S1.

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1319 Table 1. Summary results showing the percentage of studies that identified a negative
1320 association between sperm DNA damage and parameters used to assess (A) IVF
1321 outcome or (B) ICSI outcome, classified according to DNA damage assessment method
1322 used.

1323

(A) IVF

	SCSA		**TUNEL**		**SCD**		**Alkaline Comet**		**Total %**	***P* value (Chi-squared)**
Clinical parameters	***N***	**%**	***N***	**%**	***N***	**%**	***N***	**%**		
Implantation rate	9	78%	4	75%	3	33%	3	100%	74%	0.299
Pregnancy rate	17	59%	15	73%	13	31%	6	100%	61%	0.020 *
Live-birth rate	5	80%	0		3	0%	2	100%	60%	0.036 *
Laboratory parameters										
Fertilization rate	8	63%	10	90%	6	50%	5	100%	76%	0.130
Embryo quality	8	63%	3	100%	7	43%	4	100%	68%	0.141
Blastocyst formation	2	50%	2	100%	1	0%	1	100%	67%	0.290

(B) ICSI

	SCSA		**TUNEL**		**SCD**		**Alkaline Comet**		**Total %**	***P* value (Chi-squared)**
Clinical parameters	***N***	**%**	***N***	**%**	***N***	**%**	***N***	**%**		
Implantation rate	10	70%	4	50%	6	50%	5	60%	60%	0.841
Pregnancy rate	23	43%	19	58%	18	33%	6	50%	45%	0.505
Live-birth rate	5	80%	1	100%	5	20%	2	50%	54%	0.165
Laboratory parameters										
Fertilization rate	7	43%	9	44%	8	63%	3	67%	52%$^{\Psi}$	0.789
Embryo quality	8	63%	5	80%	8	25%	5	80%	58%	0.132
Blastocyst formation	3	33%	2	100%	2	100%	2	50%	67%	0.290

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1325 * Values for different methods measuring DNA damage are statistically different (P<0.05).
1326 $^{\Psi}$ Values are statistically different from IVF (P<0.05).
1327 SCD, sperm chromatin dispersion; SCSA, sperm chromatin structure assay; TUNEL, terminal
1328 deoxynucleotidyl transferase-mediated nick end labelling.

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1329 Table 2. Summary of the findings of recent systematic reviews (SR) and meta-analyses (MA) regarding the effect of sperm DNA damage on

1330 clinical outcomes of *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI).

Reference	Search conducted	Quality score?	Studies included	Subjects included in meta-analysis	DNA damage methods	Fertilization methods	Parameters analysed	Conclusions
Li *et al.* (2006)	April 2006	No	8	1115	TUNEL, SCSA	IVF and ICSI	Pregnancy rate, fertilization rate	Clinical pregnancy rate decreased with sperm DNA damage in IVF when DNA damage measured using TUNEL, but not when measured with SCSA. No differences were found in ICSI.
Evenson & Wixon (2006)	2006	No	17	2109	SCSA	IUI, IVF and ICSI	Pregnancy rate	Low levels of sperm DNA damage resulted in a 1.6 times higher likelihood of pregnancy after IVF/ICSI, although the results were non-significant.
J.A. Collins *et al.* (2008)	2006	No	22	2162	TUNEL, SCSA	IVF and ICSI	Pregnancy rate	DNA damage had a small effect on IVF/ICSI pregnancy rates, but the effect was not strong enough to recommend routine sperm DNA damage testing.
Zini (2011)	May 2010	No	24	2976	TUNEL, SCSA	IUI, IVF and ICSI	Pregnancy rate and miscarriage	Sperm DNA damage was associated with a lower pregnancy rate in IVF, but no clear association was found in ICSI.
Zhao *et al.* (2014)	October 2013	No	16	3106	TUNEL, SCSA, Alkaline Comet	IVF and ICSI	Pregnancy rate and miscarriage	Sperm DNA Fragmentation had a detrimental effect on pregnancy rate in IVF but not in ICSI cycles.
Zhang *et al.* (2015)	Until June 2014	Yes	20	5871	TUNEL, SCSA	IVF and ICSI	Pregnancy rate and miscarriage	Pregnancy rate was affected by DNA damage only in IVF; insufficient evidence to support an

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								effect of sperm DNA damage on IVF and ICSI outcomes.
Osman *et al.* (2015)	January 2014	No	6	998	SCSA, TUNEL, Alkaline Comet	IVF and ICSI	Live birth rate	In IVF, live-birth rate was higher if DNA fragmentation was low. No significant association in ICSI.
Cissen *et al.* (2016)	January 2016	Yes	67 for SR 30 for MA	7672	TUNEL, SCSA, SCD, Alkaline Comet	IVF and ICSI	Pregnancy rate	Insufficient evidence for pregnancy rate reduction to recommend routine use of sperm DNA damage assessment in couples undergoing IVF/ICSI.
Simon *et al.* (2017)	April 2014	No	67 for SR 41 for MA	8068	SCSA, TUNEL, SCD, Alkaline Comet	IVF and ICSI	Pregnancy rate	Sperm DNA damage has a negative effect on clinical pregnancy rate following IVF and ICSI treatment.
Deng *et al.* (2019)	June 2018	Yes	29	9645	SCSA, TUNEL, SCD, Alkaline Comet	IVF and ICSI	Pregnancy rate, live birth rate, miscarriage	Sperm DNA fragmentation associated with a lower pregnancy rate in IVF; no significant results found in ICSI. No significant association with live birth rate after IVF.

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1332 SCD, sperm chromatin dispersion; SCSA, sperm chromatin structure assay; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labelling.

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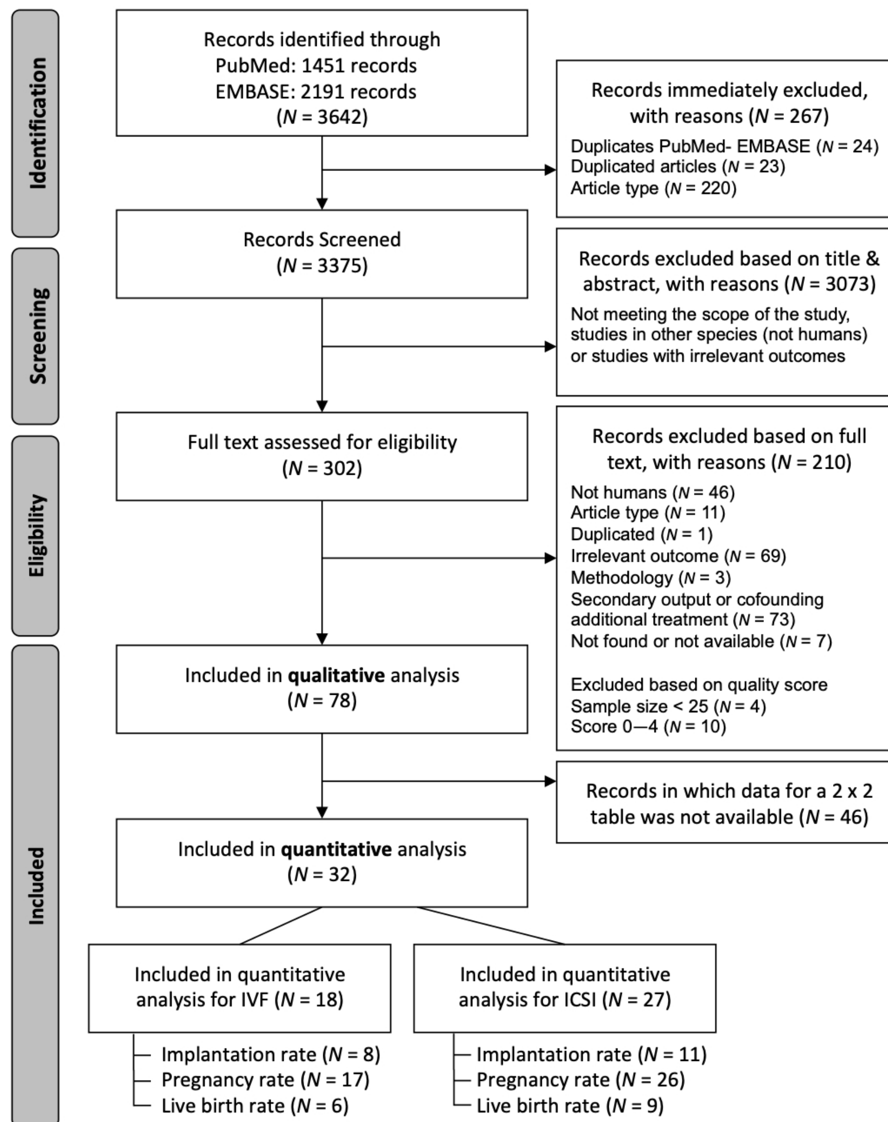


Fig. 1. Flowchart of the literature search and selection process. ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization.

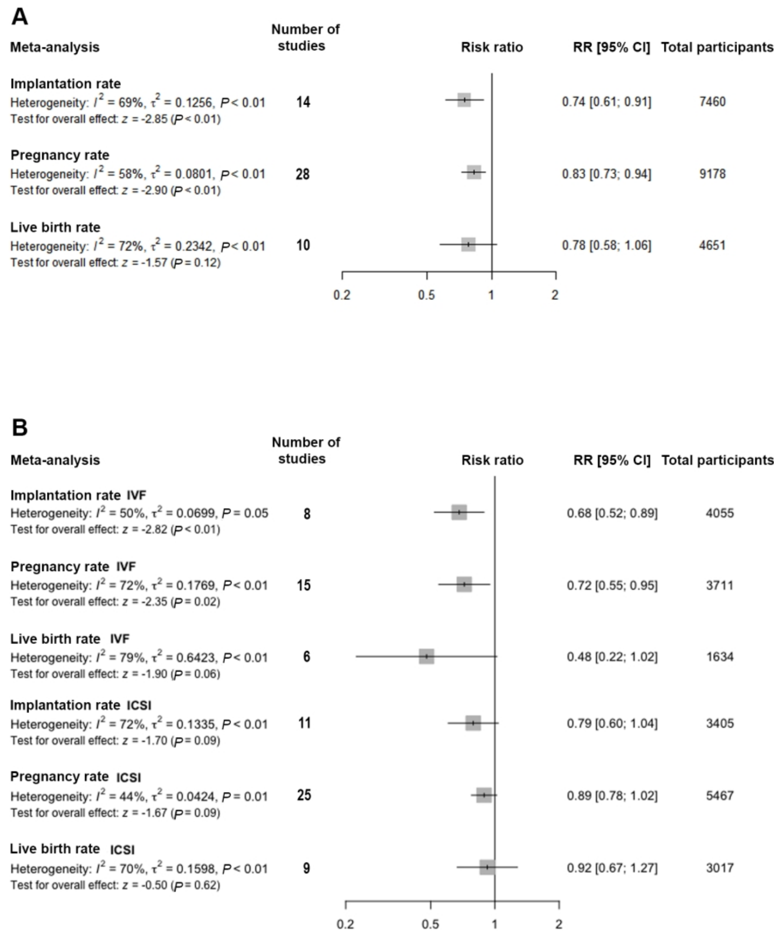
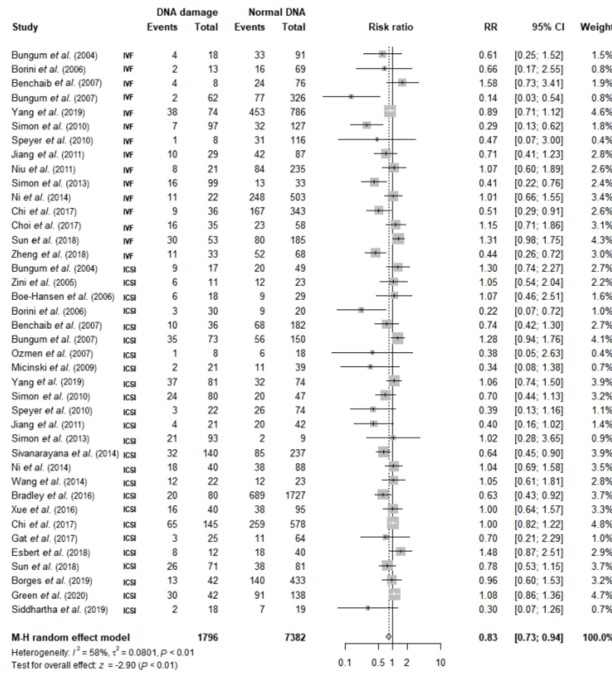
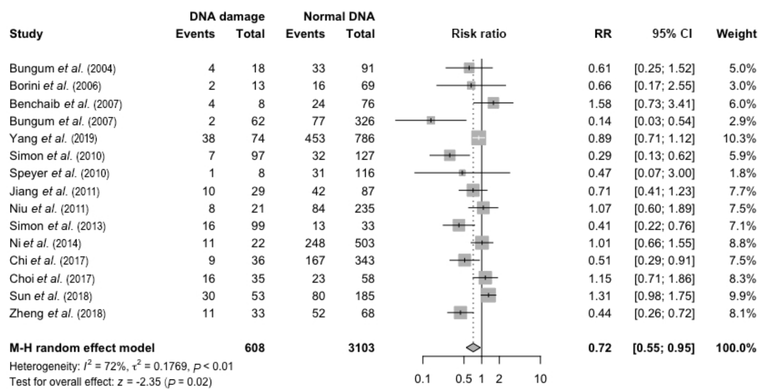


Fig. 2 . Forest plot of pooled risk ratios (RRs) for studies assessing the association between DNA damage and implantation rate, pregnancy rate and live birth rate for (A) all IVF and ICSI treatments, and (B) IVF and ICSI treatments separately. A RR value <1 indicates a greater risk of a negative outcome on the measured parameter in the DNA-damage group compared with the normal DNA group. ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization.



Pregnancy rate – all studies
DNA damage
Normal DNA
Study Events Total Events Total Risk ratio RR 95% CI Weight
Bungum et al. (2004) IVF 4 18 33 91 0.61 [0.25; 1.52] 1.5%
Borini et al. (2006) IVF 2 13 16 69 0.66 [0.17; 2.55] 0.8%
Benchaib et al. (2007) IVF 4 8 24 76 1.58 [0.73; 3.41] 1.9%
Bungum et al. (2007) IVF 2 62 77 326 0.14 [0.03; 0.54] 0.8%
Yang et al. (2019) IVF 38 74 453 786 0.89 [0.71; 1.12] 4.6%
Simon et al. (2010) IVF 7 97 32 127 0.29 [0.13; 0.62] 1.8%
Speyer et al. (2010) IVF 1 8 31 116 0.47 [0.07; 3.00] 0.4%
Jiang et al. (2011) IVF 10 29 42 87 0.71 [0.41; 1.23] 2.8%
Niu et al. (2011) IVF 8 21 84 235 1.07 [0.60; 1.89] 2.6%
Simon et al. (2013) IVF 16 99 13 33 0.41 [0.22; 0.76] 2.4%
Ni et al. (2014) IVF 11 22 248 503 1.01 [0.66; 1.55] 3.4%
Chi et al. (2017) IVF 9 36 167 343 0.51 [0.29; 0.91] 2.6%
Choi et al. (2017) IVF 16 35 23 58 1.15 [0.71; 1.86] 3.1%
Sun et al. (2018) IVF 30 53 80 185 1.31 [0.98; 1.75] 4.3%
Zheng et al. (2018) IVF 11 33 52 68 0.44 [0.26; 0.72] 3.0%
Bungum et al. (2004) ICSI 9 17 20 49 1.30 [0.74; 2.27] 2.7%
Zini et al. (2005) ICSI 6 11 12 23 1.05 [0.54; 2.04] 2.2%
Boe-Hansen et al. (2006) ICSI 6 18 9 29 1.07 [0.46; 2.51] 1.6%
Borini et al. (2006) ICSI 3 30 9 20 0.22 [0.07; 0.72] 1.0%
Benchaib et al. (2007) ICSI 10 36 68 182 0.74 [0.42; 1.30] 2.7%
Bungum et al. (2007) ICSI 35 73 56 150 1.28 [0.94; 1.76] 4.1%
Ozmen et al. (2007) ICSI 1 8 6 18 0.38 [0.05; 2.63] 0.4%
Micinski et al. (2009) ICSI 2 21 11 39 0.34 [0.08; 1.38] 0.7%
Yang et al. (2019) ICSI 37 81 32 74 1.06 [0.74; 1.50] 3.9%
Simon et al. (2010) ICSI 24 80 20 47 0.70 [0.44; 1.13] 3.2%
Speyer et al. (2010) ICSI 3 22 26 74 0.39 [0.13; 1.16] 1.1%
Jiang et al. (2011) ICSI 4 21 20 42 0.40 [0.16; 1.02] 1.4%
Simon et al. (2013) ICSI 21 93 2 9 1.02 [0.28; 3.65] 0.9%
Sivanarayana et al. (2014) ICSI 32 140 85 237 0.64 [0.45; 0.90] 3.9%
Ni et al. (2014) ICSI 18 40 38 88 1.04 [0.69; 1.58] 3.5%
Wang et al. (2014) ICSI 12 22 12 23 1.05 [0.61; 1.81] 2.8%
Bradley et al. (2016) ICSI 20 80 689 1727 0.63 [0.43; 0.92] 3.7%
Xue et al. (2016) ICSI 16 40 38 95 1.00 [0.64; 1.57] 3.3%
Chi et al. (2017) ICSI 65 145 259 578 1.00 [0.82; 1.22] 4.8%
Gat et al. (2017) ICSI 3 25 11 64 0.70 [0.21; 2.29] 1.0%
Esbert et al. (2018) ICSI 8 12 18 40 1.48 [0.87; 2.51] 2.9%
Sun et al. (2018) ICSI 26 71 38 81 0.78 [0.53; 1.15] 3.7%
Borges et al. (2019) ICSI 13 42 140 433 0.96 [0.60; 1.53] 3.2%
Green et al. (2020) ICSI 30 42 91 138 1.08 [0.86; 1.36] 4.7%
Siddhartha et al. (2019) ICSI 2 18 7 19 0.30 [0.07; 1.26] 0.7%
M-H random effect model 1796 7382 0.83 [0.73; 0.94] 100.0%
Heterogeneity: I^2 = 58%, τ^2 = 0.0801, P < 0.01
Test for overall effect: z = -2.90 (P < 0.01)
0.1 0.5 1 2 10


Pregnancy rate – IVF
DNA damage
Normal DNA
Study
Events
Total
Events
Total
Risk ratio
RR
95% CI
Weight
Bungum et al. (2004) 4 18 33 91 0.61 [0.25; 1.52] 5.0%
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Yang et al. (2019) 38 74 453 786 0.89 [0.71; 1.12] 10.3%
Simon et al. (2010) 7 97 32 127 0.29 [0.13; 0.62] 5.9%
Speyer et al. (2010) 1 8 31 116 0.47 [0.07; 3.00] 1.8%
Jiang et al. (2011) 10 29 42 87 0.71 [0.41; 1.23] 7.7%
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Sun et al. (2018) 30 53 80 185 1.31 [0.98; 1.75] 9.9%
Zheng et al. (2018) 11 33 52 68 0.44 [0.26; 0.72] 8.1%
M-H random effect model 608 3103 0.72 [0.55; 0.95] 100.0%
Heterogeneity: I^2 = 72%, τ^2 = 0.1769, p < 0.01
Test for overall effect: z = -2.35 (p = 0.02)
0.1
0.5
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