SEMMELWEIS EGYETEM

DOKTORI ISKOLA

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Urológia

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MALE INFERTILITY: THE FUNCTIONAL SIDE OF THE SPERMATOZOA

Ph.D. Thesis

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Translational Medicine Program

Surgical Medicine Division

SEMMELWEIS UNIVERSITY

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Budapest

2025

"This is the first day of the rest of your life, live it in a way that makes it worthwhile to continue."

David Safier

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1 LIST OF ABBREVIATIONS

BMI	Body mass index
CENTRAL	Cochrane Central Register of Controlled Trials
CI	Confidence interval
DFI	DNA fragmentation index
DSBss	Double-strand breaks
dUTP	Deoxyuridine triphosphate
FSH	Follicle-stimulating hormone
GRADEpro	Grading of Recommendations Assessment, Development and Evaluation
hCG	Human chorionic gonadotropin
HPV	Human papilloma virus
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilizastion
MD	Mean difference
MINORS	Methodological Index for Non-Randomized Studies
OR	Odds ratio
PICO	Population, intervention, comparison, outcome
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
QUIPS	Quality of prognostic studies
RCT	Randomized controlled trial
ROBINS-I	Risk Of Bias In Non-randomized Studies
RoB2	Risk of Bias 2
ROS	Reactive oxygen species
SCD	Sperm chromatin dispersion
SCSA	Sperm chromatin structure assay
SD	Standard deviation
SDF	Sperm DNA fragmentation
SSBs	Single-strand breaks
TUNEL	Terminal deoxynucleotidyl transferase (dUTP) nick end labeling
WHO	World Health Organization

2 STUDENT PROFILE

2.1 Vision and mission statement, specific goals

My vision is to educate patients regarding our findings on risk factors impacting fertility. My mission is a larger-scale education of the population on how fertility can be enhanced.

Number of publications:	13
Cumulative IF:	46.7
Av IF/publication:	3.6
Ranking (SCimago):	D1: 4, Q1: 7, Q2: 2
Number of publications related to the subject of the thesis:	2
Cumulative IF:	8.1
Av IF/publication:	4.1
Ranking (SCImago):	D1:1, Q1:1
Number of citations on Google Scholar:	137
Number of citations on MTMT:	108
H-index:	6

2.2 Scientometrics

The detailed bibliography of the student can be found on pages 104-106.

2.3 Future plans

As a continuation of these two research topics, we are currently planning a collaboration with the University of Veterinary Medicine Budapest to further investigate the role of antioxidants, initially in animal models and, subsequently, in human subjects.

In parallel, I am involved in several other projects related to male infertility. In collaboration with the Budapest University of Technology and Economics, we have developed an artificial intelligence-based software tool with exceptionally high predictive accuracy for forecasting sperm retrieval success in patients with non-obstructive azoospermia. Building on this work, we have established a prospective registry to collect additional clinical data. This will allow us to further refine the model's accuracy by incorporating new variables and a larger patient population.

As Hungary's largest andrology centre, we perform a significant number of surgeries, including resections of non-palpable testicular tumors. We are preparing a publication summarizing our experience and reviewing the literature, with a particular focus on advocating organ-sparing surgery as the optimal approach, given that the vast majority of these tumors are benign.

In summary, we are actively exploring multiple facets of male infertility and its treatment, with several additional projects underway that aim to improve both diagnostic accuracy and therapeutic outcomes.

3 SUMMARY OF THE THESIS

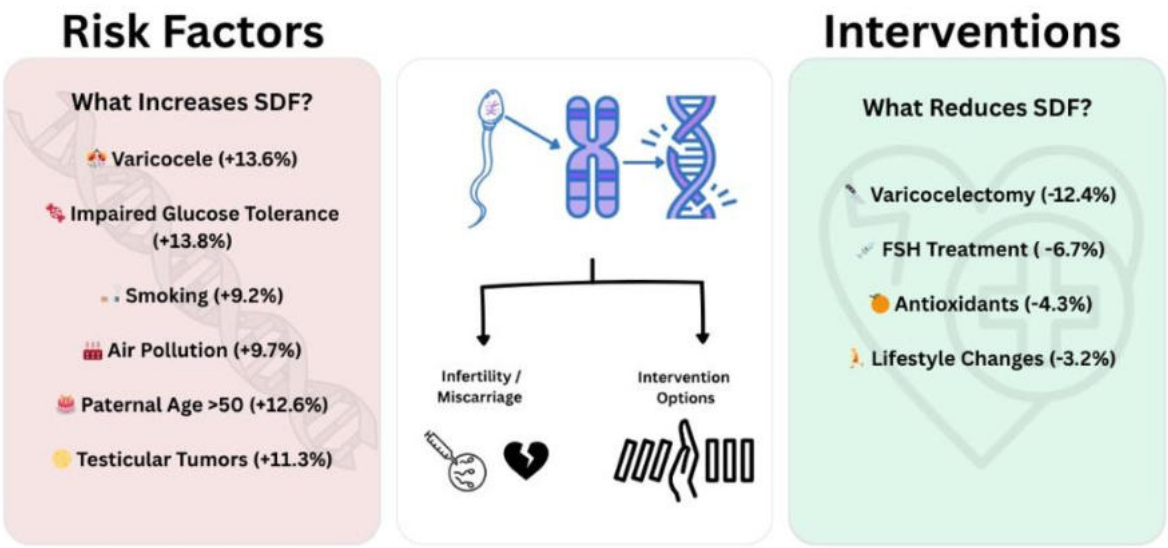
Male infertility represents a major and growing public health concern, contributing to more than half of all infertility cases worldwide. Traditional semen analysis often fails to identify the underlying cause of infertility and cannot distinguish between fertile and infertile males. Therefore, new biomarkers are needed to improve the accuracy of diagnosing the underlying causes of male infertility. Sperm DNA fragmentation (SDF) has emerged as a key biomarker reflecting sperm DNA integrity, with elevated levels associated with poorer natural conception rates, reduced success in assisted reproductive technologies, higher miscarriage risks, and an increased likelihood of foetal abnormalities. During my Ph.D. work, I aimed to better understand the causes and management of elevated SDF by conducting two comprehensive studies – one focusing on identifying risk factors, and the other on evaluating potential interventions.

In our first study, we examined over 200 potential risk factors across the literature and identified several key contributors elevating SDF. Among these, varicocele, impaired glucose tolerance, smoking, environmental pollution, and paternal age over 50 stood out as having the most significant impact. While certain non-modifiable factors such as age, cannot be altered, others – like smoking and varicocele – can be targeted. This meta-analysis helped to clarify which lifestyle, environmental, and health-related factors clinicians should prioritize when evaluating male fertility.

The second study evaluated the effectiveness of various interventions designed to lower SDF. This analysis included 86 studies and over 8,000 men. Of the interventions studied, varicocelectomy yielded the most consistent and clinically significant improvement in SDF levels, particularly in patients with grade II and III varicoceles. Follicle-stimulating hormone (FSH) treatment showed promising results, while antioxidant therapies fell short of clinical relevance. Lifestyle modifications also showed limited efficacy, in part due to study heterogeneity and inconsistent intervention protocols. These findings highlight the importance of personalized, evidence-based treatment strategies, while also underscoring the urgent need for standardization in diagnostic methods and clinical trial design.

Together, these two studies provide a robust overview of both the aetiology and management of elevated sperm DNA fragmentation. The results have immediate clinical implications for male infertility care and offer a valuable foundation for future research aimed at optimizing diagnosis of underlying causes and refining treatment strategies.

4 GRAPHICAL ABSTRACT



5 INTRODUCTION

5.1 Overview of the topic

5.1.1 What is the topic?

The topic of this thesis is the role of SDF in male infertility, currently the only evidence-based sperm functional parameter incorporated into clinical guidelines. (1) This work focuses on identifying risk factors associated with elevated SDF and evaluating interventions aimed at reducing SDF levels. The research is based on two comprehensive meta-analyses – one examining the contributing risk factors and the other assessing the effectiveness of various therapeutic strategies to lower SDF.

5.1.2 What is the problem to solve?

Infertility affects approximately 15% of couples globally, with male factors implicated in more than half of these cases. (2) Traditional semen parameters often fail to identify underlying causes. SDF has emerged as a functional biomarker with strong predictive value for fertility outcomes, yet the exact risk factors contributing to increased SDF, and the effectiveness of treatments to reduce it, remain unclear. Other limitations can also be mentioned, as there is no gold standard laboratory method for measurement, nor a universal threshold to differentiate fertile from infertile men based on SDF. Thus, better understanding both the risk factors and therapeutic options for high SDF is crucial for targeted clinical management.

5.1.3 What is the importance of the topic?

SDF is associated with decreased fertility, lower success rates in assisted reproductive techniques, increased miscarriage rates and higher foetal abnormalities. (3, 4) By identifying risk factors such as varicocele, smoking, pollution, age, and impaired glucose tolerance, and by evaluating treatments like varicocelectomy, antioxidant therapy, FSH administration, and lifestyle modifications, this research provides critical insights for personalized fertility care. Addressing SDF may improve reproductive outcomes, and guide future guideline recommendations.

5.1.4 What would be the impact of our research results?

The results of our research have the potential to significantly influence both clinical practice and future scientific work. By identifying the most relevant risk factors

contributing to elevated sperm DNA fragmentation and assessing the efficacy of various interventions aimed at reducing it, our findings offer valuable guidance for the individualized management of male infertility. Clinicians will be better equipped to make evidence-based decisions regarding which patients may benefit from specific treatments, such as varicocelectomy or FSH therapy. Additionally, our work highlights the limitations of antioxidant therapy and the need for more robust studies on lifestyle interventions. Importantly, our research also draws attention to the current lack of standardization in measuring and evaluating SDF, highlighting the necessity of establishing reliable, reproducible diagnostic protocols.

5.2 Sperm DNA fragmentation assays

Several assays have been developed to evaluate SDF, each with distinct methodologies and diagnostic characteristics. The Sperm Chromatin Structure Assay (SCSA) is a flow cytometry-based method that detects DNA denaturation using acridine orange staining and calculates the DNA fragmentation index (DFI) based on fluorescence ratios. (5) It offers high reproducibility, large detection capacity, and low mutation rates, although it assesses DNA susceptibility rather than direct strand breaks. (6-8)

The Comet assay, or single-cell gel electrophoresis, directly visualizes DNA strand breaks as a "comet tail" formed during electrophoresis under alkaline or neutral conditions. It is relatively cheap, sensitive, and adaptable, though results are dependent on operating conditions and detection thresholds. (5, 8-10)

Terminal deoxynucleotidyl transferase deoxynucleotidyl transferase (dUTP) nick end labelling (TUNEL) is another direct assay that labels DNA strand breaks with fluorescent markers and is considered highly accurate. However, it requires careful handling and has limited sensitivity under microscopy. (5, 8, 11)

The Sperm Chromatin Dispersion (SCD) test evaluates DNA integrity by visualizing halo formation around sperm nuclei under fluorescence microscopy. This method is affordable, and easy to perform, though it indirectly assesses DNA damage. (8, 12, 13)

While these assays are largely comparable in identifying elevated SDF, they vary in sensitivity, specificity, and what aspect of DNA damage they assess. (13)

6 OBJECTIVES

6.1 Study I. – Risk factors associated with sperm DNA fragmentation

Our goal was to conduct a systematic review and meta-analysis assessing the effect of all studied risk factors on SDF.

6.2 Study II. – Efficacies of interventions aiming to improve sperm DNA fragmentation

Our aim was to conduct a comprehensive meta-analysis and systematic review to summarise the effects of all interventions studied in relation to SDF.

7 METHODS

7.1 Study I.

Our systematic review and meta-analysis were conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines and the recommendations outlined in the Cochrane Handbook. (14, 15) The study protocol was prospectively registered on PROSPERO (registration number: CRD42021282533), and the research was carried out in full compliance with the registered protocol.

7.1.1 Eligibility criteria

We formulated our research question using the PICO framework. Eligible studies included all male participants, regardless of their fertility status (P), and compared the SDF values

(O) between groups with and without a particular risk factor (I and C). The examined risk factors included lifestyle, environmental, and additional health-related influences. All types of SDF assessment methods were considered, including the SCSA, TUNEL, SCD, and both neutral and alkaline Comet assays.

Studies were included if they reported either the mean difference (MD) in SDF between exposed and unexposed groups or the proportion of individuals with high SDF based on defined cut-off values. A change of approximately 10% in SDF was considered clinically meaningful; however, interpretations were made based on consensus, given the absence of established guideline thresholds.

We included both prospective and retrospective cohort studies, without imposing any language restrictions. Studies were excluded if they (1) contained inaccurate or unprocessable data, (2) were conference abstracts, or (3) were reviews, case series, or case reports.

7.1.2 Information sources and search strategy

We performed a comprehensive systematic search in Embase, MEDLINE (via PubMed), and the Cochrane Central Register of Controlled Trials (CENTRAL) on October 17, 2021. The search strategy included the terms: “sperm DNA fragmentation” OR “SDF” OR “DNA fragmentation index” OR “DFI”. No filters or additional restrictions were applied to ensure the broadest possible inclusion of relevant studies.

7.1.3 Selection process

The reference management software Endnote v9.0 (Clarivate Analytics, Philadelphia, PA, USA) was used for the selection process. Following both automatic and manual duplicate removal, four independent review authors worked in pairs to screen titles, abstracts, and full-text articles, with each pair responsible for one half of the records. Any disagreements at any stage were resolved by a third reviewer. Interrater reliability was assessed at each step using Cohen's kappa coefficient (κ). (16)

7.1.4 Data collection process and data items

Data extraction from the eligible studies was performed by two authors using a predefined data collection sheet. The following variables were extracted: first author, year of publication, study design and period, sample size and demographic characteristics, fertility status, identified risk factors and their groupings, type of SDF assay used, cut-off values for dichotomous outcomes, MD values with corresponding distributions, incidence of high SDF within risk factor groups, and any additional outcomes such as pregnancy or birth rates linked to either the risk factor or SDF levels. Information relevant for risk of bias assessment was also collected.

In cases of missing or incomplete data, the original study authors were contacted. Participants were categorized by fertility status wherever possible – into general population, fertility clinic patients, fertile individuals, or mixed groups. Studies reporting similar SDF cut-off thresholds were grouped together for consistency.

The preferred format for SDF reporting was mean with standard deviation (SD). When data were presented as medians with interquartile ranges, they were converted to mean and SD using the method described by Wan et al. (17) In studies with multiple treatment arms, intervention groups were pooled according to recommendations from the Cochrane Handbook. (15)

For variables such as sexual abstinence, we used standard reference categories recommended for semen analysis (e.g., 2–7 days, 2–5 days, or 3–5 days of abstinence). In repeated-measure studies where the SD of change from baseline was not reported, a conservative approach was taken by assuming a correlation of minus one, resulting in a calculated SD equivalent to the sum of the individual SDs.

7.1.5 Study risk of bias assessment

Risk of bias assessment was conducted independently by two authors using the Quality in Prognostic Studies (QUIPS) tool. (18) Assessment criteria were predefined for each domain. Any disagreements between reviewers were resolved by consultation with a third author.

7.1.6 Synthesis methods

All statistical analyses were conducted using R software (R Core Team 2022, version 4.2) with the *meta* (v5.5.0) and *dmetar* (v0.0.9) packages. (19) A random-effects model was applied, using the inverse variance method for weighting. For dichotomous outcomes derived from 2×2 contingency tables (presence or absence of a risk factor vs. SDF above or below the cut-off), odds ratios (OR) with 95% confidence intervals (CI) were pooled using the Mantel–Haenszel method. For continuous outcomes (e.g., mean SDF values in exposed vs. unexposed groups), MD with 95% CIs were calculated using models based on restricted maximum likelihood estimation. (20)

Forest plots were generated to visually present the results of the meta-analyses, regardless of the number of included studies. However, results from forest plots based on fewer than three studies were interpreted with caution. Where appropriate, prediction intervals – representing the expected range of effect sizes in future studies – were reported in line with the recommendations of IntHout et al. (21)

To evaluate statistical heterogeneity, Cochrane's Q test was used, with a p-value of <0.1 indicating statistical significance. The I^2 statistic was also calculated to quantify the degree of heterogeneity among studies. For analyses involving at least ten studies, publication bias was assessed using Egger's test and visualized with funnel plots.

In addition to heterogeneity testing, statistical significance was set at a p-value <0.05. Subgroup analyses were performed based on the fertility status of the study population and the specific SDF assay used.

7.2 Study II.

Our second meta-analysis and systematic review were also conducted in accordance with the PRISMA 2020 guidelines and followed the methodological recommendations outlined in the Cochrane Handbook. (15) The study protocol was prospectively registered

on PROSPERO (registration number: CRD42021283784), and the review was carried out in full compliance with the registered protocol.

7.2.1 Eligibility criteria

The clinical question was structured using the PICO (Population, Intervention, Comparison, Outcome) framework. Eligible studies included male participants of any fertility status who underwent interventions aimed at improving SDF. These individuals were compared to control groups consisting of men who received no intervention, a placebo, or who served as their own controls through pre-treatment data. The most frequently studied interventions included surgical procedures – primarily varicocelectomy – as well as lifestyle changes, hormonal treatments, and antioxidant therapies.

All methods for measuring SDF were considered acceptable, including the SCD, SCSA, Comet assay, and TUNEL assay. Studies were included if they reported SDF either as a continuous percentage or as the proportion of patients with high SDF based on specific cut-off values. However, studies using cut-off-based reporting were ultimately excluded from pooled analyses due to insufficient data for meta-analysis. A 10% change in SDF was considered clinically relevant, based on expert judgment, in the absence of a universally accepted threshold.

We included randomized controlled trials (RCTs), as well as retrospective and prospective cohort studies with either single-arm or two-arm designs. No language restrictions were applied. Studies were excluded if they were conference abstracts, case series, case reports, or reviews, or if they presented conflicting or incomplete data, or reported outcomes in a non-quantifiable format.

7.2.2 Information sources and search strategy

A comprehensive systematic search was conducted in MEDLINE (via PubMed), Embase, and the CENTRAL on October 17, 2021, and was updated on January 3, 2023. The search strategy included the terms: “sperm DNA fragmentation” OR “SDF” OR “DNA fragmentation index” OR “DFI”. No filters or additional restrictions were applied to maximize the inclusion of relevant studies.

7.2.3 Selection process

Endnote v9.0 (Clarivate Analytics, Philadelphia, PA, USA) was used to manage references and remove duplicates. The selection process was carried out independently by two pairs of reviewers, who screened records at the title-abstract level followed by full-text review. Interrater agreement was assessed using Cohen's kappa coefficient (κ). Any discrepancies were resolved by two senior reviewers at each stage of the selection process.

7.2.4 Data collection process and data items

Two authors independently extracted data from the eligible full-text articles using a predefined data collection form. The extracted information included: first author, publication year, study period and study design, number of participants and demographic data, fertility status, type of intervention, type of control used, type of SDF assay, cut-off values for dichotomous outcomes, MD with their respective distributions for continuous data, and information to assess risk of bias or grade, if applicable.

When essential data were missing or incomplete, the original study authors were contacted. Participants were categorized based on fertility status – fertile, infertile, or general population of unknown fertility status – based on the classification used in the original studies.

Studies reporting only cut-off values, without MDs, were excluded from the meta-analysis. The preferred format for reporting SDF was mean with SD. When data were provided as mean and SE, SE was converted to SD. For studies reporting medians with ranges or interquartile ranges, means and SDs were estimated using the method described by Wan et al. (17, 22)

When original studies included multiple intervention groups or grouped participants by criteria other than fertility status, data were merged based on the recommendations of the Cochrane Handbook. (14, 15) Importantly, SDF measurements obtained using different assay types were analysed separately.

7.2.5 Study risk of bias and grade assessment

Two review authors independently assessed the risk of bias using tools appropriate to the study design: the Methodological Index for Non-Randomized Studies (MINORS) for single-arm studies, the Risk Of Bias In Non-randomised Studies – of Interventions

(ROBINS-I) for two-arm studies, and the Risk of Bias 2 (RoB2) tool for RCTs. For RCTs, the quality of evidence was further evaluated using the Grading of Recommendations Assessment, Development and Evaluation (GRADEpro) framework. Assessment criteria were predefined for each tool. Any discrepancies between reviewers were resolved by a third author.

7.2.6 Synthesis methods

Statistical analyses were conducted using the statistical software R (version 4.1.2), following the methodological guidance outlined by Harrer et al. (20, 23) The meta-analysis focused on comparing pre- and post-treatment mean SDF values within the intervention groups. In several studies, control group data from either fertile or infertile populations were also available. These control measurements varied in timing – some were recorded concurrently with the pre-intervention values, others at later time points, and some at multiple time points. Since these control groups did not undergo any intervention, only random variation was expected; therefore, a single available mean SDF value from the control group was used in each study, regardless of time point.

We meta-analysed the mean difference between intervention and control groups using the classical inverse variance random-effects model, applying the restricted maximum likelihood estimator along with the Hartung-Knapp adjustment. Prediction intervals were reported where applicable. To assess heterogeneity, the I^2 statistic and its confidence interval were calculated, along with the Cochrane Q test. To further explore sources of heterogeneity, a leave-one-out sensitivity analysis was performed, as recommended by Harrer et al. (20)

In most cases, SDs for pre- and post-intervention outcomes were available or could be estimated; however, the SD of the change between time points was typically missing. In line with Cochrane Handbook recommendations, we tested multiple correlation values to estimate this. All tested correlations yielded consistent results, and the published findings were based on an input correlation of 0.6. It should be noted that while pooled outcomes and their confidence intervals remained stable, the confidence intervals of individual study results varied depending on the assumed correlation.

For key outcomes involving at least ten studies, potential publication bias was evaluated using funnel plots and Egger's test to identify small-study effects.

8 RESULTS

8.1 Study I: Meta-analysis

8.1.1 Search and selection

Our systematic search yielded a total of 26,901 records. After screening, 190 studies were included in the meta-analysis or systematic reviews (Figure 1).

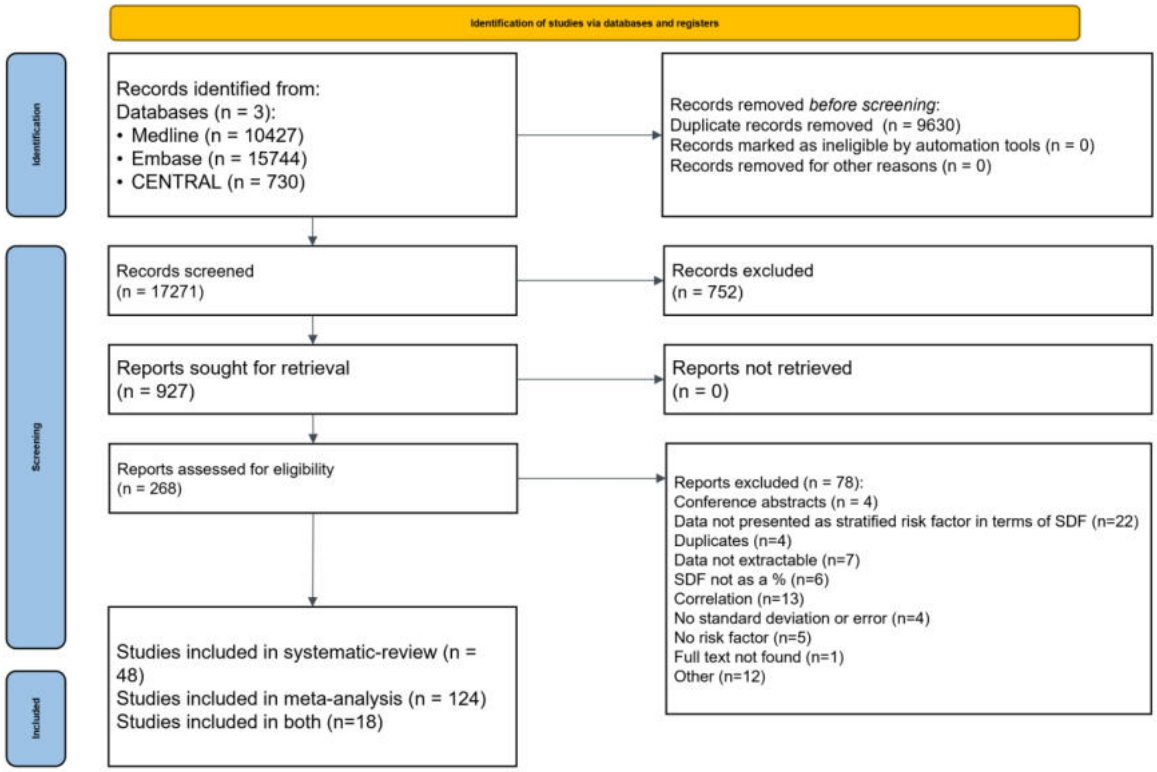


Figure 1. PRISMA 2020 flowchart showing the study selection process of the article on the risk factors of SDF

8.1.2 Basic characteristics of included studies and summary of results

Detailed baseline characteristics are presented in Table 1. The studies spanned from 2003 to 2021, with Europe being the most common study setting, followed by North America and Asia. A smaller number of studies originated from Africa, and the fewest from Australia. Most of the included studies were retrospective and primarily involved men in their 30s attending fertility clinics. Varicocele was the most frequently analysed risk factor, and the most commonly used SDF measurement techniques were SCSA, SCD, and TUNEL assays.

Table 1. Basic characteristics of the included articles in the study on risk factors of sperm DNA fragmentation

Author (year)	Study site	Study type	Population	Number of analysed patients	Age (year) ‡	Risk factors	DFI measurement
Abdelbaki (2017) (24)	Egypt	p	fertility clinic + fertile controls	80	31.5 (23-49)	varicocele	SCSA
Abdullah (2019) (25)	USA	r	varicocele	141	34.2 ± 6.2	testicular atrophy	SCD
Agarwal (2016) (26)	USA	p	fertile	7	ND (20-45)	abstinence	TUNEL
Agbaje (2008) (27)	UK	p	diabetes type 1 patients + fertile controls	19	32.6 ±1.5	diabetes type 1	alkaline Comet
Alargkof (2019) (28)	Bulgaria	r	fertility clinic + general population controls	28	33 (26.25-42)	varicocele	SCD
Albani (2019) (29)	Italy	r	fertility clinic	89	37.9 ± 3.5	age	SCSA
Alhathal (2016) (30)	Canada	p	fertility clinic + general population controls	35	ND	varicocele	SCSA

Alshahrani (2014) (31)	USA	r	fertility clinic	472	ND	age	TUNEL
Ammar (2021) (32)	Tunisia	p	fertility clinic + fertile controls	80	ND	varicocele	TUNEL
Amor (2019) (33)	Germany	r	fertility clinic	141	ND	smoking	TUNEL
Andersen (2016) (34)	Norway	r	fertility clinic + general population controls	112	36.5 (22-61)	body mass index	SCSA
Anifandis (2014) (35)	Germany	p	fertility clinic	207	37.43 ± 4.3	smoking, alcohol	SCD
Ayad (2018) (36)	South Africa	r	fertile	20	ND	abstinence	TUNEL
Bandel (2015) (37)	Sweden, Greenland, Ukraine, Poland, Norway	r	general population (mainly fertile)	1503	27.9 ± 10.9	body mass index	SCSA
Banks (2021) (38)	USA	p	fertility clinic	135	35 (32-40)	vitamin-D	SCSA

Belloc (2009) (39)	France	r	fertility clinic	1111	ND	age	TUNEL
Berg (2021) (40)	Germany	p	patients with chronic prostatitis + general population controls	63	35 (20-62)	prostatitis, age	SCSA
Bergamo (2016) (41)	Italy	r	general population	40	28 ± 6	pollution	SCD
Bian (2004) (42)	China	r	Fenvalerate (pesticide) exposed workers, office workers of factory, unexposed controls (general population)	63	30.2 ± 8	fenvalerate (pesticide)	alkaline Comet, TUNEL
Boeri (2020) (43)	Italy	r	fertility clinic	1547	37 (18-60)	age, body mass index	SCSA
Boeri (2019) (44)	Italy	r	fertility clinic	189	38.1 ± 5.6	smoking, alcohol	SCSA
Boeri (2019) (45)	Italy	r	fertility clinic	744	38 (19-50)	pre-diabetes	SCSA
Bojar (2013) (46)	Poland	r	fertility clinic	185	ND	age, smoking	SCSA

Borges (2019) (47)	Brazil	p	fertility clinic	463	38.28 ± 5.74	abstinence	SCD
Bosco (2018) (48)	Italy	r	general population	175	36.5 ± 4.8	pollution	SCD
Bozhedomov (2021) (49)	Russia	p	fertility clinic	1502	32.5 ± 5.6	varicocele	SCD
Brackett (2008) (50)	Florida	r	spinal cord injury (SCI) & non-SCI controls	22	33.3 ± 9.8	abstinence, spinal cord injury	SCSA
Brahem (2011) (51)	Tunisia	p	fertility clinic	140	ND (24-76)	age	TUNEL
Chavarro (2010) (52)	USA	r	fertility clinic	483	36.3 ± 5.4	body mass index	neutral Comet
Chigrinets (2019) (53)	Russia	r	fertility clinic	34	30.8 ± 3.9	smoking, alcohol	SCD
Comar (2017) (54)	Brazil	r	fertility clinic	2458	38 ± 6.7	abstinence	TUNEL
Cortés-Gutiérrez (2017) (55)	Mexico	r	human papilloma virus infected + fertile controls + fertility clinic controls	38	29 (19-32)	human papilloma virus	SCD

Cui (2016) (56)	China	r	fertility clinic	1128	ND	smoking	acridine orange staining
Dahan (2020) (57)	Canada	p	fertility clinic	112	41.1 ± 6.3	abstinence	SCD
Darbandi (2019) (58)	Iran	p	fertility clinic	151	34.5 ± 2	reactive oxygen species	SCD
Darbandi (2019) (59)	Iran	r	fertility clinic	70	ND	age	SCD
Das (2013) (60)	Canada	r	fertile	148	ND (20-57)	age	SCSA
De Jonge (2004) (61)	Belgium	p	fertility clinic	11	30 ± 2.9	abstinence	SCSA
De Win (2021) (62)	Belgium	p	general population	89	21.7 ± ND	varicocele	TUNEL
Dehghan Marvast (2018) (63)	Iran	r	fertility clinic	80	33 (22-49)	Chlamydia trachomatis	aniline blue, chromomycin A3, TUNEL, acridine orange
Depuydt (2021) (64)	Belgium	p	fertility clinic	180	34.9 ± ND	human papilloma virus	SCSA

Dieamant (2017) (65)	Brazil	r	fertility clinic	2399	37.8 ± 6.5	varicocele	TUNEL
Domes (2012) (66)	Canada	r	fertility clinic	1806	37.7 ± 6.1	bacteriospermia, elevated seminal leukocytes	"the DNA fragmentation assay"
Dupont (2013) (67)	France	r	fertility clinic	331	37.6 ± 6.2	body mass index	TUNEL
Eini (2021) (68)	Iran	r	fertility clinic + fertile controls	207	35 ± 20	bacteriospermia	SCD
Eisenberg (2014) (69)	USA	p	general population	459	31.8 ± 4.8	body mass index, waist circumference, sports	SCSA
Elbardisi (2021) (70)	Qatar	r	fertility clinic	269	34 (ND)	age	SCD
Elbardisi (2020) (71)	Qatar	r	fertility clinic	1068	35.98 ± 7.78	oxidation-reduction potential	SCD
Elbardisi (2018) (72)	Qatar	r	fertility clinic	1050	36 ± 0.1	geography, age	SCD
Elshal (2009) (73)	Egypt	r	general population	86	ND	smoking	SCSA

Esfaahani (2010) (74)	Iran	r	varicocele patients + fertile controls	122	ND	varicocele	SCD
Esteves (2015) (75)	Brazil	r	fertility clinic +fertile controls	289	ND	varicocele, testicular cc, Chlamydia	SCD
Evenson (2020) (76)	North America, Europe	r	fertility clinic, general population	25445	ND (21-80)	age	SCSA
Falahieh (2021) (77)	Iran	p	fertile	20	ND (20-50)	COVID	SCD
Fernandez-Encinas (2020) (78)	Spain	r	fertility clinic + fertile donors	24	ND	varicocele	alkaline Comet, neutral Comet, SCD
Finelli (2021) (79)	Italy	r	varicocele patients + fertile controls	169	30.6 ± 8	varicocele	TUNEL
Frainais (2010) (80)	France	r	fertility clinic	40	40 ± 5.85	human immunodeficiency virus	TUNEL
Gallegos (2008) (81)	Mexico	p	fertility clinic + fertile controls	193	ND (25-51)	Chlamydia trachomatis +	SCD

						Mycoplasma urealyticum	
Gao (2020) (82)	China	r	fertility clinic	102	32 (20-49)	Location of semen collection	TUNEL
Gao (2021) (83)	China	r	fertility clinic	18441	34 ± 5.66	age	SCSA
García-Ferreyra (2015) (84)	Peru	r	fertility clinic	32	ND (34-72)	age	SCD
García-Peiró (2011) (85)	Spain	r	fertility clinic + fertile controls	19	ND	varicocele	SCD
García-Peiró (2014) (86)	Spain	r	fertility clinic + fertile controls	52	ND (25-35)	varicocele	TUNEL, SCD, SCSA
Gautam (2015) (87)	India	r	general population	26	29 ± 4.9	smoking, alcohol	SCSA
Ghandehari-Alavijeh (2019) (88)	Iran	r	fertility clinic + fertile controls	40	ND	varicocele	TUNEL

Ghazavi-Khorasgani (2017) (89)	Iran	r	fertility clinic + fertile controls	55	ND	varicocele	SCSA
Gill (2019) (90)	Poland	r	general population	254	32.6 ± 5.8	low activity at work	SCD
Gill (2020) (91)	Poland	r	fertility clinic	675	32 (19-54)	age	SCD
Gill (2021) (92)	Poland	r	fertility clinic + fertile controls + healthy controls	335	ND	varicocele	SCD
Giwercman (2007) (93)	Greenland, Sweden, Poland, Ukraine	r	mainly fertile (general population)	680	34 ± 10	geography	SCSA
Gosálvez (2011) (94)	Spain	p	fertile	21	ND (25-35)	abstinence	SCD
Grosen (2021) (95)	Denmark	p	Methotrexate-treated + general population	54	ND (18-45)	Methotrexate, inflammatory bowel disease/rheumatoid arthritis/psoriatic arthritis	SCSA

Grosen (2019) (96)	Denmark	p	inflammatory bowel disease patients	56	29 (18-50)	inflammatory bowel disease	SCSA, neutral Comet
Grosen (2019) (97)	Denmark	r	inflammatory bowel disease patients + general population	55	28 (18-46)	inflammatory bowel disease, Vendolizumab	SCSA, neutral Comet
Grosen (2019) (98)	Denmark	p	inflammatory bowel disease patients + general population	80	25 (18-45)	inflammatory bowel disease, Thiopurines	SCSA, neutral Comet
Guo (2020) (99)	China	r	fertility clinic (abnormal sperm parameters), fertility clinic (normozoospermia)	429	ND	age	SCD
Håkonsen (2012) (100)	Denmark	r	general population	337	ND (18-21)	body mass index, smoking, abstinence	SCSA
Hammadeh (2010) (101)	Germany	r	fertility clinic	116	37.9 ± 5.7	smoking	TUNEL
Hammiche (2011) (102)	Netherlands	p	fertility clinic	227	36.9 (25.8-59.1)	geography	SCSA
Hansen (2012) (103)	Denmark	r	general population	345	ND (18-21)	alcohol in last 5 days	SCSA

Henkel (2003) (104)	Germany	r	fertility clinic	44	ND	reactive oxygen species	TUNEL
Homa (2019) (105)	UK	r	fertility clinic	238	38.3 ± ND	seminal oxidative stress	SCSA
Horta (2011) (106)	Chile	r	general population	62	35 ± ND	age	TUNEL, SCD
Huang (2011) (107)	Taiwan	r	general population (workers of PVC pellet manufacturing plant)	45	35.2 ± 9.2	di(2-ethylhexyl) phthalate (DEHP) exposure	SCSA
Humaidan (2021) (108)	Denmark	p	fertility clinic + general population controls	86	35 (24-55)	seminal oxidative stress	SCSA
Iommiello (2015) (109)	Italy	r	fertility clinic	56	ND	ejaculate oxidative stress	SCSA
Janghorban-Laricheh (2016) (110)	Iran	r	fertility clinic + fertile	55	ND	varicocele	SCSA
Jeng (2015) (111)	USA	r	general population (coke oven workers + administrators/security)	177	40 ± 10	Polycylic aromatic hydrocarbons	TUNEL, SCSA

Jeremias (2021) (112)	Brazil	r	fertility clinic	94	34 ± 5.5	varicocele	alkaline Comet, neutral Comet, FPG-associated alkaline assay
Ji (2011) (113)	China	r	fertility clinic	240	28.5 ± 3.6	3-phenoxybenzoic acid (3-PBA)	TUNEL
Ji (2013) (114)	China	r	fertility clinic	433	28.4 ± 3.3	Polycyclic aromatic hydrocarbons	TUNEL
Jurewicz (2018) (115)	Poland	r	fertility clinic	336	32 ± ND	diet	SCSA
Kabukçu (2021) (116)	Turkey	p	fertility clinic	106	33 ± 4.8	abstinence	TUNEL
Karimi (2012) (117)	Iran	r	diabetes patients + fertile (non-diabetic)	67	33 ± 7	diabetes	TUNEL
Kaspersen (2013) (118)	Denmark	r	general population	76	24 ± ND	Human herpes virus or human papilloma virus	SCSA
Kavoussi (2021) (119)	USA	r	patients with varicocele	141	34.2 ± 6.2	testicular atrophy	SCD

Kiwitt-Cárdenas (2021) (120)	Spain	r	general population (healthy university students)	158	20.5 (18-23)	bisphenol A	SCD
Krüger (2008) (121)	Denmark	r	fertile	300	ND	geography	SCSA
Kumar (2013) (122)	India	r	general population (hospital workers: occupationally exposed to ionizing radiation and not)	134	ND (21-50)	ionizing radiation	TUNEL, alkaline Comet, SCSA
Kumar (2015) (123)	India	r	fertile	130	ND	smoking	SCSA
La Vignera (2012) (124)	Italy	r	fertility clinic + fertile controls	60	26.5 ± 3.2	varicocele	TUNEL
Laqqan (2021) (125)	Palestine	r	general population	188	34.9 ± 5.8	smoking	TUNEL
Lara-Cerrillo (2020) (126)	Spain	r	fertility clinic + fertile	32	ND (17-44)	varicocele	alkaline Comet, neutral Comet
Le (2020) (127)	Vietnam	r	fertility clinic	290	35.26 ± 5.87	body mass index, metabolic syndrome	SCD

Le (2021) (128)	Vietnam	r	fertility clinic	534	34.7 ± 6.3	metabolic syndrome	SCD
Lenters (2015) (129)	Greenland, Poland, Ukraine	r	fertile	602	30 ± ND	geography	TUNEL, SCSA
Li (2012) (130)	Japan	r	fertility clinic + fertile controls	38	33.1 ± ND	varicocele	SCSA
Liu (2021) (131)	China	r	fertility clinic	253	31 ± 2.3	Chlamydia trachomatis + Ureaplasma urealyticum	SCD
Liu (2021) (132)	China	r	fertility clinic	108	33.6 ± 10.1	oxidative stress	SCD
Long (2007) (133)	Greenland, Poland, Ukraine, Sweden (Denmark)	r	fertile	262	ND (18-68)	geography	TUNEL
Lu (2018) (134)	China	p	fertility clinic	1010	28.89 ± ND	body mass index, waist circumference,	SCSA

						waist-to-hip ratio, waist-to-height	
Lu (2020) (135)	China	r	fertility clinic, fertility clinic (normozoospermic)	1790	ND (21-58)	age	SCSA
Lu (2017) (136)	China	r	diabetic patients + general population	60	ND (21-49)	diabetes	acridine orange
Ma (2017) (137)	China	r	fertility clinic + fertile	49	ND (24-42)	Ureaplasma urealyticum	TUNEL
Mahfouz (2010) (138)	USA	p	fertility clinic	101	37 ± 7.5	seminal reactive oxygen species	TUNEL
Mahran (2019) (139)	Egypt	r	fertility clinic + fertile	110	28.5 ± 5.5	varicocele	acridine orange
Malm (2017) (140)	Sweden	r	general population	198	28,5 ± ND	geography, season (melatonin)	SCSA
Manna (2020) (141)	Italy	p	fertile, fertility clinic	30, 35	37 ± 3.3	abstinence	SCD
Marchlewska (2016) (142)	Poland	r	fertility clinic + patients with germ cell testicular tumor	335	34 (24-58)	testicular cancer	SCD

Martínez (2021) (143)	Argentina	r	fertility clinic	163	ND (28-55)	age	TUNEL
Mayorgaa-Torres (2015) (144)	Colombia	r	general population	6	26.7 ± 4.8	abstinence	SCSA
Mayorgaa-Torres (2016) (145)	Colombia	r	general population	3	32 ± 3.6	abstinence	SCSA
McDowell (2013) (146)	Australia	r	cancer pts + fertile controls	124	31.6 ± ND	tumors	SCSA
Meseguer (2008) (147)	Spain	r	cancer patients + sperm donors + fertile + infertile patients	291	ND	tumors	SCD
Mohammed (2015) (148)	Egypt	p	fertility clinic + fertile controls	115	30.7 ± ND	varicocele	acridine orange, SCSA
Moskovtsev (2009) (149)	Canada	r	fertility clinic	84	ND	varicocele, bacteriospermia	acridine orange
Moskovtsev (2009) (150)	Canada	r	fertility clinic	2586	ND	age	SCSA

Moustafa (2004) (151)	USA	r	fertility clinic + general population	27	ND	reactive oxygen species	SCSA
Nazmara (2021) (152)	Iran	r	general population (but half are heroin users)	48	34.8 ± 1.5	heroin	SCSA
Nguyen (2019) (153)	Vietnam	p	fertility clinic + fertile controls	358	ND	varicocele	SCD
Ni (2016) (154)	China	r	fertility clinic + fertile controls	113	29 ± 3.2	varicocele	SCSA
Nijs (2011) (155)	Belgium	p	fertility clinic	278	35.1 ± 4.9	age	SCSA
Oliveira (2014) (156)	Brazil	r	fertility clinic	1500	37.7 ± 6.7	age	TUNEL
Oliveira (2018) (157)	Brazil	r	fertility clinic	1824	37.9 ± 6.6	body mass index	TUNEL
Osadchuk (2014) (158)	Russia	r	general population	44	30.5 ± 0.9	varicocele, prostatitis	SCSA
Pant (2014) (159)	India	r	general population	278	28.5 ± 4.2	lindane, p-p′-DDE	SCSA
Pearce (2019) (160)	Australia	r	fertility clinic	29	36.9 ± 5.2	body mass index	SCD

Pelliccione (2011) (161)	Italy	p	sportsmen	7	33 ± 9	body mass index, waist circumference, % body fat	TUNEL
Petersen (2018) (162)	Brazil	p	fertility clinic	2178	37.9 ± 6.4	age	TUNEL
Pons (2013) (163)	Spain	p	fertility clinic (DFI>=30)	35	38 ± 5.5	abstinence	SCD
Rago (2013) (164)	Italy	r	fertile	63	28.5 ± 5	cell phone use	TUNEL
Ranganathan (2019) (165)	India	p	fertile, fertility clinic	170, 170	30 ± 3.5	smoking	diphenylamine method (colorimetric method, aniline blue)
Ribeiro (2008) (166)	Brazil	p	fertility clinic + fertile controls	98	27 ± 5.2	tumors	TUNEL
Romerius (2010) (167)	Sweden, Norway	r	general population, childhood cancer survivors given radiotherapy	292, 12	29 (19-46)	childhood cancer and treatment	SCSA

Rosiak-Gill (2019) (168)	Poland	r	fertility clinic + general population, fertile	336, 160	33 (ND)	age	TUNEL
Rubes (2010) (169)	Czech Republic	r	general population	47	33.6 ± 5.3	smoking, pollution	SCSA
Rubes (2021) (170)	Czech Republic	r	general population	150	37.8 ± 9.1	age	SCSA
Safarinejad (2008) (171)	Iran	r	fertile	118	34.5 ± ND	selective serotonin reuptake inhibitors	SCSA
Safarinejad (2010) (172)	Iran	r	mustard gas injured and not, fertile and infertile	268	47.2 ± ND	mustard gas	SCSA
Said (2009) (173)	Canada	r	general population + fertile controls	109	31 ± ND	tumors	SCSA
Saleh (2003) (174)	USA	p	fertility clinic + fertile controls	47	32 (ND)	varicocele	SCSA
Savasi (2018) (175)	Italy	p	HIV patients	77	41 (25-54)	highly active antiretroviral therapy, age	SCD
Sepaniak (2006) (176)	France	p	fertility clinic	108	30.5 ± 6.8	smoking	TUNEL

Smit (2010) (177)	Netherlands	p	general population (cancer patients) + fertile controls	149	ND	tumors and treatment	SCSA
Smit (2010) (178)	Netherlands	p	vasectomy reversal patients + fertile controls	92	41.6 (ND)	vasectomy reversal	SCSA
Smith (2007) (179)	Chile	r	orchidopexy patients + idiopathic oligozoospermic controls + normozoospermic healthy controls	63	ND	orchidopexy	SCSA, TUNEL
Smith (2006) (180)	Chile	r	fertility clinic + fertile controls	80	28.2 ± 4.5	varicocele	SCSA, TUNEL
Spanò (2005) (181)	Italy	r	general population (mainly fertile)	707	31 (18-67.5)	pollution	SCSA
Specht (2012) (182)	Denmark	r	fertile	548	ND (18-51)	pollution	SCSA, TUNEL
Ståhl (2009) (183)	Sweden	r	general population (cancer patients) + fertile controls	258	29 (16-56)	tumors and treatment	SCSA
Ståhl (2004) (184)	Sweden	p	testicular germ cell tumor patients + general population controls	362	29 (ND)	tumor treatment	SCSA

Ståhl (2006) (185)	Sweden	r	testicular germ cell tumor patients + general population controls	355	30 (range=49)	tumor treatment	SCSA, TUNEL
Stronati (2006) (186)	Italy	r	mainly infertile	652	ND	pollution	TUNEL
Taha (2012) (187)	Egypt	r	fertile	160	ND	smoking	SCSA
Taha (2014) (188)	Egypt	r	fertility clinic	246	35 ± 4	smoking, varicocele	SCSA
Taha (2019) (189)	Egypt	r	fertile, hepatitis B surface antigen seropositive fertile men	103	ND	hepatitis B virus	SCD
Taha (2016) (190)	Egypt	r	fertile	165	36.5 ± 4.9	body mass index	SCSA
Tahamtan (2019) (191)	Iran	r	fertility clinic	38	ND	varicocele	TUNEL
Talebi (2008) (192)	Iran	p	fertility clinic + fertile controls	60	ND	varicocele	anilin blue, chromomycin A3, acridine

							orange, toluidine blue
Tanaka (2020) (193)	Japan	p	fertility clinic + fertile controls	240	ND	varicocele	SCSA
Tangal (2019) (194)	Turkey	p	fertility clinic	117	36 ± 5.4	human papilloma virus	TUNEL
Tartibian (2012) (195)	Iran	p	general population (athletes and recreationally active)	108	ND (18-28)	sports	TUNEL
Vagnini (2007) (196)	Brazil	r	fertility clinic	508	37.7 ± 6.2	age	TUNEL
van Brakel (2017) (197)	Netherlands	r	fertility clinic (follow-up on fertility) + fertile controls	121	ND	undescended testes	SCSA
Vargas-Baquero (2020) (198)	Spain	r	general population (spinal cord injury patients) + fertile controls	37	32.2 ± 9	spinal cord injury	SCD
Vaughan (2020) (199)	Spain	r	fertility clinic	16 945	37.6 ± 6.8	age	SCSA
Vellani (2013) (200)	Italy	r	fertility clinic	179	38.91 ± 4.54	anxiety from in vitro fertilization	TUNEL

Vinnakota (2019) (201)	New Zealand	r	fertility clinic + fertile controls	1219	41 ± 4	age	SCSA
Vivas-Acevedo (2014) (202)	Venezuela	p	fertility clinic + fertile controls	90	ND	varicocele	SCD
Vujkovic (2009) (203)	Netherlands	p	fertility clinic	161 v 252	36 (28.5-53.9)	diet	SCSA
Wang (2018) (204)	China	r	general population	707	20 (ND)	sleep	SCSA
Wang (2012) (205)	China	r	fertility clinic + fertile controls	76	30.2 ± 5.6	varicocele	SCD
Wijesekara (2020) (206)	Sri Lanka	r	fertility clinic	40	34.8 ± 5.34	lead exposure	SCD
Winkle (2008) (207)	Germany	r	fertility clinic, fertile	320, 84	36.62 ± ND	age	SCSA
Wyrobek (2006) (208)	USA	r	general population	88	41.5 (20-80)	age	SCSA
Yang (2016) (209)	China	r	fertility clinic	104	34.4 ± 4	body mass index	SCD

Zequiraj (2019) (210)	Kosovo	r	general population	169	ND	age	SCD
Zeyad (2018) (211)	Germany	r	fertility clinic	120	ND	bacteriospermia	TUNEL
Zhang (2021) (212)	China	r	fertility clinic	5114	32 (ND)	age	SCSA
Zhu (2021) (213)	China	r	fertility clinic	54	ND (22-40)	body mass index	SCSA

‡ parameters represented as mean with standard deviation, or median with range (minimum and maximum)

SCSA: sperm chromatin structure assay, TUNEL: terminal deoxynucleotidyl transferase (dUTP) nick end labeling, SCD: sperm chromatin dispersion test

ND: not defined

8.1.3 Risk factors – associated health conditions

Figure 2 displays pooled SDF values linked to specific health conditions. Varicocele, regardless of the assay used, was associated with an average increase of more than 10% in SDF (MD = 13.62%, CI: 9.39–17.84). When grouped by palpability, non-palpable and palpable varicocele had a lower but still clinically relevant MD of 7.95% (CI: 3.93–11.97), reaching a maximum of 11.32% (CI: 3.47–19.17) when measured with SCSA. Impaired glucose tolerance also showed a marked increase in SDF (MD = 13.75%, CI: 6.99–20.51). Among malignancies, testicular cancer demonstrated a clinically and statistically significant elevation in SDF, with a maximum MD of 11.3% (CI: 7.84–14.76) using SCD. Hodgkin's lymphoma had a statistically significant but smaller effect (MD = 3.65%, CI: 0.71–6.58), while other lymphomas and leukemias showed less clear or non-significant associations.

Regarding infections, *Chlamydia* and human papilloma virus (HPV) showed no statistically or clinically meaningful differences in SDF. Viral infections overall had a negligible effect (MD = 2.36%, CI: -0.82–5.54), while bacterial infections and sexually transmitted infections (STIs) yielded mixed results (MD = 8.98%, CI: 2.45–15.52 and MD = 5.54%, CI: -0.18–11.26, respectively).

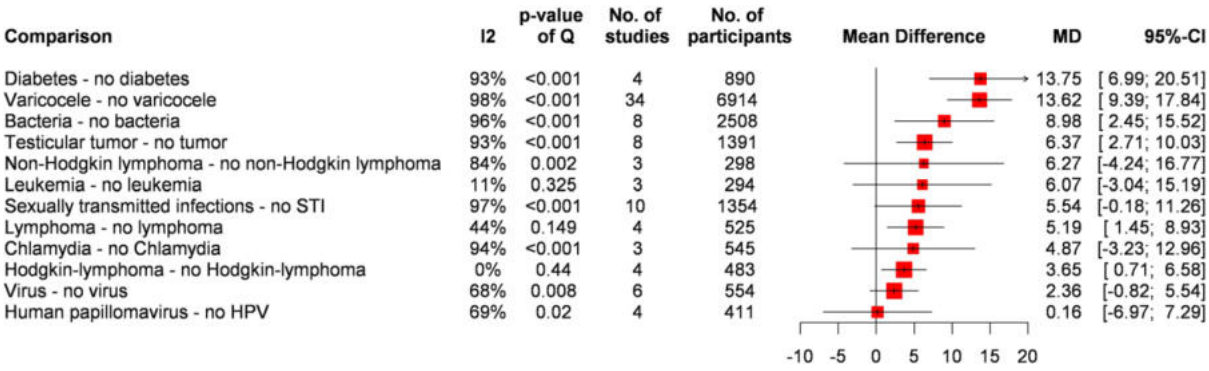


Figure 2. Summary forest plot of associated health conditions' effects on SDF

8.1.4 Risk factors – lifestyle factors

Figure 3 summarizes the findings for lifestyle-related factors. Smoking was associated with a significant increase in SDF (MD = 9.19%, CI: 4.33–14.06), with a clear dose-dependency pattern: light smokers had a modest increase (MD = 2.93%, CI: -1.30–7.15), while heavy smokers exhibited a more pronounced effect (MD = 9.60%, CI: 3.80–15.40).

Alcohol consumption was linked to a slight, non-clinically relevant elevation in SDF (MD = 1.88%, CI: -1.93–5.69), with no meaningful differences when moderate (MD = 0.86%, CI: -2.43–4.15) or heavy drinkers (MD = 2.92%, CI: -2.51–8.34) were compared to abstainers.

Body mass index (BMI) had little effect on SDF, with overweight/obese men showing a negligible increase (MD = 0.88%, CI: -1.73–3.49), and underweight individuals demonstrating a minor, non-significant decrease (MD = -1.54%, CI: -3.08–0.01).

Sexual abstinence durations outside the recommended window did not appear to significantly improve SDF compared to standard days of recommendations

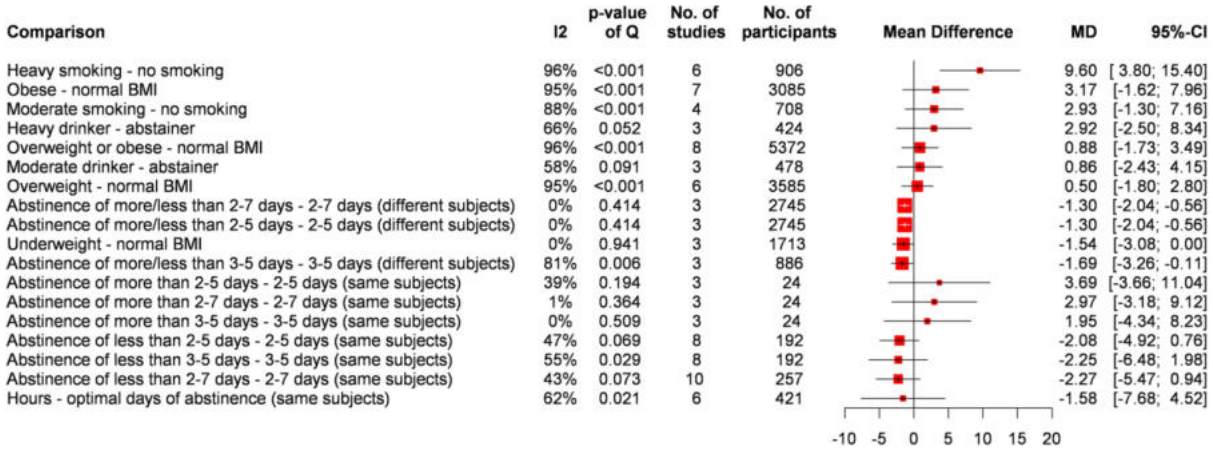


Figure 3. Summary forest plot of lifestyle factors' effects on SDF

8.1.5 Risk factors – other risk factors

Figure 4 illustrates other risk factors. Age over 50 was linked to a clinically important increase in SDF (MD = 12.58%, CI: 7.31–17.86). Exposure to pollutants resulted in a substantial rise in SDF (MD = 9.68%, CI: 6.85–12.52), though pollutant types varied considerably. Pesticide and insecticide exposure also significantly elevated SDF (MD = 6.02%, CI: 3.66–8.38). Higher levels of reactive oxygen species (ROS) were also associated with greater SDF values (MD = 6.10%, CI: 4.65–7.55). Notably severe effects were observed for spinal cord injuries (MD = 60.8%, CI: 53.94–67.66 and MD = 49.8%, CI: 35.66–63.94) and heroin use (MD = 31.79%, CI: 29.09–34.49). Chronic prostatitis and previous orchidopexy were also linked to SDF elevations of approximately 10%.

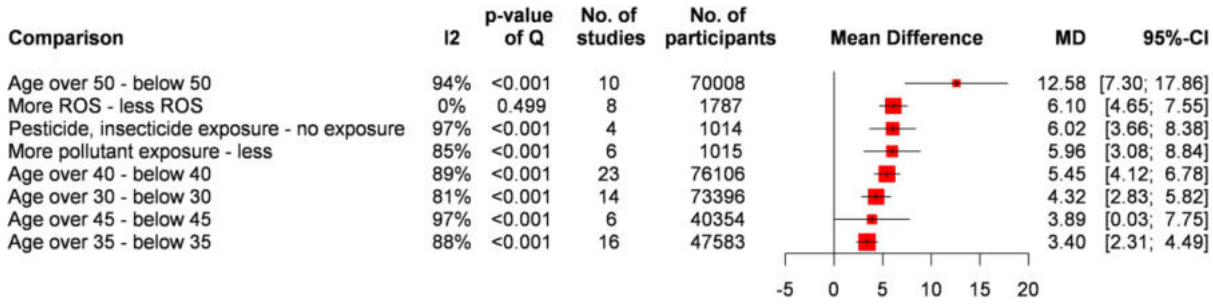


Figure 4. Summary forest plot of other factors' effects on SDF

8.1.6 Risk of bias assessment

Most studies had a low risk of bias in terms of study participation and risk factor measurement. Attrition bias was generally not applicable due to the retrospective nature of the majority of studies. However, the highest risk of bias was observed in the study confounding domain.

8.1.7 Publication bias and heterogeneity

Egger's test for publication bias was only feasible for the varicocele and age analyses based on SDF cut-off values, yielding p-values of 0.548 and 0.405, respectively. High heterogeneity was observed across nearly all risk factors, largely due to inconsistencies in the definitions of the exposures.

8.2 Study II: Meta-analysis

8.2.1 Search and selection

Our search resulted in 36,531 records, from which 86 studies met the inclusion criteria and were selected for systematic review or meta-analysis (Figure 5).

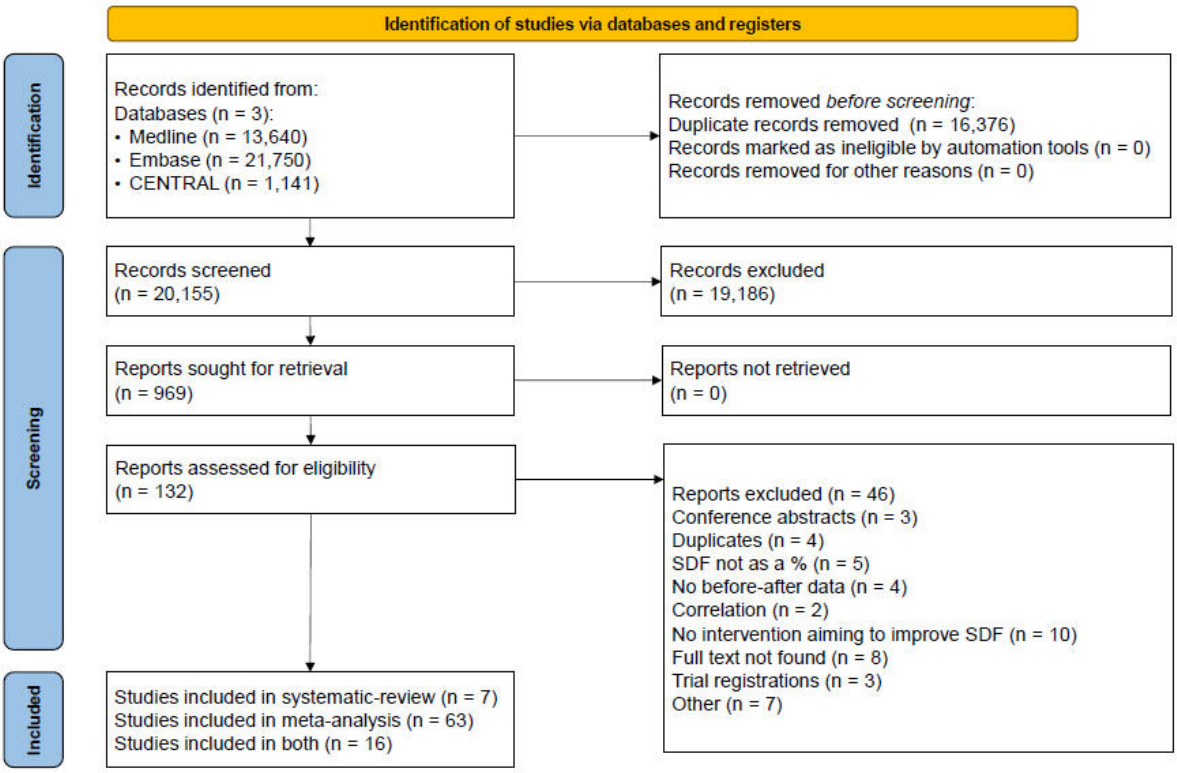


Figure 5. PRISMA flowchart showing the study selection process of the article on the interventions aiming to improve SDF

8.2.2 Basic characteristics of included studies and summary of results

Details of the included studies are available in the original article. The studies spanned from 2005 to 2022 and were conducted mainly in Asia and Europe. Single-arm studies were the most frequently encountered, followed by non-randomized studies and, less commonly, RCTs. Most studies included infertile men, with the most common interventions being varicocelectomy, antioxidant supplementation, FSH treatment, and lifestyle modification. TUNEL was the most frequently used assay, followed by SCSA and SCD.

8.2.3 Interventions – varicocelectomy

Twenty-seven studies involving 1,818 men evaluated the impact of varicocelectomy. Subgroup analyses were based on follow-up measurement time point, assay type, fertility status of the comparison group, and grade of varicocele. The largest reduction in SDF was observed six months after surgery (MD = -12.39%, CI: -22.41, -2.36) (Figure 6). Compared to fertile controls, baseline SDF levels were elevated by 14.70% (CI: 8.09–21.30), while post-surgical values showed a smaller difference (7.34%, CI: -8.68, 23.37). Surgical outcomes varied with grade of varicocele: grade II showed a reduction of -4.55% (CI: -5.87, -3.22), while grade III showed a greater reduction of -7.35% (CI: -9.28, -5.43) post-surgery.

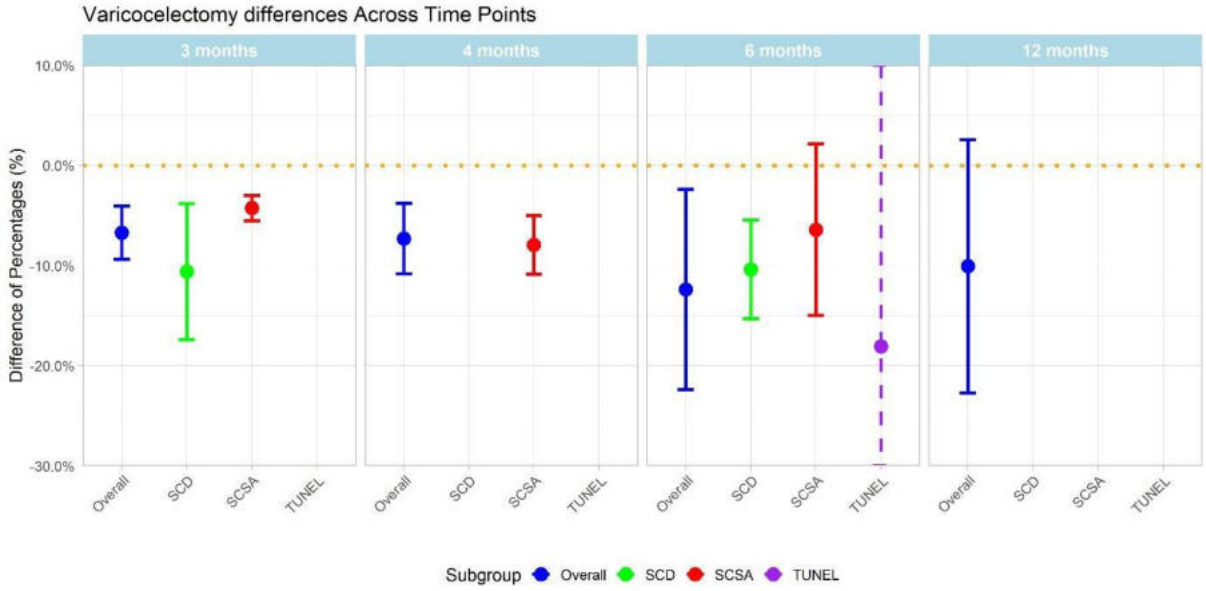


Figure 6. Pooled effect of varicocelectomy on sperm DNA fragmentation with 95% confidence intervals at three, four, six and twelve months pooling results of all assays, SCD, SCSA and TUNEL

8.2.4 Interventions – antioxidants

A total of 39 studies involving 4,958 men evaluated the impact of antioxidant therapy. Subgroup analyses considered the timing of SDF measurement after antioxidant use, assay types, fertility status of control groups, and whether antioxidants were administered as a single agent or in combination.

After three months of treatment, the reduction in SDF was similar across groups: combined and single-agent antioxidants together (MD = -4.27%, CI: -6.11, -2.43), combined antioxidant therapy alone (MD = -4.51%, CI: -6.81, -2.20), and monotherapy alone (MD = -3.36%, CI: -4.44, -2.28) (Figure 7).

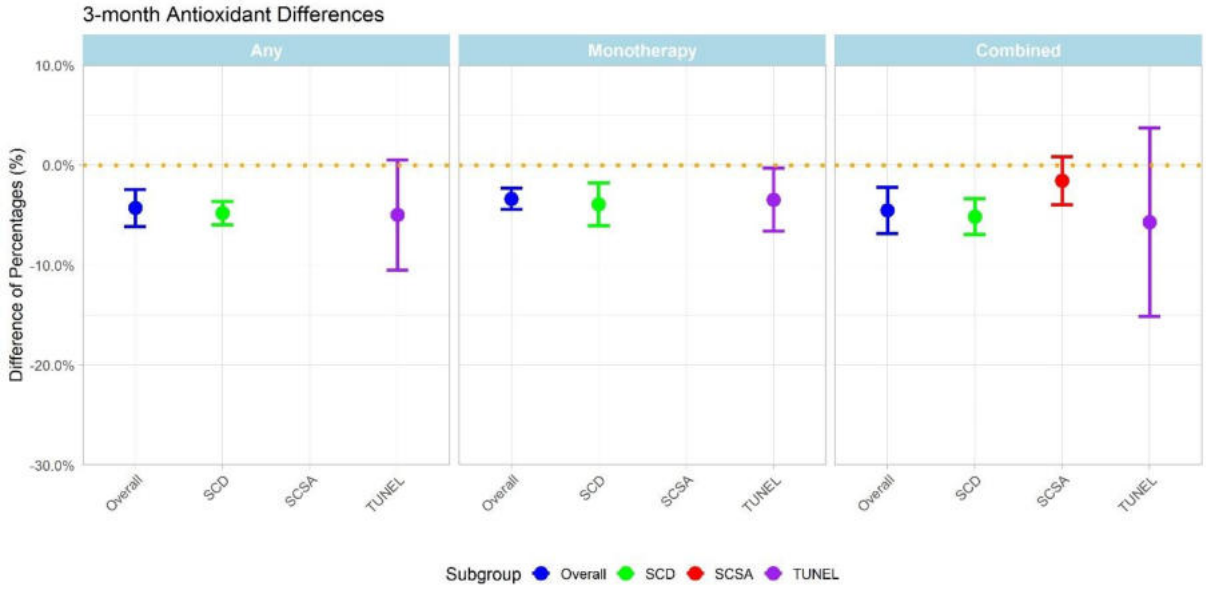


Figure 7. Pooled effect of antioxidant treatment with 95% confidence intervals after three months of mono-, combined or any therapy pooling the results of all assays, SCD, SCSA and TUNEL

8.2.5 Interventions – follicle stimulating hormone

Eight studies comprising 637 men investigated the effect of FSH. Subgroup analyses were based on the assays used and FSH dosage. At three months post-treatment, SDF showed a mean reduction of -6.66% (CI: -9.64, -3.69) from baseline levels (Figure 8).

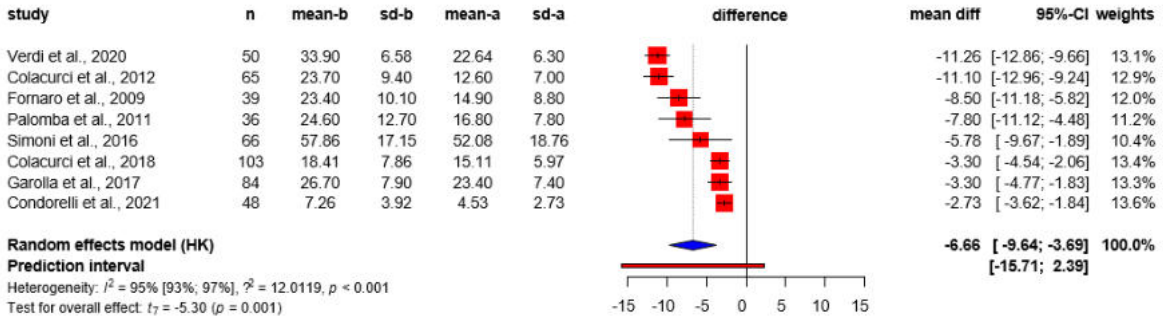


Figure 8. Comparison of mean difference in sperm DNA fragmentation values of patients before FSH treatment and 3 months after FSH treatment, with an input correlation of 0.6

8.2.6 Interventions – lifestyle changes

Lifestyle interventions were assessed in seven studies involving 587 men, with subgroup analyses based on the timing of follow-up. Exercise-based lifestyle modifications resulted in an SDF reduction of -2.94% (CI: -4.94, -0.95) compared to baseline, and -3.24% (CI: -5.33, -1.16) at three months.

8.2.7 Interventions – other interventions

Figure 9 presents data on interventions evaluated in only one or two studies, or those that could not be grouped in the above categories. Among these, the most notable effect was observed in men with genitourinary infections treated with antibiotics and anti-inflammatory agents, showing a mean SDF reduction of -13.45%.

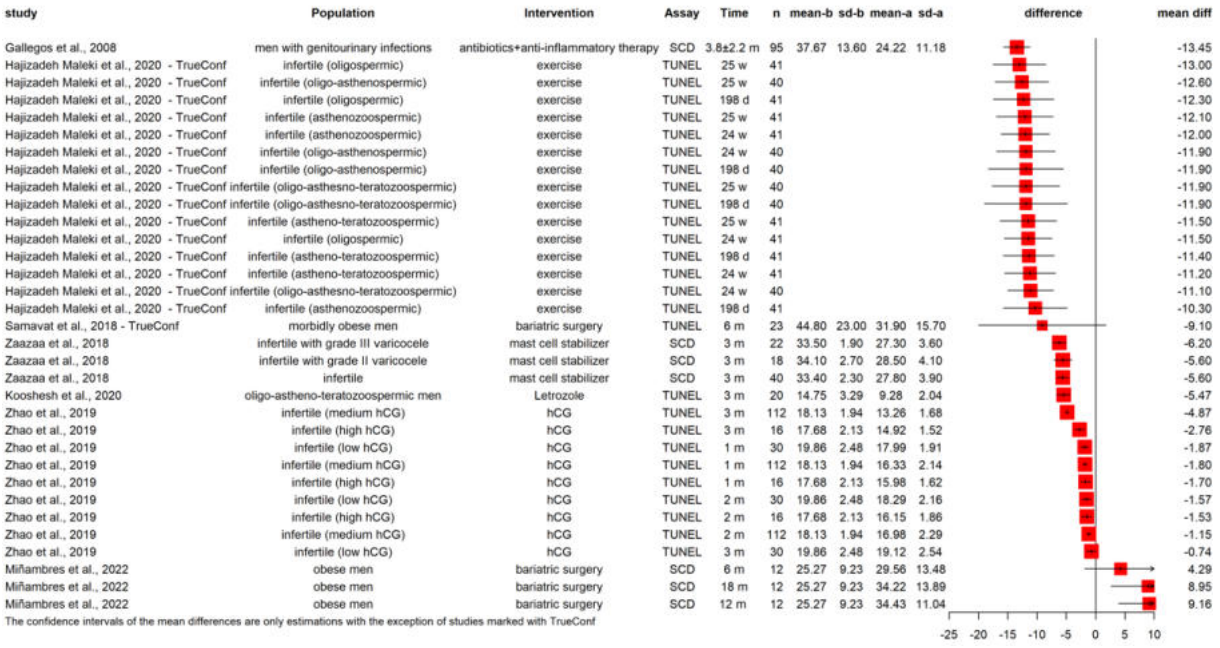


Figure 9. Summary figure of interventions not categorised otherwise with a 0.6 input correlation and 95% CI (n: number of men involved, m: months, w: weeks, d: days, hCG: human chorionic gonadotropin, TrueConf: true confidence intervals defined by the original authors)

8.2.8 Risk of bias and grade assessment

Most of the 46 single-arm studies were assessed as having a low risk of bias. In contrast, many of the 19 two-arm studies were downgraded primarily for not adjusting for confounding variables. The 21 included RCTs were generally rated as having a low risk of bias. GRADE assessments were conducted separately for the four primary intervention types. Varicocelectomy received a low certainty rating, while all other interventions were rated as very low certainty.

8.2.9 Publication bias and heterogeneity

Publication bias could not be evaluated due to the fact that standard errors of mean differences were calculated using imputed correlation coefficients. High heterogeneity was observed across nearly all intervention types. This was likely due to differences in intervention protocols, baseline population characteristics, the use of diverse assays, reproducibility challenges, and the inherent variability of sperm parameters.

9 DISCUSSION

9.1 Summary of findings, international comparisons

In our two studies, we evaluated the effect of modifiable and non-modifiable risk factors on SDF values, and subsequently the effect of interventions that aimed to reduce SDF.

Our first study found the presence of varicocele, impaired glucose tolerance, testicular tumors, smoking, pollution and paternal age above 50 years to be the most harmful on SDF.

The origins of elevated SDF seem to be multifactorial, including testicular and post-testicular insults. Intrinsic contributors include defective spermatogenesis, abortive apoptosis, and chromatin remodelling abnormalities, all of which can result in DNA strand breaks. (214) Apoptosis is particularly important, as it is responsible for eliminating defective germ cells. When this mechanism fails, sperm with fragmented DNA may enter the ejaculate. (215) Double-strand DNA breaks (DSBs) are considered more detrimental than single-strand breaks (SSBs), as they are more difficult to repair and have greater potential to impair embryo development and implantation. (216) While SSBs may be corrected by the oocyte's repair mechanisms, DSBs are more likely to persist. (217, 218)Poor chromatin packaging during histone-protamine transition also increases susceptibility to oxidative stress, which is a major driver of SDF. (5)

However, post-testicular damage is thought to be the main contributor of elevated SDF. Oxidative stress (OS) has been extensively studied as a primary mechanism leading to DNA damage. Immature spermatozoa are particularly susceptible to ROS due to their lack of cytoplasm, which limits antioxidant capacity, and their inability to activate DNA repair pathways. (219) Environmental stressors such as smoking, heat exposure, infections, and environmental pollutants contribute to excessive ROS production, exacerbating sperm DNA damage. (215, 220) On its own however, evidence of excessive amounts of ROS did not seem to greatly impact SDF, although specific evidence-based measurement of oxidative-reductive balance are not yet available.

Among clinical conditions, varicocele has been identified as one of the most consistent and well-studied risk factors for elevated SDF. It induces testicular hyperthermia and hypoxia, leading to increased ROS and subsequent DNA damage. (81, 221-224) Notably, men with varicocele exhibit significantly higher SDF than controls, regardless of fertility status. (215) Our study found a consistent difference of more than 10% in the SDF of

patients with varicocele compared to non-varicocele patients regardless of DNA fragmentation assay used.

Paternal age is now also well recognized to play a significant role in fertility, contrary to earlier assumptions that primarily emphasized maternal age. Based on classical semen parameters, it was previously believed that age-related decline in male fertility began after the age of 40.

However, our analysis of age and SDF values should a clear cut-off of a sudden deterioration in SDF at 50 years of age. Recent evidence indicates that sperm DNA fragmentation (SDF) increases much earlier, with DNA fragmentation index (DFI) values rising by approximately 2% per year between the ages of 19 and 59. (219) By age 60, SDF levels may be nearly double those seen in younger men. (31, 225, 226) This progressive increase is likely driven by the accumulation of oxidative damage over time and a gradual decline in the sperm cell's DNA repair capacity. (219) While advancing paternal age clearly impacts fertilization potential through increased SDF, maternal age remains the more critical determinant of reproductive success due to its stronger influence on oocyte quality and embryo viability. (227)

Lifestyle and environmental exposures play a central role in the aetiology of SDF as well. Smoking negatively affects SDF due to toxic metabolites, including nicotine, benzopyrene and heavy metals. (228-231) Our analysis found that smokers had almost 10% higher levels of SDF compared to non-smokers with a clear dose-dependency. Alcohol consumption was also thought to have harmful effects on male fertility, mainly through the induction of apoptosis. (232, 233) However, the difference in SDF values was minor, although a dose-response was also noted when heavy drinkers and moderate drinkers were compared to abstainers. Obesity, another common scapegoat of declining fertility is thought to lead to higher levels of ROS through increased scrotal temperature, chronic inflammatory processes and endocrine disruptors. (67, 160, 209, 234, 235) It often associates with comorbidities such as diabetes, leading to the formation of advanced glycation end products. Body weight on its own did not have a clinically relevant impact on SDF based on our results. Higher ranges of BMI however, showed statistically significant deterioration of SDF, whereas BMI values below the normal range showed a slight improvement. On the other hand, impaired glucose tolerance has one of the most detrimental effects on SDF.

Infections have also been implicated as significant contributors to increased SDF. Genitourinary tract infections can lead to leukocytospermia and elevated levels of ROS. (81, 221-224, 236) Bacterial infections may exert a more direct effect via self-produced restriction endonucleases that cleave sperm DNA. (214) Ghadikolaei et al. that mumps infection was associated with the deterioration of both classical parameters and SDF. (219) Our findings align with this evidence, as we also observed a strong link between the presence of infectious conditions and elevated SDF levels, supporting the role of infection-mediated oxidative stress in the pathophysiology of sperm DNA damage.

An increase in SDF has also been reported in men with malignancies, particularly testicular cancer, likely due to associated endocrine disruptions and heightened oxidative stress. (142, 147, 173) This aligns with our findings, where testicular tumors were associated with the highest SDF levels, followed closely by non-Hodgkin lymphoma and, to a slightly lesser extent, leukaemia.

Prolonged ejaculatory abstinence has been consistently associated with higher SDF, even in the absence of changes in conventional semen parameters. (26, 145, 237) Short-term recurrent ejaculation has been proposed as a simple, non-invasive strategy to lower SDF. (47, 237) Despite this compelling evidence, our findings did not reveal significant differences in SDF levels across varying abstinence durations.

Exposure to environmental toxins such as heavy metals and pesticides are strongly associated with increased DNA damage. (238) These data align with our results, although due to the highly heterogenous data, firm conclusions cannot be drawn. In a study by Ghadikolaei et al. men in rural areas were found to have higher levels of SDF, than urban men, likely due to agricultural chemicals having a more profound effect than pollution. (219)

Ghadikolaei et al. also studied seasonal variation and found that higher fragmentation indices were reported in the spring and summer months, possibly due to increased ambient temperature and UV exposure. (219)

Elevated levels of SDF have been linked to adverse reproductive outcomes. While sperm with DNA damage can still – with a lower chance – fertilize oocytes, the resulting embryos are at greater risk for impaired development and miscarriage. (5, 239) A strong inverse relationship exists between SDF and natural pregnancy rates, particularly when DFI exceeds 30%. (225) Similarly, higher miscarriage rates have been consistently

reported in in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles utilizing sperm with high SDF, with odds ratios for pregnancy loss ranging from 2.16 to 2.48. (215, 240)

Embryo development is also compromised, particularly beyond the cleavage stage when the paternal genome becomes activated. This is evidenced by lower rates of blastocyst development and poorer embryo quality in cycles involving sperm with high SDF. (47) SDF also appears to delay the time required to reach the blastocyst stage. (104, 241, 242) Nonetheless, the impact of SDF may be modulated by the oocyte's capacity to repair sperm DNA damage, which diminishes with advancing maternal age or diminished ovarian reserve. (47)

Therefore, to be able to tackle these effects, in our next study we assessed the effect of interventions aiming to reduce SDF. Amongst strategies that have been explored to mitigate SDF, varicocelectomy appears to be the most consistently effective intervention. Multiple studies have demonstrated significant post-operative reductions in SDF and improved reproductive outcomes following surgical repair in appropriately selected patients with clinical varicocele and abnormal semen parameters. (215, 243) Our investigation also found a consistent reduction in SDF of over 10% following varicocelectomies with surgeries leading to a greater reduction in patients with higher grade of varicocele.

Hormonal interventions, namely FSH therapy, may benefit selected patients, particularly those with idiopathic infertility or subclinical hypogonadism. (244-246) While the improvements in SDF and reproductive outcomes are more modest compared to varicocelectomy, FSH therapy remains a reasonable option in specific cases.

Antioxidant supplementation has also been widely studied, based on its potential to neutralize ROS. Supplements such as vitamin C, beta-carotene, and L-carnitine have demonstrated some benefit in reducing oxidative stress and improving sperm quality. (214, 247) However, the results across trials remain inconsistent, likely due to heterogeneity in formulations, dosages, patient populations, and baseline SDF levels. Moreover, excessive antioxidant use may paradoxically induce reductive stress, with potentially similar harmful effects on sperm function. (238)

While lifestyle modifications, including weight loss, smoking cessation, and reduced exposure to toxins, are broadly beneficial, their isolated impact on reducing SDF tends to

be limited. (67, 235, 238) Their effects are likely to be gradual and may only become apparent after several months, rather than within a short-term timeframe. In contrast, while antibiotic therapy may have potential benefits in men with confirmed infections or leukocytospermia – conditions in which inflammation and oxidative stress contribute to elevated SDF – evidence supporting its effectiveness remains limited. (81) In our analysis, which included only a single study assessing antibiotic treatment, a mean reduction in SDF of 13.45% was observed. However, given the scarcity of data, these findings should be interpreted with caution, and further research is needed before antibiotics can be recommended as a routine intervention for elevated SDF!

When conventional interventions fail to sufficiently reduce SDF, additional strategies such as sperm selection techniques and the use of testicular sperm may offer alternative solutions. Various sperm selection methods – including density gradient centrifugation, magnetic sorting, and hyaluronic acid-binding – aim to isolate better quality sperm for use in MAR. (248-251) However, none of these methods can fully eliminate sperm with DNA fragmentation or chromosomal abnormalities and based on recent guideline none of the sperm selection methods is superior and neither of them can select based on DNA fragmentation. Testicular sperm, on the other hand, has consistently been shown to exhibit significantly lower SDF compared to ejaculated sperm – up to fivefold lower in some studies. (249, 252) In men with persistently high SDF, the use of testicular sperm for ICSI has been associated with improved live birth rates. Nonetheless, testicular sperm may carry a higher risk of aneuploidy, particularly for chromosomes 13, 18, 21, X, and Y. (253) This remains a subject of ongoing debate, and its use is recommended only in select cases based on clear indication criteria; therefore, the potential benefits and risks should be thoroughly discussed with the patient during clinical decision-making. Although current data do not indicate increased congenital anomalies in offspring, continued surveillance is recommended. These findings suggest that in cases where standard medical or lifestyle interventions are inadequate, advanced sperm retrieval and selection techniques can be considered as part of an individualized fertility treatment approach, although with low evidence.

In conclusion, the aetiology of sperm DNA fragmentation is complex and multifactorial, involving physiological, pathological, and environmental contributors; therefore, available interventions are diverse and variable in effectiveness. Both in vivo and ex vivo

strategies exist, and the most appropriate approach – or a combination thereof – should be carefully selected based on the underlying cause and individual patient characteristics.

9.2 Strengths

The strengths of our analyses included following our pre-registered protocols. Rigorous methodologies were applied, both studies included a large number of articles and a high number of patients, which resulted in the generalizability of our results. Moreover, we have included all risk factors and interventions that have been studied so far. In addition, subgroup analyses led to more precise conclusions.

9.3 Limitations

There were several limitations to our studies. First, the included studies have different study designs, data collection methods, inclusion and exclusion criteria, definitions of fertility, risk factors outcome measures and intervention types. Many studies did not account for confounding factors. Moreover, SDF is a biologically variable parameter, and changes may occur independent of any intervention. This inherent variability represents a potential source of bias and should be considered when interpreting the findings. These factors contributed to the substantial heterogeneity observed.

10 CONCLUSIONS

Our studies identified several factors associated with increased SDF, including varicocele, impaired glucose tolerance, testicular tumors, smoking, environmental pollution, and advanced paternal age. These findings highlight the need to assess underlying health and lifestyle factors in the evaluation of male fertility. In terms of management, we evaluated four major interventions for reducing SDF. Varicocelectomy – when correctly indicated – showed the most consistent and clinically meaningful improvement, particularly six months postoperatively. Appropriately indicated FSH therapy demonstrated moderate efficacy, while antioxidant supplementation and lifestyle modifications produced limited and inconsistent results. Together, these findings offer evidence-based guidance for a more individualized and targeted approach to the diagnosis and treatment of elevated SDF in men presenting with infertility.

11 IMPLEMENTATION FOR PRACTICE

Healthcare providers should focus on identifying modifiable contributors of elevated SDF such as varicocele, smoking, and metabolic disorders, and encourage patients to address these through appropriate lifestyle changes. Medical interventions (e.g., varicocelectomy) should be prioritized in eligible individuals, given their proven efficacy when performed under the right indications. Although antioxidant supplementation and other lifestyle modifications may offer some benefits, their use should be tailored to each patient and guided by current evidence, with an emphasis on setting realistic expectations.

12 IMPLEMENTATION FOR RESEARCH

Future research should focus on standardizing SDF assay methodologies and establishing validated reference ranges to improve the clinical applicability of SDF testing. There is also a critical need for high-quality randomized controlled trials to assess the long-term reproductive outcomes associated with various interventions, including pregnancy and live birth rates. Moreover, research should aim to clarify the mechanisms by which modifiable risk factors influence SDF and evaluate potential synergistic effects of combination therapies. Investigating biomarkers and standardizing laboratory method to differentiate between oxidative and reductive stress could further refine antioxidant treatment strategies.

13 IMPLEMENTATION FOR POLICYMAKERS

Policymakers should support the integration of SDF testing into public reproductive health guidelines. Given the public health implications of rising infertility rates, policies that promote early detection and management of high-risk individuals through screening for varicocele, diabetes, and other factors could reduce healthcare costs and improve reproductive outcomes. Funding should be directed toward standardizing diagnostic tools and supporting large-scale trials to refine male fertility interventions.

14 FUTURE PERSPECTIVES

In the management of male infertility, particularly cases associated with elevated SDF, the future offers promising directions for both diagnostic and therapeutic advancements. Continued innovation in assay development – in particular, more precise, rapid, and standardized techniques – may improve the clinical utility of SDF testing and enable better stratification of patients. Exploring novel interventions, including targeted antioxidant formulations, FSH supplementation to a broader range of subjects or agents that enhance DNA repair pathways, could offer more effective and individualized treatment options. Advances in sperm selection technologies and the refinement of testicular sperm use in assisted reproduction may further optimize outcomes in patients with persistently high SDF. Investigating the specific molecular mechanisms that contribute to DNA damage – such as oxidative stress, chromatin remodelling defects, and apoptotic dysregulation – will be crucial for developing targeted therapies. By deepening our understanding of the causes and consequences of SDF and integrating these insights into clinical practice, we can move toward more precise, effective, and patient-centred management of male infertility.

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16.1 Publications related to the thesis

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D1, IF: 4.2

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Q1, IF: 3.9

16.2 Publications not related to the thesis

Réka Eszter Sziva, Júlia Ács, Anna-Mária Tőkés, Ágnes Korsós-Novák, György L Nádasy, Nándor Ács, Péter Gábor Horváth, Anett Szabó, Haoran Ke, Eszter Mária Horváth, Zsolt Kopa, Szabolcs Várbíró. Accurate Quantitative Histomorphometric-Mathematical Image Analysis Methodology of Rodent Testicular Tissue and Its Possible Future Research Perspectives in Andrology and Reproductive Medicine. Life. 2022 January
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