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The role of iron in the pathogenesis of endometriosis – a systematic review

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Keywords:	ENDOMETRIOSIS, OXIDATIVE STRESS, FEMALE INFERTILITY, ANTIOXIDANTS, APOPTOSIS, METABOLISM

Decision Letter (HROPEN-22-0162.R1)

From: editorial@humanreproduction.co.uk

To: james.wyatt@liverpool.ac.uk

CC:

Subject: Human Reproduction Open - Decision on Manuscript ID HROPEN-22-0162.R1

Dear Mr. Wyatt:

I have now received and given full consideration to the appended reports of the reviewers and Associate Editor on your manuscript HROPEN-22-0162.R1 entitled "The role of iron in the pathogenesis of endometriosis – a systematic review" that you submitted to Human Reproduction Open.

We have recommended publication, but also suggest some minor revisions to your manuscript. Each of their comments must be answered in a revised manuscript before formal acceptance can be given.

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Once again, thank you for submitting your manuscript to Human Reproduction Open and I look forward to receiving your revision.

Yours sincerely,
Dr. Edgardo Somigliana
Editor in Chief, Human Reproduction Open
editorial@humanreproduction.co.uk

Comments to the Authors

Reviewer: 1
Comments to the Authors:
After reviewing 'The role of iron in the pathogenesis of endometriosis – a systematic review' the authors have to be congratulated for a nice and comprehensive systematic review.

We thank the reviewer for their kind recognition of our work

Please consider the comments below as suggestions to improve and complete the paper. As mentioned before, these might result to some extent from a personal bias. Considering that endometriosis lesions start after a series of genetic or epigenetic have reached a threshold, the initiation and the growth of lesions need to be separated. The importance of this for this manuscript is that
- Oxidative stress is primarily important for initiating endometriosis
- Prevention of initiation of endometriosis can be considered eventually also by anti-oxidants and by oral contraceptives reducing retrograde menstruation

- Endometriosis initiation then become comparable with malignant transformation (l60)(l588)(l620) explaining that endometriosis lesions are clonal

We thank the reviewer for sharing their thoughts on this aspect.
We believe that there is not solid evidence to support the theory that oxidative stress is primarily important for initiation of the disease. Many observational studies, including some of ours using human tissue and interventional animal models have demonstrated that ongoing inflammation and resulting oxidative stress to be present with propagation and maintenance of active endometriosis (Scutiero et al., 2017). We agree that most contraceptive hormonal agents will reduce retrograde menstruation since they prevent/ reduce menstrual bleeding from the eutopic endometrium. However, there is yet to be conclusive studies to demonstrate that anti-oxidants will prevent initiation of endometriosis. Although contraceptive hormones reduce the symptoms (thus assumed reduction in inflammation/ oxidative stress at the lesions) of endometriosis, there is also yet to be conclusive evidence for their ability to prevent endometriosis (reviewed extensively by Vercellini et al. (2011)).

Considering these controversies, we wish to remain unbiased, and have stated in our manuscript "The theory that the presence of oxidative stress alone may permit the maintenance and proliferation of endometriotic lesions has been supported by a murine model'. But further research will be needed to prove that this is the case.

References:
Scutiero, G., Iannone, P., Bernardi, G., Bonaccorsi, G., Spadaro, S., Volta, C.A., Greco, P. and Nappi, L., 2017. Oxidative stress and endometriosis: a systematic review of the literature. Oxidative medicine and cellular longevity, 2017.
Vercellini, P., Eskenazi, B., Consonni, D., Somigliana, E., Parazzini, F., Abbiati, A. and Fedele, L., 2011. Oral contraceptives and risk of endometriosis: a systematic review and meta-analysis. Human reproduction update, 17(2), pp.159-170.

L258: higher ferritin concentrations in peritoneal fluid. This is surprising and potentially important. We know since the early 80ies that peritoneal concentrations of large molecules are much lower than in plasma although the peritoneal fluid volume is normal, but highly variable during the menstrual cycle. Therefore, a higher concentration of ferritin with a MW of 450 da suggests that this must reflect more abundant retrograde menstruation in women with endometriosis. Bleeding of superficial lesions seems less likely since rarely observed clinically.

We thank the reviewer for this insight which lends further support to the evidence of a local source of iron excess in the peritoneal cavity, as summarised in this section.

L263 and 480: disease severity. It is suggested to replace severity with cystic ovarian endometriosis. Differences between women with superficial and cystic ovarian endometriosis seem logical. Deep endometriosis is a clinically more severe disease.

The papers referenced in line 263 (267 in this submission) use the revised American Fertility Society classification of disease severity which scores on presence of adhesions, depth of penetration and cul-de-sac obliteration in addition to the presence of ovarian endometriomas. As such, we feel it would be inaccurate to report alterations in iron levels by presence of endometriomas alone. The term 'severity' may better represent what the primary papers describe.
Line 480 (now 484) describes symptom severity and not disease severity.

L372 not clear 'cellular hypoxia in endometriosis'

We thank the reviewer for highlighting this and the wording has been revised for clarity (line 376)

L440-442: not clear whether this indicates endometrial cells from women with and without endometriosis.

We thank the reviewer for highlighting this and the wording of this sentence has been altered for clarity (line 445)

L468 PF contain many other substances than Fe

We agree that this sentence reads as a presumption that iron concentration is the primary causative factor of reduced fertility in this model and have therefore re-written this line accordingly (line 472).

L488: mice with ovarian endometriomas?

We thank the reviewer for highlighting this and this line has been altered for clarity (now line 492)

Discussion
- It is not surprising that plasma Fe concentrations are not different

We agree that widespread changes in systemic iron levels are unlikely but this is a fundamental question without a satisfactory answer. We have expanded our discussion to reflect this (line 691)

- Altered iron transport might indicate the (genetic) predisposition of endometriosis

The cause of altered iron transport in women with endometriosis is indeed unclear, and certainly a genetic predisposition is logical. We have expanded our discussion to reflect this (line 582)

- Murine models have no menstruation

Murine models are indeed limited by the requirement for surgical induction of a process akin to endometriosis. Some of the findings in this review, particularly those related to deferoxamine, are reliant on these models. We have reiterated this in our discussion (line 712)

Associate Editor
Comments to the Authors:
Please respond to several points raised by the reviewer and revise the paper.

Editor-in-Chief
Comments to the Authors:
Please can the authors address the points suggested by Reviewer 1 and also the minor administrative issues that have been identified by the Editorial Office (below).

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The tile page has been edited accordingly

2. Please add the 'What does this mean for patients?' (lay summary) to the main manuscript file; it should be placed immediately after the key words.

The lay summary section has been inserted into the correct location

3. HROpen articles do not include abbreviation lists (as per journal style). Please remove the list of abbreviations and ensure that they are defined at first use in the abstract, again in the main text and in each table and figure, as necessary.

The abbreviations section has been removed

4. Please renumber your figures using Arabic numbering (i.e. Figure 1, Figure 2, etc) as per journal style when mentioned in the manuscript text and in the Figure legends. Owing to a recent journal style change, we would be grateful if you could also renumber your tables using Arabic numbering (i.e. Table 1, Table 2, etc) both in the manuscript text file and on the tables themselves.

The figure/table numbering system has been changed from Roman to Arabic.

5. Please note that recent changes to journal policy mean that inclusion of a Data availability statement, which (where ethically possible) provides details of the status of, and access to, research data underlying the article, is now required. Please provide text for this statement, which should be placed immediately after the Conclusions.

A data availability statement has been added in the required location.

6. Please ensure that all references are formatted in accordance with the style of Human Reproduction Open. Please see our information for authors for more details: https://academic.oup.com/hropen/pages/systematic_reviews.

References have been amended to follow the rules laid out the provided link. Specifically, the year of publication has been moved, issue has replaced volume and author lists have been limited to 10, after which, et al. is used to abbreviate.

7. Please add a copy of the figure legends to the main manuscript document; they should be placed after the references. Please ensure each legend is self-contained, with all symbols and abbreviations used in the figure defined (at the end of the legend).

A figure and table legend has been inserted.

8. Please upload non-pdf versions of your tables. Our production team requires the original (editable) file (in a Word format) for typesetting purposes. Please include a brief descriptive title at the top of your tables (to ensure they are self-explanatory). Please see the attached guide to preparing final table files for publication.

The tables have now been submitted as editable word.docx files.

9. High quality, editable figure files are required for publication. Ideally, all figures should be in TIFF, EPS or AI format at a resolution of 600dpi. Care should be taken when preparing figures to ensure that all text is readable at print size.

The figures have now been uploaded as high-resolution (600dpi) .tif/.tiff files.

Further to the suggested changes, the following is a list of additional changes that we, as authors, felt improved the submission.

1. Addition of references
2. Correction of typographical errors in table 1
3. Addition of missing studies to table 2
4. Correction of number of studies included (line 222)
5. General grammar and sentence structure improvements throughout the manuscript

The role of iron in the pathogenesis of endometriosis – a systematic review

Running title – The role of iron in the pathogenesis of endometriosis

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Abstract

Study question

What is the role of iron in the pathophysiology of endometriosis?

Summary answer

Iron excess is demonstrated wherever endometriotic tissues are found and is associated with oxidative stress, an inflammatory microenvironment and cell damage. Iron-mediated oxidative stress is independently linked to subfertility, symptom severity and malignant transformation.

What is known already?

Iron is found in excess in endometriotic tissues, and multiple mechanisms have been studied and posited to explain this. It is clear that iron excess plays a vital role in promoting oxidative stress and cell damage. The evidence base is large, but no comprehensive reviews exist to summarise our understanding and highlight the overarching themes to further our understanding and suggest future directions of study for the field.

Study design, size, duration

This systematic review with a thematic analysis retrieved studies from the PubMed, Embase, Web of Science and Cochrane Library databases and searches were conducted from inception through to August 2022. Human and animal studies published in the English language were included and identified using a combination of exploded MeSH terms ('Iron' and 'Endometriosis') and free-text search terms ('Iron', 'Ferric', 'Ferrous', 'Endometriosis', 'Endometrioma').

Participants/Materials, setting, methods

This review was reported in accordance with the PRISMA guidelines. All studies reporting original data concerning the role of iron or iron complexes in the pathophysiology of

endometriosis were included. Studies which did not report original data or provided a review of the field were excluded. Bias analysis was completed for each included study using the Newcastle-Ottawa scoring system.

Main results and the role of chance

Seven hundred seventy-six records were identified and screened down to 53 studies which met the eligibility criteria, including nine animal and 44 human studies, with 3,556 individual participants. Iron excess is demonstrated in various tissues and fluids, including ovarian endometriomas, ovarian follicles, ectopic endometriotic lesions and peritoneal fluid. Markers of oxidative stress are strongly associated with high iron levels, and aberrant expression of iron-transport proteins has been demonstrated. Abnormal resistance to ferroptosis is likely. Iron-mediated oxidative stress is responsible for a pro-inflammatory micro-environment and is linked to subfertility, symptom severity and malignant transformation.

Limitations, reasons for caution

A minority of the included studies were of objectively low quality with a high-risk of bias and may lead to misleading conclusions. Additionally, multiple studies failed to appropriately characterise included patients by known confounding variables such as menstrual cycle phase, which may introduce bias to the findings.

Wider implications of the findings

Current literature depicts a central role of aberrant iron mechanics and subsequent oxidative stress in endometriosis. It is likely that iron excess is at least partly responsible for the persistence and proliferation of ectopic endometriotic lesions. As such, iron mechanics represent an attractive target for novel therapeutics, including iron chelators or effectors of the iron-oxidative stress pathway. There are significant gaps in current understanding, and this review highlights and recommends several topics for further research. These include the role of iron chelation, resistance to ferroptosis, the relationship between iron excess and

localised hypoxia, systemic iron pathophysiology in endometriosis, and oxidative stress's role in malignant transformation.

Study funding/ Competing interests

The authors acknowledge support from Royal Liverpool University Hospital (Clinical Research Fellowships (JW, SP) and the authors have no conflicts of interest to declare.

PROSPERO registration number

A protocol was prospectively registered with the PROSPERO database in August 2021 (CRD42021272818)

Keywords

Endometriosis, iron, oxidative stress, ferroptosis, systematic review, iron chelation, iron excess

What this means for patients

The causes of endometriosis are not yet fully understood. Previous research has shown that iron levels appear to be high in endometriosis tissues, but we do not fully understand the significance of this.

This review has gathered all the current research into the role of iron in endometriosis, to better understand what happens in patients with the disease and identify areas that need further study. The findings confirm that iron levels are abnormally high in endometriosis lesions and this is likely due to repeated episodes of bleeding. The red blood cells then break down and the iron contained within is released. High levels of iron causes inflammation and leads to damage to the surrounding cells. High levels of iron are linked to worse symptoms and infertility.

Several methods of potentially treating endometriosis are also highlighted. Binding excess iron appears to partially treat the effects of endometriosis in animals and different methods of

altering the way iron interacts with cells could lead to new treatments but this requires further research.

Introduction

Endometriosis is a common, chronic, gynaecological inflammatory condition affecting approximately 10% of women of reproductive age (Shafrir et al., 2018), equating to 1.5 million women in the United Kingdom alone (WHO, 2022). The histopathological definition of the disease centres on the establishment of extra-uterine endometrium-like tissue, primarily found in the anatomical pelvis. Typical symptoms consist of chronic pelvic pain, dysmenorrhoea and dyspareunia, and there is a strong association with subfertility and negative psychosocial impacts (Delanerolle et al., 2021). The economic productivity cost has been estimated at a loss of £8.2 billion in the United Kingdom per annum; a figure which will only have risen since its estimation in 2012 (Simoens et al., 2012)

Despite the high societal and individual burden, the precise pathophysiological pathways leading to disease remain uncertain (Sourial et al., 2014). Sampson's theory of 'retrograde menstruation and transtubal migration' (Sampson, 1927), whereby viable fragments of physiologically-shed endometrium are deposited onto the peritoneal surface (Tempest et al., 2022, Tempest et al., 2020), probably represents only a small piece of the puzzle. Retrograde menstruation can be considered a normal physiological process, identifiable in 90% of women (Halme et al., 1984). Therefore, pathways which allow the establishment and maintenance of seeded endometrium have been posited. These include altered immune, hormonal and metabolic responses (Hapangama et al., 2010, Sourial et al., 2014, Zondervan et al., 2018). Genetics, hormonal exposure, diet, toxins and BMI have all been implicated as endometriosis-associated factors. The theories of coelomic metaplasia, lymphatic or vascular metastases and neonatal uterine bleeding have also been developed to explain processes Sampson's theory alone cannot. The answer to the question is likely to be a complex interplay between multiple pathogenic mechanisms.

Endometriotic lesions demonstrate hormonal responses similar to healthy eutopic endometrium (Chantalat et al., 2020). Ectopic lesions undergo a cycle of ovarian hormone-sensitive proliferation, haemorrhage, inflammation and fibrosis, leading to adhesion formation and, ultimately, clinical symptoms (Lin et al., 2018, Reis et al., 2013). Repeated localised haemorrhage and an abnormal peritoneal response to retrograde menstruation are theorised to precipitate a cumulative deposition of erythrocytes in endometriosis (Allavena et al., 2015, Defrère et al., 2008, Ng et al., 2020). As a critical constituent of haem and haemoglobin, iron is released during subsequent erythrocytic degradation, leading to iron excess in endometriotic tissues (Maines, 2005, Ganz and Gordon, 2016).

Aberrant iron mechanics are widely demonstrated in endometriosis, and are an established pathophysiological factor. Iron is an essential element in human physiology and is required for vital mechanisms, including oxygen transport, cellular energy production and DNA synthesis (Muñoz et al., 2009). However, iron is toxic in excess. Via the formation of hydroxyl radicals, iron excess leads to oxidative stress, cellular damage, DNA dysregulation and eventual organ dysfunction (Kohgo et al., 2008). As there is no iron-specific excretion pathway, iron homeostasis is tightly regulated by multiple sophisticated mechanisms (Anderson and Frazer, 2017). Despite this, localised iron excess is common in endometriotic lesions (Defrère et al., 2008, Ng et al., 2020).

The oxidative-antioxidative balance exists in healthy tissues and is maintained to avoid excess oxidation and subsequent oxidative stress (Kisaoglu et al., 2013). Oxidative stress is defined by free radical and reactive oxygen species (ROS) induced lipid, protein and DNA oxidation, a process which is cytotoxic and mutagenic (Pizzino et al., 2017). Oxidative stress is prevalent in various human pathologies, including cancer development, atherosclerosis, neurological degradation and, importantly for endometriosis, initiation and maintenance of chronic inflammation (Pizzino et al., 2017).

Iron exists in the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states but can only be absorbed as ferrous iron and cannot be transported independently (Papanikolaou and Pantopoulos, 2005, Aisen et al., 1999). Transferrin is the major iron transport protein, and ferritin is the storage protein which maintains iron in a soluble, non-toxic form, mostly within the liver and bone marrow. Ferritin is composed of both H-Ferritin and L-Ferritin. H-Ferritin has a greater capacity to oxidise iron molecules and is more protective against oxidative stress. Total iron levels are a measure of iron bound to transferrin and ferritin. Free or catalytic iron represents non-transferrin-bound iron, highly capable of producing oxidative stress via the generation of hydroxyl radicals in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$) (Fenton, 1894, Leonard et al., 2004). Haem iron refers to haem, Fe^{2+} iron bound with a protoporphyrin IX complex, an essential constituent of haemoglobin. Total iron binding capacity (TIBC) is an indirect measure of serum transferrin levels and relates to the maximum amount of Fe^{3+} iron that that blood sample can carry. *Figure 1* demonstrates the storage and transport of iron in health in addition to the role of the Fenton reaction and its effects on the cell.

Multiple individual studies have examined iron mechanics in endometriosis but are focused in scope and, therefore, limited in their ability to demonstrate the overall picture. Several reviews have been published on this topic but are now largely outdated, and none are systematic in their design (Defrère et al., 2008, Kobayashi et al., 2009, Ng et al., 2020).

This review aims to collate and summarise the evidence base regarding aberrant iron mechanics in endometriosis to inform readers and identify areas requiring further research.

Materials and methods

This systematic review has been reported according to the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) guidelines (Page et al., 2021). A prospective protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO) database on 10th August 2021 (Registration number: CRD42021272818).

Systematic Search

A systematic search was performed using the PubMed, Embase, Web of Science and Cochrane Library databases. All databases were searched from inception to August 2022. The search string utilised a combination of exploded MeSH terms ('Iron' and 'Endometriosis') and free-text search terms ('Iron', 'Ferric', 'Ferrous', 'Endometriosis', 'Endometrioma'). Results were filtered to English language studies only. Grey literature was not searched.

Eligibility criteria

Inclusion criteria

All human and animal studies reporting original data concerning the role of iron or iron complexes in the pathophysiology of endometriosis were included.

Exclusion criteria

Studies which did not report original data or provided a review of the field only were excluded. All studies without a full-text English language version were excluded. Studies not published in an established journal with a peer-review process were excluded.

Study selection

Results from the initial searches were collated, and duplicates were deleted. Screening, data extraction, theme identification and bias analysis were completed independently by two authors (J.W. and S.M.F.), and disagreements were resolved through discussion. The online software Rayyan (Ouzzani et al., 2016) was used for the title and abstract screening.

Full texts were retrieved and assessed for inclusion using the pre-determined eligibility criteria.

Additional studies were then identified via forwards, and backward chaining of all studies included thus far. Similar articles, as suggested by the PubMed search engine, were also

screened for inclusion. References of all relevant literature and systematic reviews identified by the initial search were also screened.

Data extraction and synthesis

Data extracted included but was not limited to title, author, journal, year of publication, population studied, interventions, results, comparisons and outcomes.

The results were synthesised thematically. Recurring themes were identified from the final list of included studies. Two authors (J.W. and S.M.F) confirmed this final list of themes, which encompasses the titles presented in the results section of this review. Given the heterogeneity of the methods and results found throughout this review, no statistical meta-analysis was possible.

Bias analysis

The Newcastle-Ottawa scale (NOS) was used to assess the quality of each study included in this review (Wells et al., 2000).

Results

Study selection

A total of 776 records were identified from database searches (*Figure 2*). Two hundred eighty-seven duplicate records were excluded, and screening excluded 350 irrelevant records. One hundred and thirty-nine studies underwent full-text review, and the subsequent studies were excluded: 33 reviews of the field did not present any original data. Sixteen records were conference abstracts only, and 27 studies were irrelevant.

A further three studies were identified via forward and backward chaining, and all were included, leaving 53 studies eligible for inclusion.

Study characteristics

Six studies used non-human experimental models; the remaining 47 used human bio-samples or cell lines derived from humans. A total of 3,556 patients are included in the human studies. Publication dates range from 1994 to 2021, and various tissue types and experimental techniques are utilised (*Table 1*).

Bias and quality analysis

A formal methodological quality assessment was completed using the NOS. All studies were non-randomised and susceptible to selection bias. Just 18 of 47 human studies account for the cycle phase in the reported methodology, and 31 describe controlling for any other confounding variable such as age, comorbidity or previous surgery, suggesting a high risk of confounding bias. A breakdown of the NOS scoring is presented in *Table 2*.

Systemic iron levels

Seven studies report on systemic iron levels (Al-Shammaa, 2020, Alizadeh et al., 2015, Chmaj-Wierzchowska et al., 2013, Kokot et al., 2021, Osman et al., 2012, Liu et al., 2022). Five studies compare serum iron levels in women with and without endometriosis, and one uses an animal model of endometriosis (Atkins et al., 2018).

Two small case-control studies with significant methodological weaknesses (*Table 2)* report higher serum iron levels in women with endometriosis (Al-Shammaa, 2020, Alizadeh et al., 2015) while contrastingly, another study reported lower serum iron levels (Osman et al. (2012).. Iron deficiency and secondary anaemia has been demonstrated in Macaques with naturally occurring endometriosis Atkins et al. (2018), where duodenal, bone marrow and liver sampling, supported a systemic deficiency and attempted correction through increased gastrointestinal absorption, as evidenced by ferroportin-1 upregulation despite high dietary iron.

The remaining three studies found no significant difference in serum iron levels between women with endometriosis and controls (Chmaj-Wierzchowska et al., 2013, Kokot et al.,

2021, Liu et al., 2022). Of particular note, however, the only study which considered disease severity, did demonstrate serum iron deficiency in women with revised American Fertility Society (rAFS) grade IV endometriosis (Kokot et al. (2021). Finally, one study (Liu et al. (2022)) included a comparison of iron levels in serum and ovarian endometriomas. The iron excess found in endometriomas was not observed in the serum, suggesting iron overload is limited to the locality of endometriotic tissues Therefore, the included studies' findings are contradictory and marred by low quality. Specifically, none characterise the patient population by menstrual cycle phase or for hormonal treatments. At most, there is possible evidence of an association between increased disease severity and systemic iron deficiency.

Iron in peritoneal fluid

Despite using different methodology and patient characteristics, six studies found evidence of iron overload in the peritoneal fluid of endometriosis patients, compared to healthy controls (Arumugam and Yip (1995), Lousse et al. (2009), Osman et al. (2012), Polak et al. (2018, 2007), Van Langendonckt et al. (2002)).

Free ion and ferritin levels were significantly higher in patients with endometriosis compared with healthy controls (Arumugam et al. (1995); Van Langendonckt et al. (2002); Lousse et al. (2009)). Furthermore, a local rather than systemic source had been suggested for the observed peritoneal iron overload, as evidenced by comparatively low serum iron levels (Van Langendonkt et al. (2002); Osman et al. (2012)).

Increasing disease severity significantly correlated with iron excess (stage III-IV vs. stage I-II (rAFS classification) in some studies (Arumugam et al. (1995) Polak et al. (2007, 2018)) while others found no significant difference (Lousse et al. (2009)). The high Iron and ferritin levels were reported to be specific to the secretory phase by some studies (Van Langendonkt et al. (2002)) while others did not detect such a difference in any marker of iron metabolism (Lousse et al. (2009) Polak et al. (2007, 2018)).

While all studies reported iron overload in the peritoneal fluid of women with endometriosis, there is no consensus on the effect of the menstrual cycle stage or disease severity on iron concentrations. Moreover, multiple studies suggest that excess iron is produced locally rather than systemically.

Iron in the peritoneum and peritoneal deposits

The available data on iron in the peritoneum and peritoneal deposits in endometriosis is limited, with only three studies reporting iron levels in these tissues. Two studies examined the peritoneum of women (Fassbender et al., 2011, Van Langendonckt et al., 2002), while one used a nude mice model (Defrère et al., 2006).

Higher iron and ferritin levels were reported in the peritoneum adjacent to established endometriotic lesions (Van Langendonckt et al. (2002)). When lesions were divided into newer and older, as defined by their visual appearances, all demonstrated raised iron levels, suggesting persistent but minimally variable iron excess throughout the natural history of peritoneal disease. "Typical features" of iron excess are also seen in peritoneal lesions and adjacent tissues in a mouse model of endometriosis (Defrère et al. (2006)). Furthermore, the authors suggest iron overload, secondary to the lysis of erythrocytes, likely by local macrophages, due to the comparably high concentration of siderophages (hemosiderin-laden macrophages). A reduced expression of ferritin mRNA in macroscopically normal peritoneum was detected in women with endometriosis, suggesting iron overload is limited to peritoneal lesions and does not extend into surrounding tissues (Fassbender et al. (2011)). Overall, all studies support the presence of localised iron overload in peritoneal endometriotic lesions.

Iron content in ovarian endometriomas

The iron content of ovarian endometriomas is well-studied, with eleven papers reporting on the iron concentrations in this tissue (Benaglia et al., 2015, Guo et al., 2015, Iizuka et al.,

1998, Imanaka et al., 2021a, Nagayasu et al., 2020, Sanchez et al., 2014, Singh et al., 2013, Takahashi et al., 1996, Yamaguchi et al., 2008, Yoshimoto et al., 2015, Imanaka et al., 2021b). The findings of Benaglia et al. (2015), Sanchez et al. (2014), Nagayasu et al. (2020) and Singh et al. (2013) are summarised elsewhere in this review.

While some studies have compared iron levels in endometriomas to other benign ovarian cysts, others have compared them with malignant ovarian lesions. Endometriomas had significantly higher levels of total, haem and free iron when compared with serous/mucinous adenomas and mature teratomas (Imanaka et al. (2021a); Iizuka et al. (1998)). They also have higher iron levels (total, haem and free) compared with clear cell ovarian cancers, serous/mucinous adenomas (Yamaguchi et al. (2008)); and with a pooled group of endometriosis-associated ovarian cancers (EAOCs) (Yoshimoto et al. (2015)). Alternatively, comparably high iron levels were found in endometrioid ovarian adenocarcinomas, haemorrhagic corpus luteum, and lutein cysts (Iizuka et al. (1998)).

Taking a temporal approach, when "older" and "younger" endometriomas were compared based on their visual appearance during surgery, a significantly higher level of free iron and ferritin were observed within "older" cysts Guo et al. (2015). The accuracy of this categorisation however, remains to be verified, since the appearance may be a mere reflection of hormone responsiveness or aberrant angiogenesis of the lesions. Two studies investigating specific iron-sensitive MRI techniques as a diagnostic tool for endometriomas (Takahashi et al. (1996) and Imanaka et al. (2021b) also confirmed higher iron levels in endometriomas via cyst fluid sampling.

Overall, all studies on endometriomas have reported elevated levels of iron and iron-related proteins in endometriotic fluid compared to almost all other ovarian cyst subtypes. The only exception was alternative haemorrhage-associated cysts, which suggest endometriotic bleeding and haem catabolism, to be the causative pathway for the subsequent iron excess. Furthermore, the reported temporal association with older, more established endometriomas

and higher iron levels suggest accumulation due to failed iron sequestration mechanisms over time. Since the origin of iron in endometriomas is thus localised bleeding at the time of menstruation, it appears to be related to the presumed cyclical hormone responsiveness in this sub-type of endometriosis.

Ovarian follicle iron content

Four studies reported iron levels within ovarian follicles (Benaglia et al., 2015, Li et al., 2020a, Sanchez et al., 2014, Singh et al., 2013). All studies included a subfertile population undergoing in-vitro fertilisation (IVF) and examined follicular fluid sampled at the stage of oocyte retrieval.

Significantly higher levels of follicular free iron (Singh et al. (2013)) and ferric iron in addition to lower transferrin levels with transferrin saturation indicated "iron overload" (Li et al. (2020)) in women with endometriosis compared to those with tubal infertility. These findings suggest that high local iron levels may lead to transferrin saturation with subsequent insufficiency in endometrioma-adjacent follicles.

In women with unilateral endometriomas, higher levels of free iron and ferritin was observed in affected ovaries compared to healthy ones (Benaglia et al. (2015)) and a stepwise increase has been reported in iron levels within the normal ovary through to spatially distant follicles in the diseased ovary and, finally, endometrioma-adjacent follicles (Sanchez et al. (2014)).

Overall, these four studies confirm localised iron overload in and adjacent to endometriotic lesions, which may contribute to subfertility in women with endometriosis.

Iron and macrophages

Macrophage iron concentration has been examined in three studies (Akashi et al., 2021, Kobayashi et al., 2012, Lousse et al., 2009). The observation of a higher ferritin levels in peritoneal macrophages, particularly in the secretory phase in women with endometriosis,

has been interpreted as a progressive overwhelming of the iron-detoxification mechanisms during the menstrual cycle (Lousse et al. (2009)). Eutopic endometrial stroma of women with endometriosis also had a high deposition of iron-laden macrophages (Kobayashi et al. (2012)).

Iron-laden macrophages were also found in the epithelial layers of ovarian endometriomas and ovarian clear-cell carcinomas which concomitantly but predictably expressed significantly raised Ki-67 levels (Akashi et al. (2021)). .

Iron regulation and dysregulation

Iron levels reach excess when the mechanisms controlling iron homeostasis fail or are overwhelmed. Five studies examined alterations in iron transport and inflammatory pathways in endometriotic tissues (Akashi et al., 2021, Alvarado-Díaz et al., 2016, Kobayashi et al., 2012, Takenaka et al., 2017, Alvarado-Díaz et al., 2015).

Iron regulatory genes have demonstrated alterations in ectopic endometriotic cell lines (Kobayashi et al. (2012), where divalent metal transporter 1 (*DMT1*), F-box and leucine rich repeat protein 5 (*FBXL-5)*, Cullin 1 (*CUL1),* Hypoxia-inducible factor 1 beta *(HIF1B),* Iron regulatory proteins 1 and 2 (*IRP1, IRP2)* and Ferroportin *(FPN)* were upregulated while Hypoxia-induced factor 2A (*HIF2A)* had been down-regulated.

Iron overload induced greater expression of two subtypes of *DMT1,* which is responsible for iron influx into cells (Alvarado-Diaz et al. (2016)). Upregulation of *DMT1* was observed in ovarian endometriomas and clear-cell adenocarcinomas (Akashi et al. (2021)). However, the levels of proteins encoded by the genes *DMT-1, FPN, and IRP1* showed no difference between endometriomas and normal endometrium (Takenaka et al. (2017)). *IRP2* was the only gene to show consistent upregulation. *IRP2* plays a key role in cellular iron homeostasis by altering transferrin levels dependent on intracellular iron levels (Zhang et al., 2014). In cell lines with proven iron excess, *IRP2* expression decreased, as would be expected. However, in a hypoxic environment, *IRP2* remained unaltered, suggesting that in endometriosis,

altered iron metabolism and failure of the normal homeostatic pathways may directly result from tissue hypoxia.

Furthermore, when isolated endometrial stromal cells from healthy women are exposed to iron excess, stimulation of the pro-inflammatory NF-κB pathway is evidenced (Alvarado-Diaz (2015) et al). Taken together, these studies suggest aberrant iron regulation and transport in endometriotic tissues, with increased iron import and decreased iron export.

Oxidative-antioxidative balance in endometriosis

Iron-mediated oxidative stress (IMOS) occurs due to the formation of toxic hydroxyl radicals in environments of iron excess and has been explored in 13 studies (Al-Shammaa, 2020, Alizadeh et al., 2015, Arumugam and Yip, 1995, Hayashi et al., 2020, Kokot et al., 2021, Polak et al., 2018, Singh et al., 2013, Thézénas et al., 2020, Woo et al., 2020, Yamaguchi et al., 2008, Milewski et al., 2021, Zhou et al., 2022, Shigetomi et al., 2021).

Systemic studies

Three studies examined systemic markers of oxidative stress (Al-Shammaa, 2020, Alizadeh et al., 2015, Kokot et al., 2021). Some studies reported no significant differences in the oxidative stress markers such as malondialdehyde (MDA) and carbonyl (Alizadeh et al. (2015)) between patients and controls, while others reported significantly higher serum levels of MDA and 8-Hydroxy-2-deoxy guanosine (8-HdG) in the disease cohort (Al-Shammaa et al. (2020)). Some other non-endometriosis specific systemic antioxidants such as ferric-reducing antioxidant power, advanced oxidation protein products and telomerase levels were also reported to be higher in endometriosis patients compared to controls but they were also raised in other benign inflammatory gynaecological pathologies (Kokot et al. (2021)). Multiple other antioxidant markers were reported to be unchanged. Therefore, the limited existing evidence related to systemic oxidative stress provides no consensus.

Studies examining local oxidative stress

The data related to localised oxidative stress in endometriosis is robust, with studies examining IMOS in bio-samples local to endometriotic lesions, including peritoneal fluid and endometriotic deposits. These studies report on multiple markers of oxidative stress, including MDA, 8-HdG, 4-Hydroxynonenal (4-HNE), lactate dehydrogenase (LDH), lipid peroxidation (LPO), total oxidative status (TOS), reactive oxygen species (ROS), and nitric oxide (NO), as well as antioxidants such as total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR).

MDA levels in women with mild or severe endometriosis and controls were similar in one study (Arumugam et al. (1995)), yet another reported a significantly higher TOS in stage I, III, and IV endometriosis patients compared to controls and a significant correlation between TOS and iron levels (Polak et al. (2018)). Conversely, the antioxidant marker TAS was significantly lower in endometriosis patients, but this finding was limited to patients with stage IV disease.

Oxidative stress markers, including LDH, LPO, and 8-HdG, were significantly higher in endometriotic cysts and positively correlated with higher free iron levels (Yamaguchi et al. (2008)). Iron overload in endometriotic stromal cells was associated with oxidative stress but iron excess appeared to inhibit cell proliferation and increase autophagy of endometriotic cells (Zhou et al. (2022)). IMOS has shown to exceed the ability of a bilirubin-dependent antioxidant pathway to maintain oxidative-antioxidative balance in endometriotic tissue (Shigetomi et al. (2021)).

In the context of endometriosis-related infertility, markers of oxidative stress, such as ROS, NO, and MDA, were significantly raised (Singh et al. (2013)), while antioxidant markers TAC, SOD, catalase, GPx, and GR were all significantly lower. Haem oxygenase 1 (HMOX-1), an enzyme responsible for the catabolism of haemoglobin and known to be protective of inflammation and oxidative stress, was also found to have a functional polymorphism in women with endometriosis (Milewski et al. (2021)). In a murine model of endometriosis,

increased levels of 8-HdG and 4-HNE (a more IMOS-specific marker) were associated with lower follicle-stimulating hormone levels and the number of viable foetuses, suggesting a link with endometriosis-related subfertility (Hayashi et al. (2020)).

Overall, the available studies suggest that oxidative stress is prevalent in endometriosis and there is consensus evidence of deviation in the oxidative-antioxidative balance. While excess iron in the lesions appears to be associated with this alteration, direct causation of oxidative stress is hard to prove, and non-iron-mediated pathways may also be involved.

Ferroptosis

Ferroptosis, defined as a distinct form of regulated cell death via iron-dependent lipid peroxidation (Jiang et al., 2021), represents a recent area of interest in endometriosis pathophysiology. The overproduction of iron-induced reactive oxygen species is the defining event in ferroptosis and is the cause of this recently identified mode of cell death. This review includes eight studies published in the last three years which report on the role of ferroptosis in endometriosis (Li et al., 2021a, Li et al., 2022, Li et al., 2021b, Liang et al., 2022, Ni et al., 2022, Wan et al., 2022a, Wan et al., 2022b, Li et al., 2020b).

Ferroptosis in endometriosis pathogenesis

Erastin, an established inducer of ferroptosis (Zhao et al., 2020), was found to increase the rate of ferroptosis in ectopic endometriotic stromal cells but not in normal eutopic endometrial stroma, suggesting pathophysiological limited resistance to ferroptosis as a pathway allowing the establishment of ectopic endometrium (Li et al. (2020b)).

Several studies have investigated the mechanisms underlying resistance to ferroptosis in endometriotic stromal cells. Downregulation of the gene *MALAT1* (Cai et al., 2020) in erastin-induced ferroptosis in these cells (Liang et al. (2022)) suggests that ferroptosis is suppressed by a MALAT1-mediated mechanism. Overexpressing the long noncoding RNA ADAMTS9-AS1 in ectopic endometrial tissue was associated with enhanced cell viability via a reduction in ferroptosis (Wan et al. (2022a)). Inhibiting ferroptosis with ferrostatin-1

reversed the ADAMTS9-AS1-mediated cell survival in stromal cells, suggesting a potential treatment target (Wan et al. (2022a)).

Interestingly, ferroptosis may unexpectedly lead to inflammation and neovascularisation in endometriotic stromal cells. Induction of ferroptosis in endometriotic stromal cells, upregulated the expression of pro-inflammatory and angiogenic cytokines, such as IL-8 and vascular endothelial growth factor A (VEGFA), suggesting that ferroptosis may support the establishment and growth of endometriotic lesions (Li et al. (2022)).

Finally, Fibulin-1, a glycoprotein involved in extracellular matrix stabilization, may play a role in the resistance to ferroptosis in endometriotic stromal cells (Forti et al., 2002, Holmila et al., 2017, Liu et al., 2016, Timpl et al., 2003), since overexpressing Fibulin-1 in endometriotic stromal cells inhibited ferroptosis. Conversely, inhibition of Fibulin-1 increased ferroptosis within endometriotic stromal cells (Wan et al. (2022b)), suggesting a potential therapeutic strategy for endometriosis. These studies propose several mechanisms for altered regulation of ferroptosis in patients with endometriosis and suggest an aberrant resistance to ferroptosis as a critical factor allowing ectopic endometrial establishment and growth. They also suggest ferroptosis is involved in cell proliferation, survival and angiogenesis, thereby contributing to the establishment of ectopic endometriotic lesions

Ferroptosis in endometriosis-associated subfertility

Two studies in murine models, explored the role of ferroptosis in endometriosis-associated subfertility (Li et al., 2021b, Ni et al., 2022). When mouse embryos were exposed to the peritoneal fluid of women with endometriosis, mouse fertility reduced. Ostensibly, due to increased levels of ferroptosis (Li et al., 2021b). Ferrostatin-1, a ferroptosis inhibitor (Cao and Dixon, 2016, Miotto et al., 2020) and HMOX1 (Li et al., 2021b) may have a possible protective role in reversing the effect on fertility. Similarly, murine oocytes exposed to peritoneal fluid from endometriosis women caused iron overload-induced ferroptosis in vitro and in vivo and exosomes released from granulosa cells affected by ferroptosis, further

suppressed the maturation of oocytes (Ni et al., 2022).This limited data suggests that ferroptosis is involved in initiating inflammation and affects oocytes and blastocysts, thus promoting the common symptoms associated with the disease.

Iron and symptoms

Total, haem, and free iron levels in endometriomas were found to correlate with the severity of dysmenorrhoea (Imanaka et al., 2020). Total and haem median iron concentrations in endometrioma content were significantly associated with symptom severity, while a similar but non-significant trend was observed for free iron concentrations, suggesting that iron may play an important role in the pro-inflammatory pain pathways in endometriosis.

Iron and infertility

Eight studies examined the association between iron levels and infertility in women with endometriosis (Arumugam, 1994, Benaglia et al., 2015, Hayashi et al., 2020, Li et al., 2020a, Nagayasu et al., 2020, Sanchez et al., 2014, Singh et al., 2013, Chen et al., 2021).

A novel murine model replicating ovarian endometriosis was found to have significantly higher levels of iron within the ovarian tissue and these mice were less fertile than controls (Hayashi et al. (2020)), proposing that oxidative stress from iron excess directly and negatively impacts folliculogenesis, reducing fertility. These findings are discordant with the human studies, which found no significant differences in oocyte quality or retrieval rate between women with high and low iron levels (Benaglia et al. (2015), Sanchez et al. (2014)).

Significantly higher levels of follicular fluid iron in women with endometriosis undergoing IVF were reported when compared with women with tubal infertility (Singh et al. (2013)). Follicular fluid from women with endometriosis caused a significantly lower oocyte maturation rate compared to controls, and the addition of transferrin to bind excess iron proved reversibility, demonstrated by an improved maturation rate (Li et al. (2020)).

Iron exposure significantly impaired murine embryo development in vitro, with rates of both apoptosis and ferroptosis positively associated with iron concentration (Chen et al. (2021)). Women with endometriosis associated infertility had significantly higher levels of iron within their endometriomas (Nagayasu et al. (2020), suggesting a role of iron in endometriosis-associated infertility. The infertile group in this study was significantly younger, and thus, this observational study may have demonstrated age-related iron levels in endometriomas as opposed to a true association with infertility.

When the effect of iron on male fertility was investigated by exposing healthy spermatozoa from men with proven fertility to the peritoneal fluid extracted from women with and without endometriosis, significantly lower rates of successful acrosome reactions alongside significantly higher concentrations of free iron in the peritoneal fluid were observed in the endometriosis group (Arumugam et al. (1994)). These findings were limited to stage III and IV endometriosis.

Although there is some evidence to suggest that iron excess may play a role in reducing fertility, overall, the studies investigating the relationship between iron levels and infertility in endometriosis have produced mixed results. .

Iron chelation

Given the key role of iron in the pathogenesis of endometriosis, it is an attractive target for potential therapeutics. Four animal studies report the action of iron chelators in endometriosis (Defrère et al., 2006, Kizilay et al., 2017, Ni et al., 2022, Chen et al., 2021). Iron chelators, like deferoxamine (DFO), bind ferric iron, forming stable inactive complexes. Injections of DFO in a murine endometriosis model found no change in the total number of endometriotic lesions but demonstrated reduced levels of iron in those lesions and a decreased proliferative index (Ki-67 immunostaining) (Defrere et al. (2006)). Furthermore, intra-peritoneally injected DFO and curcumin demonstrated implant size to significantly decrease with curcumin alone or with a combination of DFO and curcumin in a

mouse model (Kizilay et al. (2017)). Curcumin is the active molecule within the turmeric plant, which has established antioxidant and iron-binding properties.

When DFO and vitamin E were used in conjunction, iron-mediated oocyte dysmaturity was ameliorated in mice via a reduction in ferroptosis (Ni et al. (2022)). Similarly, iron chelation also partially reversed murine blastocyst dysfunction suggesting excess peritoneal iron is likely to play a role in endometriosis-associated infertility (Chen et al. (2021)).

Discussion

This review presents a summation of the current evidence regarding the role of iron in the pathophysiology of endometriosis. Localised iron excess appears to be an established feature of all ectopic endometriosis lesions. Within these lesions, oxidative stress is strongly associated with elevated iron levels, and aberrant expression of iron-transport proteins appears to be one mechanism responsible for maintaining iron excess. Iron-mediated oxidative stress is implicated in the development of a pro-inflammatory micro-environment, which is linked to subfertility, symptom severity, and, possibly, malignant transformation. The role of iron in the systemic circulation is less clear, with limited studies suggesting conflicting results. *Figure 3* presents the pathophysiological mechanisms highlighted by this review.

The overarching viewpoint afforded through this systematic review enables a greater appreciation of the interplay between pathways and mechanisms relevant to iron, which may facilitate endometriosis establishment and progression, thus, allowing the postulation of novel theories for the pathogenesis and identification of potential therapeutic strategies.

Pathophysiological changes at the peritoneal-endometriosis interface are posited to play an important role in allowing endometriosis deposits to develop and iron appears to play a significant role in this process. We propose that the presence of retrograde menstruation and subsequent hormonally-influenced recurrent bleeding from endometriotic tissue, leads to iron excess via erythrocyte degradation. Consequential oxidative stress produces a pro-inflammatory state, associated with an abnormal resistance to ferroptosis, which encourages

554 homeostatic dysregulation and hypoxia resistance, inciting endometriotic tissue to proliferate
555 at an ectopic site. This review provides evidence for the existence of each step in this
556 pathway.

557 Iron overload is amply demonstrated in ovarian endometriomas, peritoneal endometriosis,
558 and in the peritoneal fluid of women with endometriosis. Elevated levels of erythrocytes and
559 haemoglobin are found in the peritoneal fluid of endometriosis patients and have previously
560 been ascribed to either haemorrhage from ectopic lesions or aberrant processing of
561 menstrual effluent during retrograde menstruation (D'Hooghe and Debrock, 2002, Halme et
562 al., 1984, Polak et al., 2018, Van Langendonckt et al., 2004). Bleeding, as a source of iron,
563 is supported by the findings in in this review, whereby all ovarian lesions associated with
564 haemorrhage, including endometriosis, endometrioid-type adenocarcinomas and a
565 haemorrhagic corpus luteum, demonstrated an iron-rich micro-environment.

566 The above is logical, considering that senescent erythrocytes release iron during
567 erythrophagocytosis where they are engulfed by peritoneal macrophages and undergo
568 degradation and recycling (Gupta et al., 2015). Haem is catabolised via interaction with
569 HMOX-1 to release free iron, which within normal physiological conditions, is rapidly stored
570 within the stable ferritin complex or transported extracellularly via transferrin for further
571 processing (Ganz and Gordon, 2016). However, given the excessive levels of free and
572 stable iron complexes demonstrated in the included studies, we can conclude that these
573 homeostatic processes are either overwhelmed or defective in endometriosis.

574 Altered iron transport may also have a role in the maintenance of iron excess. As outlined,
575 cellular iron importers such as DMT1 appear upregulated in endometriosis, while the iron
576 exporter ferroportin is downregulated. Increased IL-1β levels are also associated with the
577 upregulation of DMT1, leading to a pathological circular pathway of DMT1 upregulation
578 leading to cellular iron influx and induction of oxidative stress (Alvarado-Díaz et al., 2016).
579 This, in turn, leads to IL-1β-mediated inflammation and over-expression of DMT1. Coupled

with the finding of ferroportin downregulation in endometriosis (Li et al. (2021b)), abnormal iron transport does appear to play a role in iron excess. The cause of altered iron transport regulation is unclear but may suggest a genetic predisposition to endometriosis. However, studies are limited, and the suggested mechanisms remain primarily theoretical. Alternative explanations for these findings have not been investigated and may be a fruitful avenue for further research.

Oxidative stress

Iron disrupts redox homeostasis and leads to the formation of hydroxyl radicals. Hydroxyl molecules are highly toxic and, on formation, oxidise any nearby chemical group capable of reaction, including DNA, lipids and proteins, leading to cell death or DNA mutations (Galaris et al., 2019). Cellular and tissue damage is the result, and oxidative stress is implicated in malignancies, atherosclerosis and chronic inflammation (Pizzino et al., 2017). Within endometriosis, oxidation has been linked with infertility, inflammation and malignant transition (Scutiero et al., 2017, Gupta et al., 2006).

The included studies are primarily in accord with one another in describing high levels of oxidative stress in and around endometriotic lesions. The findings of equivalent TOS but deficient TAS in endometriosis suggest a deficiency in the defence against oxidation rather than an overwhelming formation of oxidative radicals (Polak et al. (2018)). Furthermore, the progressive and cumulative deterioration in the oxidative balance demonstrated with disease severity, and the volume of ectopic tissue within the peritoneum is in keeping with the cumulative snowballing effect of initial lesion establishment to facilitate disease progression reported in primate studies (Fazleabas et al., 2002, Hapangama et al., 2010).

The theory that the presence of oxidative stress alone may permit the maintenance and proliferation of endometriotic lesions (Pirdel and Pirdel, 2014) has been supported by a murine model (Defrère et al. (2006)) where iron levels were not associated with the establishment of lesions but iron excess supported their maintenance and proliferation.

606 Macrophages produce pro-inflammatory cytokines in response to haem and iron (Simoni et
607 al., 1994) and stimulate carbon monoxide (CO) production. CO is a potent vasodilator and
608 has been theorised to stimulate angiogenesis in endometriosis (Polak et al., 2018), thereby
609 creating a hospitable environment for the development of lesions. This may partly explain
610 why some lesions proliferate and thrive whilst others do not. The relationship between iron
611 excess and oxidative stress is well described in the included papers and the broader
612 literature (Donnez et al., 2016, Galaris et al., 2019, Hayashi et al., 2020, Ng et al., 2020).
613 The evidence however seems to be incongruous suggesting that iron excess and oxidative
614 stress could play a causative role as well as being a consequence of endometriosis. We
615 postulate that menstrual effluent after retrograde menstruation will initiate an iron-excess and
616 oxidative stress at the initial ectopic sites, facilitating the establishment of new lesions, while
617 the established lesions with an iron over-load (even between menses) maintain oxidative
618 stress and thus, cause lesion progression and contribute to symptoms. Oxidative stress and
619 ferroptosis represent the two major pathways through which iron excess may feed into pro-
620 inflammatory and apoptotic-resistant pathways and are worth exploring in greater detail.
621 However, combination therapy to reduce iron overload and anti-inflammatory medications is
622 likely to produce cumulative benefit for the patients and this is an important avenue of future
623 research.

624 Beyond the pro-inflammatory effects of oxidative stress are the potential genetic mutations
625 noted in malignancies (Hayes et al., 2020). Oxidative stress has been suggested as a direct
626 cause of malignant transformation (Yamaguchi et al. (2008)) and EAOCs, such as
627 endometrioid and clear cell ovarian malignancies, have been linked to oxidative stress
628 (Dahiya et al., 2021). DNA damage from IMOS has been proposed as the likely causative
629 factor (Taniguchi, 2017, Iwabuchi et al., 2015). The pathway from iron excess to oxidative
630 stress is, therefore, a potential preventative target for malignant transformation. Although
631 highly proliferative ovarian cancers contained iron laden macrophages, in contrast, EAOCs
632 generally seem to have lower iron levels than endometriomas and the malignant

transformation of endometriosis is a relatively rare event. Until a comprehensive cellular transcriptomic, metabolomic, proteomic and mutational signature of ectopic endometriotic cells in different endometriosis sub-types are directly compared against the sub-regions of the eutopic endometrium, it is difficult to conclude the exact and specific cellular differences in endometriosis lesions. Therefore, with the current evidence it is difficult to conclude if chelation of iron could reduce the risk of the relatively rare, malignant transformation of endometriomas and further research is required to clarify this possibility.

Oxidative stress has also been implicated in endometriosis-associated subfertility by several high-quality studies (Hayashi et al., 2020, Li et al., 2020a, Singh et al., 2013). Ovarian follicles with iron-rich environments demonstrated lower-quality immature embryos, and animal models confirmed fewer viable foetuses (Hayashi et al., 2020, Li et al., 2020a, Nagayasu et al., 2020). Furthermore, there was evidence of reversibility; since oocyte maturation rates were significantly improved with the introduction of transferrin to bind and stabilise free iron (Li et al. (2020)). IMOS is, therefore, a significant factor in endometriosis-associated subfertility and a highly attractive pathway to target in future research.

Ferroptosis

Iron-mediated cell death was recognised as a novel mechanism as late as 2012 (Dixon et al., 2012), and as such, this is an evolving area of research. Iron overload is the primary driver for this form of regulated cell death, and endometriotic lesions establish and thrive in an iron-rich environment. This review highlights the original work studying the role of ferroptosis in endometriosis and the potential mechanisms leading to possible resistance.

The available data suggests aberrant resistance to ferroptosis in endometriotic tissues. The abnormal regulation or resistance to ferroptosis in endometriosis has been suggested (Ng et al. (2020)) and a link between hypercholesterolaemia and ferroptosis has been posited. Since cholesterol-derived lipophilic antioxidants is a source of protection from ferroptosis (Shimada et al., 2016), elevated cholesterol levels in peritoneal fluid of women with

endometriosis (Sharma et al., 2010) has been proposed to be a potential mechanism of abnormal ferroptosis resistance (Ng et al. (2020)). This theory was supported by the studies, demonstrating 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or Statins, in the treatment of endometriosis (Sokalska et al., 2019, Taylor et al., 2017, Villanueva et al., 2013).

The ferroptosis pathway holds promise as a potential treatment target. Inhibition of ferroptosis with ferrostatin-1 was associated with improved fertility outcomes in mice (Li et al., 2021b), but resistance to ferroptosis appears to be associated with increased viability of endometriotic cells (Wan et al., 2022a). Therefore, the relationship between ferroptosis resistance and clinically apparent symptoms remains poorly delineated and requires further research.

Hypoxia-resistance

The uterus is a highly vascular organ and eutopic endometrial physiology is finely regulated by changes in oxygen concentration (Maybin et al., 2018). Eutopic endometrial cells have high oxygen levels for normal physiological function (Reavey et al., 2021) and thus unsurprisingly, hypoxia has been proposed to play a role in abnormal iron mechanics in endometriosis (Takenaka et al. (2017)). In the peritoneal cavity, the vascularisation of endometriotic lesions via neo-angiogenesis may be sub-optimal and ectopic lesions are thus likely to be susceptible to high levels of hypoxic stress (Powell et al., 2023). In order to thrive, lesions need to develop processes such as inflammation, angiogenesis and steroidogenesis (Hsiao et al., 2015). There is an emerging link between iron physiology and hypoxic conditions (Renassia and Peyssonnaux, 2019), but only one study commented on this topic (Takenaka et al., 2017); therefore, further research is required to clarify the relationship between iron physiology and hypoxia, in the context of endometriosis.

Systemic iron

Whether localised iron excess translates into abnormal systemic iron metabolism remains unclear. The available studies present contradictory results and primary studies are generally of low quality. Within the published data, there is no convincing evidence of either systemic iron deficiency or excess, except for an association between stage IV disease and iron deficiency (Kokot et al., 2021, Hsiao et al., 2015). Considering the local haemorrhage into endometriotic lesions (particularly with endometriomas), we can postulate that women with severe endometriosis will lose iron from the circulation and heavy bleeding is also a common complaint in these women. However, it is also possible that this finding relates to excess menstrual losses or dietary insufficiency rather than any generalised metabolic changes in women with endometriosis. It is perhaps unsurprising that systemic iron levels may be unaltered in women with endometriosis but it is unusual for such a fundamental question to remain without a satisfactory answer. Systemic iron deficiency shares many clinical manifestations with endometriosis, including headache, dizziness or light-headedness and symptoms of restless leg syndrome (Tempest et al., 2021, Allen et al., 2013). If iron deficiency is confirmed to be prevalent in symptomatic women with endometriosis, iron replacement is a readily available treatment option to alleviate at least some of the symptoms that negatively affect quality of life. Further research is therefore required to delineate both the prevalence of abnormal systemic iron levels and the mechanisms controlling this.

Iron chelation

If iron excess is accepted to play an important role in initiating and propagating endometriosis, targeting this pathway for potential treatments is an attractive option. Thus far, this has primarily been explored via iron chelation. Iron chelation involves the introduction of an iron-binding compound into an iron-rich environment to bind toxic, free iron into stable iron complexes, rendering it inactive and suitable for recycling or storage. Deferoxamine has primarily been utilised within the included studies. DFO has an established role in the clinical management of other diseases characterised by iron excess,

including B-thalassaemia (Borgna-Pignatti and Marsella, 2015). In endometriosis, studies are limited to animal models, in which there is surgical induction of an endometriosis-like process in species which do not physiologically menstruate (Defrère et al., 2006, Kizilay et al., 2017). As such, findings are speculative but intra-peritoneal injections of DFO do demonstrate significant reductions in iron levels, lesion size and proliferative activity. In theory, a reduction in local iron levels could decrease oxidative stress, inflammation and lesion proliferation. As such, further research is required to delineate any therapeutic role for iron chelation.

It is also important to examine how current medical therapy, primarily aimed at reducing or stopping menstrual bleeding both from the endometrium, thus reducing retrograde menstruation and locally at the lesion-site by manipulating the ovarian cycle, affects local iron overload. This is a further avenue of interest for future study.

Limitations to this review exist, despite the methodological precautions taken throughout. Namely, any review is reliant on the quality of the primary literature. In this case, a minority of the included studies were of objectively low quality with a high risk of bias and may lead to misleading conclusions. Furthermore, multiple studies failed to appropriately characterise included patients by known confounding variables such as the menstrual cycle phase, which may introduce bias to the findings. In addition, studies present significant heterogeneity in patient population, experimental techniques and research focus. It is, therefore, challenging to compare results directly. However, this review provides a contemporary summary of understanding and an overarching viewpoint allowing greater clarity when describing the pathophysiological pathways allowing endometriotic proliferation.

Weather all ectopic endometriosis lesion sub-types go through the same changes and bleed in synchrony with the eutopic endometrium is not yet fully established. The available evidence is limited and typically does not contain matched full thickness eutopic and different types of ectopic lesions from the same woman with comprehensive assessment of bleeding.

This is a further area of study, which will facilitate the understanding of the lesion-specific influence of iron in pathogenesis and thus, the therapeutic significance.

Conclusions

Degradation of erythrocytes originating from shedding endometrium via retrograde menstruation or ectopic endometriosis lesions leads to localised iron excess in endometriotic lesions. In turn, protective physiological mechanisms are either overwhelmed or primarily defective, allowing toxic iron-mediated oxidative stress to form and maintain a pro-inflammatory environment. Iron excess is associated with and directly impacts endometriotic lesion proliferation, subfertility, symptom severity and rarely, malignant transformation. Further research is required, and specific topics highlighted by this review include the role of iron chelation, ferroptosis, the relationship between iron excess and localised hypoxia, systemic iron mechanics in endometriosis and the role of IMOS in malignant transformation.

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author

Author's Roles

J.W. and D.H. conceived of the review and developed the systematic review protocol. J.W. and S.F. were responsible for database searches and data extraction. J.W. wrote the first draft of the manuscript. C.H. was responsible for creating the figures. J.W. and S.F. created the tables. All authors have provided critical review and feedback on the first draft of the manuscript with substantial input into the analysis and interpretation of the findings. All authors have subsequently reviewed and approved the final version.

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Conflicts of interest

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Figure and table legend

Figure 1 - Iron transport and homeostasis. Schematic diagram depicts major iron transport and storage proteins. Reactive iron is capable of generating hydroxyl radicals, thus iron accumulation increases the risk of oxidative stress. Abbreviations: Fe2+ (ferrous iron), Fe3+ (ferric iron), Tf (transferrin), TfR1 (transferrin receptor 1), DMT1 (divalent metal transporter 1), ZIP8/14 (ZRT/IRT-like protein 8 and 14), Fpn (ferroportin).

Figure 2 – PRISMA flow diagram. Abbreviations: WofS (Web of Science)

Figure 3 - Pathophysiology of iron in endometriosis. Schematic diagram highlighting the most established pathways involved in aberrant iron physiology in endometriotic tissues. 1. Conflicting evidence regarding systemic iron levels 2. Oxidative stress and inflammation 3. Abnormal iron transport. Abbreviations: Fe (Iron), OS (Oxidative stress), TAS (Total

antioxidant status), Fpn (Ferroportin), DMT1 (Divalent metal transporter 1), IL-1β (Interleukin 1 beta).

Table 1 – Summary table of included studies characteristics, findings and conclusions. Abbreviations: nr (not reported), PF (peritoneal fluid), MRI (magnetic-resonance imaging), LDH (lactate dehydrogenase), 8-OHdG (8-hydroxy-2'-deoxyguanosine), mRNA (messenger ribonucleic acid), RANTES (regulated upon activation, normal T cell expressed and presumably secreted), CRP (c-reactive protein), CA-125 (cancer antigen 125), ROS (reactive oxygen species), NO (nitric oxide), LPO (lipid peroxidation), TAC (total antioxidant capacity), IVF (in-vitro fertilisation), L-Ferritin (light ferritin), H-Ferritin (heavy ferritin), MDA (malondialdehyde), NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), DNA (deoxyribonucleic acid), ESC (endometrial stromal cell), DMT1 (divalent metal transporter-1), DFO (deferoxamine), IRP2 (iron-responsive element-binding protein 2), O2 (oxygen), Hb (haemoglobin), NCO4 (nuclear receptor coactivator 4), PCV (packed cell volume), ESR (erythrocyte sedimentation rate), FSH (follicle stimulating hormone), FPN (ferroportin), AOC3 (amine oxidase, copper containing 3), CF (cyst fluid), MMP-2 (matrix-metalloproteinase-2), TfR (transferrin receptor), ATP (adenosine triphosphate), OMA (ovarian endometrioma), oxyHb (oxyhaemoglobin), metHb (methaemoglobin), TAS (total antioxidant status), FRAP (ferric reducing antioxidant power), EM (endometriosis), SIRT (Sirtuin), AOPP (advanced oxidation protein products), UIBC (unsaturated iron-binding capacity), HMOX1 (haem oxygenase-1), VEGFA (vascular endothelial growth factor A), IL8 (interleukin-8), HUVEC (human umbilical vein endothelial cells), MALAT1 (metastasis associated lung adenocarcinoma 1), MUC1 (mucin-1), ADAMTS9-AS1 (ADAMTS9 antisense RNA 1), FAC (ferric ammonium citrate), Ki67 (Antigen KI-67), PARP1 (poly [ADP-ribose] polymerase 1)

Table 2 – Summary table of Newcastle-Ottawa scoring. Abbreviations: Nil

The role of iron in the pathogenesis of endometriosis – a systematic review

Running title – The role of iron in the pathogenesis of endometriosis

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Abstract

Study question

What is the role of iron in the pathophysiology of endometriosis?

Summary answer

Iron excess is demonstrated wherever endometriotic tissues are found and is associated with oxidative stress, an inflammatory microenvironment and cell damage. Iron-mediated oxidative stress is independently linked to subfertility, symptom severity and malignant transformation.

What is known already?

Iron is found in excess in endometriotic tissues, and multiple mechanisms have been studied and posited to explain this. It is clear that iron excess plays a vital role in promoting oxidative stress and cell damage. The evidence base is large, but no comprehensive reviews exist to summarise our understanding and highlight the overarching themes to further our understanding and suggest future directions of study for the field.

Study design, size, duration

This systematic review with a thematic analysis retrieved studies from the PubMed, Embase, Web of Science and Cochrane Library databases and searches were conducted from

inception through to August 2022. Human and animal studies published in the English language were included and identified using a combination of exploded MeSH terms ('Iron' and 'Endometriosis') and free-text search terms ('Iron', 'Ferric', 'Ferrous', 'Endometriosis', 'Endometrioma').

Participants/Materials, setting, methods

This review was reported in accordance with the PRISMA guidelines. All studies reporting original data concerning the role of iron or iron complexes in the pathophysiology of endometriosis were included. Studies which did not report original data or provided a review of the field were excluded. Bias analysis was completed for each included study using the Newcastle-Ottawa scoring system.

Main results and the role of chance

Seven hundred seventy-six records were identified and screened down to 53 studies which met the eligibility criteria, including nine animal and 44 human studies, with 3,556~~2,608~~ individual participants. Iron excess is demonstrated in various tissues and fluids, including ovarian endometriomas, ovarian follicles, ectopic endometriotic lesions and peritoneal fluid. Markers of oxidative stress are strongly associated with high iron levels, and aberrant expression of iron-transport proteins has been demonstrated. Abnormal resistance to ferroptosis is likely. Iron-mediated oxidative stress is responsible for a pro-inflammatory micro-environment and is linked to subfertility, symptom severity and malignant transformation.

Limitations, reasons for caution

A minority of the included studies were of objectively low quality with a high-risk of bias and may lead to misleading conclusions. Additionally, multiple studies failed to appropriately characterise included patients by known confounding variables such as menstrual cycle phase, which may introduce bias to the findings.

Wider implications of the findings

Current literature depicts a central role of aberrant iron mechanics and subsequent oxidative stress in endometriosis. It is likely that iron excess is at least partly responsible for the persistence and proliferation of ectopic endometriotic lesions. As such, iron mechanics represent an attractive target for novel therapeutics, including iron chelators or effectors of the iron-oxidative stress pathway. There are significant gaps in current understanding, and this review highlights and recommends several topics for further research. These include the role of iron chelation, resistance to ferroptosis, the relationship between iron excess and localised hypoxia, systemic iron pathophysiology in endometriosis, and oxidative stress's role in malignant transformation.

Study funding/ Competing interests

The authors acknowledge support from Royal Liverpool University Hospital (Clinical Research Fellowships (JW, SP) and the authors have no conflicts of interest to declare.

PROSPERO registration number

A protocol was prospectively registered with the PROSPERO database in August 2021 (CRD42021272818)

Keywords

Endometriosis, iron, oxidative stress, ferroptosis, systematic review, iron chelation, iron excess

What this means for patients

The causes of endometriosis are not yet fully understood. Previous research has shown that iron levels appear to be high in endometriosis tissues, but we do not fully understand the significance of this.

This review has gathered all the current research into the role of iron in endometriosis, to better understand what happens in patients with the disease and identify areas that need further study. The findings confirm that iron levels are abnormally high in endometriosis lesions and this is likely due to repeated episodes of bleeding. The red blood cells then break down and the iron contained within is released. High levels of iron causes inflammation and leads to damage to the surrounding cells. High levels of iron are linked to worse symptoms and infertility.

Several methods of potentially treating endometriosis are also highlighted. Binding excess iron appears to partially treat the effects of endometriosis in animals and different methods of altering the way iron interacts with cells could lead to new treatments but this requires further research.

~~Abbreviations –~~

~~BMI – Body Mass Index, TIBC – Total Iron Binding Capacity, ROS – Reactive oxygen species, NOS – Newcastle-Ottawa Scale, rASRM – revised American Society for Reproductive Medicine score, rASF – revised American Fertility Society classification, EAOCs – Endometriosis-Associated Ovarian Cancers, PF – Peritoneal Fluid, IMOS – Iron-mediated oxidative stress, TOS – Total oxidative status, TAS – Total Antioxidant Status, IVF – In-Vitro Fertilisation, DFO - Deferoxamine~~

Introduction

Endometriosis is a common, chronic, gynaecological inflammatory condition affecting approximately 10% of women of reproductive age (Shafrir et al., 2018), equating to 1.5 million women in the United Kingdom alone (WHO, 2022). The histopathological definition of the disease centres on the establishment of extra-uterine endometrium-like tissue, primarily found in the anatomical pelvis. Typical symptoms consist of chronic pelvic pain, dysmenorrhoea and dyspareunia, and there is a strong association with subfertility and negative psychosocial impacts (Delanerolle et al., 2021). The economic productivity cost has been estimated at a loss of £8.2 billion in the United Kingdom per annum; a figure which will only have risen since its estimation in 2012 (Simoens et al., 2012)

Despite the high societal and individual burden, the precise pathophysiological pathways leading to disease remain uncertain (Sourial et al., 2014). Sampson's theory of 'retrograde menstruation and transtubal migration' (Sampson, 1927), whereby viable fragments of physiologically-shed endometrium are deposited onto the peritoneal surface (Tempest et al., 2022, Tempest et al., 2020), probably represents only a small piece of the puzzle. Retrograde menstruation can be considered a normal physiological process, identifiable in 90% of women (Halme et al., 1984). Therefore, pathways which allow the establishment and maintenance of seeded endometrium have been posited. These include altered immune, hormonal and metabolic responses (Hapangama et al., 2010, Sourial et al., 2014, Zondervan et al., 2018). Genetics, hormonal exposure, diet, toxins and BMI have all been implicated as endometriosis-associated factors. The theories of coelomic metaplasia, lymphatic or vascular metastases and neonatal uterine bleeding have also been developed to explain processes Sampson's theory alone cannot. The answer to the question is likely to be a complex interplay between multiple pathogenic mechanisms.

Endometriotic lesions demonstrate hormonal responses similar to healthy eutopic endometrium (Chantalat et al., 2020). Ectopic lesions undergo a cycle of ovarian hormone-

147 sensitive proliferation, haemorrhage, inflammation and fibrosis, leading to adhesion
148 formation and, ultimately, clinical symptoms (Lin et al., 2018, Reis et al., 2013). Repeated
149 localised haemorrhage and an abnormal peritoneal response to retrograde menstruation are
150 theorised to precipitate a cumulative deposition of erythrocytes in endometriosis (Allavena et
151 al., 2015, Defrère et al., 2008, Ng et al., 2020). As a critical constituent of haem and
152 haemoglobin, iron is released during subsequent erythrocytic degradation, leading to iron
153 excess in endometriotic tissues (Maines, 2005, Ganz and Gordon, 2016).

154 Aberrant iron mechanics are widely demonstrated in endometriosis, and are an established
155 pathophysiological factor. Iron is an essential element in human physiology and is required
156 for vital mechanisms, including oxygen transport, cellular energy production and DNA
157 synthesis (Muñoz et al., 2009). However, iron is toxic in excess. Via the formation of
158 hydroxyl radicals, iron excess leads to oxidative stress, cellular damage, DNA dysregulation
159 and eventual organ dysfunction (Kohgo et al., 2008). As there is no iron-specific excretion
160 pathway, iron homeostasis is tightly regulated by multiple sophisticated mechanisms
161 (Anderson and Frazer, 2017). Despite this, localised iron excess is common in endometriotic
162 lesions (Defrère et al., 2008, Ng et al., 2020).

163 The oxidative-antioxidative balance exists in healthy tissues and is maintained to avoid
164 excess oxidation and subsequent oxidative stress (Kisaoglu et al., 2013). Oxidative stress is
165 defined by free radical and reactive oxygen species (ROS) induced lipid, protein and DNA
166 oxidation, a process which is cytotoxic and mutagenic (Pizzino et al., 2017). Oxidative stress
167 is prevalent in various human pathologies, including cancer development, atherosclerosis,
168 neurological degradation and, importantly for endometriosis, initiation and maintenance of
169 chronic inflammation (Pizzino et al., 2017).

170 Iron exists in the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states but can only be absorbed as ferrous
171 iron and cannot be transported independently (Papanikolaou and Pantopoulos, 2005, Aisen
172 et al., 1999). Transferrin is the major iron transport protein, and ferritin is the storage protein

which maintains iron in a soluble, non-toxic form, mostly within the liver and bone marrow. Ferritin is composed of both H-Ferritin and L-Ferritin. H-Ferritin has a greater capacity to oxidise iron molecules and is more protective against oxidative stress. Total iron levels are a measure of iron bound to transferrin and ferritin. Free or catalytic iron represents non-transferrin-bound iron, highly capable of producing oxidative stress via the generation of hydroxyl radicals in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH$) (Fenton, 1894, Leonard et al., 2004). Haem iron refers to haem, Fe^{2+} iron bound with a protoporphyrin IX complex, an essential constituent of haemoglobin. Total iron binding capacity (TIBC) is an indirect measure of serum transferrin levels and relates to the maximum amount of Fe^{3+} iron that that blood sample can carry. *Figure ~~1~~ 1* demonstrates the storage and transport of iron in health in addition to the role of the Fenton reaction and its effects on the cell.

Multiple individual studies have examined iron mechanics in endometriosis but are focused in scope and, therefore, limited in their ability to demonstrate the overall picture. Several reviews have been published on this topic but are now largely outdated, and none are systematic in their design (Defrère et al., 2008, Kobayashi et al., 2009, Ng et al., 2020).

This review aims to collate and summarise the evidence base regarding aberrant iron mechanics in endometriosis to inform readers and identify areas requiring further research.

Materials and methods

This systematic review has been reported according to the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) guidelines (Page et al., 2021). A prospective protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO) database on 10th August 2021 (Registration number: CRD42021272818).

Systematic Search

A systematic search was performed using the PubMed, Embase, Web of Science and Cochrane Library databases. All databases were searched from inception to August 2022. The search string utilised a combination of exploded MeSH terms ('Iron' and 'Endometriosis') and free-text search terms ('Iron', 'Ferric', 'Ferrous', 'Endometriosis', 'Endometrioma'). Results were filtered to English language studies only. Grey literature was not searched.

Eligibility criteria

Inclusion criteria

All human and animal studies reporting original data concerning the role of iron or iron complexes in the pathophysiology of endometriosis were included.

Exclusion criteria

Studies which did not report original data or provided a review of the field only were excluded. All studies without a full-text English language version were excluded. Studies not published in an established journal with a peer-review process were excluded.

Study selection

Results from the initial searches were collated, and duplicates were deleted. Screening, data extraction, theme identification and bias analysis were completed independently by two authors (J.W. and S.M.F.), and disagreements were resolved through discussion. The online software Rayyan (Ouzzani et al., 2016) was used for the title and abstract screening.

Full texts were retrieved and assessed for inclusion using the pre-determined eligibility criteria.

Additional studies were then identified via forwards, and backward chaining of all studies included thus far. Similar articles, as suggested by the PubMed search engine, were also screened for inclusion. References of all relevant literature and systematic reviews identified by the initial search were also screened.

Data extraction and synthesis

Data extracted included but was not limited to title, author, journal, year of publication, population studied, interventions, results, comparisons and outcomes.

The results were synthesised thematically. Recurring themes were identified from the final list of included studies. Two authors (J.W. and S.M.F) confirmed this final list of themes, which encompasses the titles presented in the results section of this review. Given the heterogeneity of the methods and results found throughout this review, no statistical meta-analysis was possible.

Bias analysis

The Newcastle-Ottawa scale (NOS) was used to assess the quality of each study included in this review (Wells et al., 2000).

Results

Study selection

A total of 776 records were identified from database searches (*Figure ~~1~~2~~)~~*). Two hundred eighty-seven duplicate records were excluded, and screening excluded 350 irrelevant records. One hundred and thirty-nine studies underwent full-text review, and the subsequent studies were excluded: 33 reviews of the field did not present any original data. Sixteen records were conference abstracts only, and 27 studies were irrelevant.

A further three studies were identified via forward and backward chaining, and all were included, leaving 53 studies eligible for inclusion.

Study characteristics

~~Five~~ Nine studies used non-human experimental models; the remaining 44~~37~~ used human bio-samples or cell lines derived from humans. A total of 32,556~~457~~ patients are included in

the human studies. Publication dates range from 1994 to 2021, and various tissue types and experimental techniques are utilised (*Table ~~I~~1*).

Bias and quality analysis

A formal methodological quality assessment was completed using the NOS. All studies were non-randomised and susceptible to selection bias. Just 1~~8~~5 of 47~~37~~ human studies account for the cycle phase in the reported methodology, and 3~~2~~1 describe controlling for any other confounding variable such as age, comorbidity or previous surgery, suggesting a high risk of confounding bias. A breakdown of the NOS scoring is presented in *Table ~~II~~2*.

Systemic iron levels

Seven studies report on systemic iron levels (Al-Shammaa, 2020, Alizadeh et al., 2015, Chmaj-Wierzchowska et al., 2013, Kokot et al., 2021, Osman et al., 2012, Liu et al., 2022). Five studies compare serum iron levels in women with and without endometriosis, and one uses an animal model of endometriosis (Atkins et al., 2018).

Two small case-control studies with significant methodological weaknesses (*Table 2~~II~~)* report higher serum iron levels in women with endometriosis (Al-Shammaa, 2020, Alizadeh et al., 2015) while- contrastingly, another study reported lower serum iron levels (Osman et al. (2012).. Iron deficiency and secondary anaemia has been demonstrated in Macaques with naturally occurring endometriosis Atkins et al. (2018), where duodenal, bone marrow and liver sampling, supported a systemic deficiency and attempted correction through increased gastrointestinal absorption, as evidenced by ferroportin-1 upregulation despite high dietary iron.

The remaining three studies found no significant difference in serum iron levels between women with endometriosis and controls (Chmaj-Wierzchowska et al., 2013, Kokot et al., 2021, Liu et al., 2022). Of particular note, however, the only study which considered disease severity, did demonstrate serum iron deficiency in women with revised American Fertility

Society (rAFS) -grade IV endometriosis (Kokot et al. (2021). Finally, one study (Liu et al. (2022)) included a comparison of iron levels in serum and ovarian endometriomas. The iron excess found in endometriomas was not observed in the serum, suggesting iron overload is limited to the locality of endometriotic tissues Therefore, the included studies' findings are contradictory and marred by low quality. Specifically, none characterise the patient population by menstrual cycle phase or for hormonal treatments. At most, there is possible evidence of an association between increased disease severity and systemic iron deficiency.

Iron in peritoneal fluid

Despite using different methodology and patient characteristics, six studies found evidence of iron overload in the peritoneal fluid of endometriosis patients, compared to healthy controls (Arumugam and Yip (1995), Lousse et al. (2009), Osman et al. (2012), Polak et al. (2018, 2007), Van Langendonckt et al. (2002)).

Free ion and ferritin levels were significantly higher in patients with endometriosis compared with healthy controls (Arumugam et al. (1995); Van Langendonckt et al. (2002); Lousse et al. (2009)). Furthermore, a local rather than systemic source had been suggested for the observed peritoneal iron overload, as evidenced by comparatively low serum iron levels (Van Langendonkt et al. (2002); Osman et al. (2012)).

Increasing disease severity significantly correlated with iron excess (stage III-IV vs. stage I-II (rAFS classification) in some studies (Arumugam et al. (1995) Polak et al. (2007, 2018)) while others found no significant difference (Lousse et al. (2009)). The high Iron and ferritin levels were reported to be specific to the secretory phase by some studies (Van Langendonkt et al. (2002)) while others did not detect such a difference in any marker of iron metabolism (Lousse et al. (2009) Polak et al. (2007, 2018)).

While all studies reported iron overload in the peritoneal fluid of women with endometriosis, there is no consensus on the effect of the menstrual cycle stage or disease severity on iron concentrations. Moreover, multiple studies suggest that excess iron is produced locally rather than systemically.

Iron in the peritoneum and peritoneal deposits

The available data on iron in the peritoneum and peritoneal deposits in endometriosis is limited, with only three studies reporting iron levels in these tissues. Two studies examined the peritoneum of women (Fassbender et al., 2011, Van Langendonckt et al., 2002), while one used a nude mice model (Defrère et al., 2006).

Higher iron and ferritin levels were reported in the peritoneum adjacent to established endometriotic lesions (Van Langendonckt et al. (2002)). When lesions were divided into newer and older, as defined by their visual appearances, all demonstrated raised iron levels, suggesting persistent but minimally variable iron excess throughout the natural history of peritoneal disease. "Typical features" of iron excess are also seen in peritoneal lesions and adjacent tissues in a mouse model of endometriosis (Defrère et al. (2006)). Furthermore, the authors suggest iron overload, secondary to the lysis of erythrocytes, likely by local macrophages, due to the comparably high concentration of siderophages (hemosiderin-laden macrophages). A reduced expression of ferritin mRNA in macroscopically normal peritoneum was detected in women with endometriosis, suggesting iron overload is limited to peritoneal lesions and does not extend into surrounding tissues (Fassbender et al. (2011)). Overall, all studies support the presence of localised iron overload in peritoneal endometriotic lesions.

Iron content in ovarian endometriomas

The iron content of ovarian endometriomas is well-studied, with eleven papers reporting on the iron concentrations in this tissue (Benaglia et al., 2015, Guo et al., 2015, Iizuka et al.,

1998, Imanaka et al., 2021a, Nagayasu et al., 2020, Sanchez et al., 2014, Singh et al., 2013, Takahashi et al., 1996, Yamaguchi et al., 2008, Yoshimoto et al., 2015, Imanaka et al., 2021b). The findings of Benaglia et al. (2015), Sanchez et al. (2014), Nagayasu et al. (2020) and Singh et al. (2013) are summarised elsewhere in this review.

While some studies have compared iron levels in endometriomas to other benign ovarian cysts, others have compared them with malignant ovarian lesions. Endometriomas had significantly higher levels of total, haem and free iron when compared with serous/mucinous adenomas and mature teratomas (Imanaka et al. (2021a); Iizuka et al. (1998)). They also have higher iron levels (total, haem and free) compared with clear cell ovarian cancers, serous/mucinous adenomas (Yamaguchi et al. (2008)); and with a pooled group of endometriosis-associated ovarian cancers (EAOCs) (Yoshimoto et al. (2015)). Alternatively, comparably high iron levels were found in endometrioid ovarian adenocarcinomas, haemorrhagic corpus luteum, and lutein cysts (Iizuka et al. (1998)).

Taking a temporal approach, when "older" and "younger" endometriomas were compared based on their visual appearance during surgery, a significantly higher level of free iron and ferritin were observed within "older" cysts Guo et al. (2015). The accuracy of this categorisation however, remains to be verified, since the appearance may be a mere reflection of hormone responsiveness or aberrant angiogenesis of the lesions. Two studies investigating specific iron-sensitive MRI techniques as a diagnostic tool for endometriomas (Takahashi et al. (1996) and Imanaka et al. (2021b) also confirmed higher iron levels in endometriomas via cyst fluid sampling.

Overall, all studies on endometriomas have reported elevated levels of iron and iron-related proteins in endometriotic fluid compared to almost all other ovarian cyst subtypes. The only exception was alternative haemorrhage-associated cysts, which suggest endometriotic bleeding and haem catabolism, to be the causative pathway for the subsequent iron excess. Furthermore, the reported temporal association with older, more established endometriomas

and higher iron levels suggest accumulation due to failed iron sequestration mechanisms over time. Since the origin of iron in endometriomas is thus localised bleeding at the time of menstruation, it appears to be related to the presumed cyclical hormone responsiveness in this sub-type of endometriosis.

Ovarian follicle iron content

Four studies reported iron levels within ovarian follicles (Benaglia et al., 2015, Li et al., 2020a, Sanchez et al., 2014, Singh et al., 2013). All studies included a subfertile population undergoing in-vitro fertilisation (IVF) and examined follicular fluid sampled at the stage of oocyte retrieval.

Significantly higher levels of follicular free iron (Singh et al. (2013)) and ferric iron in addition to lower transferrin levels with transferrin saturation indicated "iron overload" (Li et al. (2020)) in women with endometriosis compared to those with tubal infertility. These findings suggest that high local iron levels may lead to transferrin saturation with subsequent insufficiency in endometrioma-adjacent follicles.

In women with unilateral endometriomas, higher levels of free iron and ferritin was observed in affected ovaries compared to healthy ones (Benaglia et al. (2015)) and a stepwise increase has been reported in iron levels within the normal ovary through to spatially distant follicles in the diseased ovary and, finally, endometrioma-adjacent follicles (Sanchez et al. (2014)).

Overall, these four studies confirm localised iron overload in and adjacent to endometriotic lesions, which may contribute to subfertility in women with endometriosis.

Iron and macrophages

Macrophage iron concentration has been examined in three studies (Akashi et al., 2021, Kobayashi et al., 2012, Lousse et al., 2009). The observation of a higher ferritin levels in peritoneal macrophages, particularly in the secretory phase in women with endometriosis,

has been interpreted as a progressive overwhelming of the iron-detoxification mechanisms during the menstrual cycle (Lousse et al. (2009)). Eutopic endometrial stroma of women with endometriosis also had a high deposition of iron-laden macrophages (Kobayashi et al. (2012)).

Iron-laden macrophages were also found in the epithelial layers of ovarian endometriomas and ovarian clear-cell carcinomas which concomitantly but predictably expressed significantly raised Ki-67 levels (Akashi et al. (2021)). .

Iron regulation and dysregulation

Iron levels reach excess when the mechanisms controlling iron homeostasis fail or are overwhelmed. Five studies examined alterations in iron transport and inflammatory pathways in endometriotic tissues (Akashi et al., 2021, Alvarado-Díaz et al., 2016, Kobayashi et al., 2012, Takenaka et al., 2017, Alvarado-Díaz et al., 2015).

Iron regulatory genes have demonstrated alterations in ectopic endometriotic cell lines (Kobayashi et al. (2012), where divalent metal transporter 1 (*DMT1*), F-box and leucine rich repeat protein 5 (*FBXL-5)*, Cullin 1 (*CUL1),* Hypoxia-inducible factor 1 beta *(HIF1B),* Iron regulatory proteins 1 and 2 (*IRP1, IRP2)* and Ferroportin *(FPN)* were upregulated while Hypoxia-induced factor 2A (*HIF2A)* had been down-regulated.

Iron overload induced greater expression of two subtypes of *DMT1*, which is responsible for iron influx into cells (Alvarado-Diaz et al. (2016)). U~~, and u~~pregulation of *DMT1* was observed in ovarian endometriomas and clear-cell adenocarcinomas (Akashi et al. (2021))~~,~~ ~~which is responsible for iron influx into cells~~. However, the levels of proteins encoded by the genes *DMT-1, FPN, and IRP1* showed no difference between endometriomas and normal endometrium (Takenaka et al. (2017)). *IRP2* was the only gene to show consistent upregulation. *IRP2* plays a key role in cellular iron homeostasis by altering transferrin levels dependent on intracellular iron levels (Zhang et al., 2014). In cell lines with proven iron excess, *IRP2* expression decreased, as would be expected. However, in a hypoxic

environment, *IRP2* remained unaltered, suggesting that in endometriosis, altered iron metabolism and failure of the normal homeostatic pathways may directly result from tissue ~~cellular~~ hypoxiaa ~~in endometriosis~~.

Furthermore, when isolated endometrial stromal cells from healthy women are exposed to iron excess, stimulation of the pro-inflammatory NF-κB pathway is evidenced (Alvarado-Diaz (2015) et al). Taken together, these studies suggest aberrant iron regulation and transport in endometriotic tissues, with increased iron import and decreased iron export.

Oxidative-antioxidative balance in endometriosis

Iron-mediated oxidative stress (IMOS) occurs due to the formation of toxic hydroxyl radicals in environments of iron excess and has been explored in 13 studies (Al-Shammaa, 2020, Alizadeh et al., 2015, Arumugam and Yip, 1995, Hayashi et al., 2020, Kokot et al., 2021, Polak et al., 2018, Singh et al., 2013, Thézénas et al., 2020, Woo et al., 2020, Yamaguchi et al., 2008, Milewski et al., 2021, Zhou et al., 2022, Shigetomi et al., 2021).

Systemic studies

Three studies examined systemic markers of oxidative stress (Al-Shammaa, 2020, Alizadeh et al., 2015, Kokot et al., 2021). Some studies reported no significant differences in the oxidative stress markers such as malondialdehyde (MDA) and carbonyl (Alizadeh et al. (2015)) between patients and controls, while others reported significantly higher serum levels of MDA and 8-Hydroxy-2-deoxy guanosine (8-HdG) in the disease cohort (Al-Shammaa et al. (2020)). Some other non-endometriosis specific systemic antioxidants such as ferric-reducing antioxidant power, advanced oxidation protein products and telomerase levels were also reported to be higher in endometriosis patients compared to controls but they were also raised in other benign inflammatory gynaecological pathologies (Kokot et al. (2021)). Multiple other antioxidant markers were reported to be unchanged. Therefore, the limited existing evidence related to systemic oxidative stress provides no consensus.

Studies examining local oxidative stress

The data related to localised oxidative stress in endometriosis is robust, with studies examining IMOS in bio-samples local to endometriotic lesions, including peritoneal fluid and endometriotic deposits. These studies report on multiple markers of oxidative stress, including MDA, 8-HdG, 4-Hydroxynonenal (4-HNE), lactate dehydrogenase (LDH), lipid peroxidation (LPO), total oxidative status (TOS), reactive oxygen species (ROS), and nitric oxide (NO), as well as antioxidants such as total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR).

MDA levels in women with mild or severe endometriosis and controls were similar in one study (Arumugam et al. (1995)), yet another reported a significantly higher TOS in stage I, III, and IV endometriosis patients compared to controls and a significant correlation between TOS and iron levels (Polak et al. (2018)). Conversely, the antioxidant marker TAS was significantly lower in endometriosis patients, but this finding was limited to patients with stage IV disease.

Oxidative stress markers, including LDH, LPO, and 8-HdG, were significantly higher in endometriotic cysts and positively correlated with higher free iron levels (Yamaguchi et al. (2008)). Iron overload in endometriotic stromal cells was associated with oxidative stress but iron excess appeared to inhibit cell proliferation and increase autophagy of endometriotic cells (Zhou et al. (2022)). IMOS has shown to exceed the ability of a bilirubin-dependent antioxidant pathway to maintain oxidative-antioxidative balance in endometriotic tissue (Shigetomi et al. (2021)).

In the context of endometriosis-related infertility, markers of oxidative stress, such as ROS, NO, and MDA, were significantly raised (Singh et al. (2013)), while antioxidant markers TAC, SOD, catalase, GPx, and GR were all significantly lower. Haem oxygenase 1 (HMOX-1), an enzyme responsible for the catabolism of haemoglobin and known to be protective of inflammation and oxidative stress, was also found to have a functional polymorphism in

447 women with endometriosis (Milewski et al. (2021)). In a murine model of endometriosis,
448 increased levels of 8-HdG and 4-HNE (a more IMOS-specific marker) were associated with
449 lower follicle-stimulating hormone levels and the number of viable foetuses, suggesting a link
450 with endometriosis-related subfertility (Hayashi et al. (2020)).

451 Overall, the available studies suggest that oxidative stress is prevalent in endometriosis and
452 there is consensus evidence of deviation in the oxidative-antioxidative balance. While
453 excess iron in the lesions appears to be associated with this alteration, direct causation of
454 oxidative stress is hard to prove, and non-iron-mediated pathways may also be involved.

455 **Ferroptosis**

456 Ferroptosis, defined as a distinct form of regulated cell death via iron-dependent lipid
457 peroxidation (Jiang et al., 2021), represents a recent area of interest in endometriosis
458 pathophysiology. The overproduction of iron-induced reactive oxygen species is the defining
459 event in ferroptosis and is the cause of this recently identified mode of cell death. This
460 review includes eight studies published in the last three years which report on the role of
461 ferroptosis in endometriosis (Li et al., 2021a, Li et al., 2022, Li et al., 2021b, Liang et al.,
462 2022, Ni et al., 2022, Wan et al., 2022a, Wan et al., 2022b, Li et al., 2020b).

463 *Ferroptosis in endometriosis pathogenesis*

464 Erastin, an established inducer of ferroptosis (Zhao et al., 2020), was found to increase the
465 rate of ferroptosis in ectopic endometriotic stromal cells but not in normal eutopic
466 endometrial stroma, suggesting pathophysiological limited resistance to ferroptosis as a
467 pathway allowing the establishment of ectopic endometrium (Li et al. (2020b)).

468 Several studies have investigated the mechanisms underlying resistance to ferroptosis in
469 endometriotic stromal cells. Downregulation of the gene *MALAT1* (Cai et al., 2020) in
470 erastin-induced ferroptosis in these cells (Liang et al. (2022)) suggests that ferroptosis is
471 suppressed by a MALAT1-mediated mechanism. Overexpressing the long noncoding RNA
472 ADAMTS9-AS1 in ectopic endometrial tissue was associated with enhanced cell viability via

a reduction in ferroptosis (Wan et al. (2022a)). Inhibiting ferroptosis with ferrostatin-1 reversed the ADAMTS9-AS1-mediated cell survival in stromal cells, suggesting a potential treatment target (Wan et al. (2022a)).

Interestingly, ferroptosis may unexpectedly lead to inflammation and neovascularisation in endometriotic stromal cells. Induction of ferroptosis in endometriotic stromal cells, upregulated the expression of pro-inflammatory and angiogenic cytokines, such as IL-8 and vascular endothelial growth factor A (VEGFA), suggesting that ferroptosis may support the establishment and growth of endometriotic lesions (Li et al. (2022)).

Finally, Fibulin-1, a glycoprotein involved in extracellular matrix stabilization, may play a role in the resistance to ferroptosis in endometriotic stromal cells (Forti et al., 2002, Holmila et al., 2017, Liu et al., 2016, Timpl et al., 2003), since overexpressing Fibulin-1 in endometriotic stromal cells inhibited ferroptosis. Conversely, inhibition of Fibulin-1 increased ferroptosis within endometriotic stromal cells (Wan et al. (2022b)), suggesting a potential therapeutic strategy for endometriosis. These studies propose several mechanisms for altered regulation of ferroptosis in patients with endometriosis and suggest an aberrant resistance to ferroptosis as a critical factor allowing ectopic endometrial establishment and growth. They also suggest ferroptosis is involved in cell proliferation, survival and angiogenesis, thereby contributing to the establishment of ectopic endometriotic lesions

Ferroptosis in endometriosis-associated subfertility

Two studies in murine models, explored the role of ferroptosis in endometriosis-associated subfertility (Li et al., 2021b, Ni et al., 2022). When mouse embryos were exposed to ~~high iron in~~ the peritoneal fluid of women with endometriosis, mouse fertility ~~was~~ reduced.~~,~~ ~~due~~ Ostensibly, due to increased levels of ferroptosis (Li et al., 2021b). Ferrostatin-1, a ferroptosis inhibitor (Cao and Dixon, 2016, Miotto et al., 2020) and HMOX1 (Li et al., 2021b) may have a possible protective role in reversing the effect on fertility. Similarly, murine oocytes exposed to peritoneal fluid from endometriosis women caused iron overload-

induced ferroptosis in vitro and in vivo and exosomes released from granulosa cells affected by ferroptosis, further suppressed the maturation of oocytes (Ni et al., 2022).This limited data suggests that ferroptosis is involved in initiating inflammation and affects oocytes and blastocysts, thus promoting the common symptoms associated with the disease.

Iron and symptoms

Total, haem, and free iron levels in endometriomas were found to correlate with the severity of dysmenorrhoea (Imanaka et al., 2020). Total and haem median iron concentrations in endometrioma content were significantly associated with symptom severity, while a similar but non-significant trend was observed for free iron concentrations, suggesting that iron may play an important role in the pro-inflammatory pain pathways in endometriosis.

Iron and infertility

Eight studies examined the association between iron levels and infertility in women with endometriosis (Arumugam, 1994, Benaglia et al., 2015, Hayashi et al., 2020, Li et al., 2020a, Nagayasu et al., 2020, Sanchez et al., 2014, Singh et al., 2013, Chen et al., 2021).

~~Mice with ovarian endometriomas~~A novel murine model replicating ovarian endometriosis ~~were~~ was found to have significantly higher levels of iron within the ovarian tissue and ~~were~~ these mice were less fertile than controls (Hayashi et al. (2020)), proposing that oxidative stress from iron excess directly and negatively impacts folliculogenesis, reducing fertility. These findings are discordant with the human studies, which found no significant differences in oocyte quality or retrieval rate between women with high and low iron levels (Benaglia et al. (2015), Sanchez et al. (2014)).

Significantly higher levels of follicular fluid iron in women with endometriosis undergoing IVF were reported when compared with women with tubal infertility (Singh et al. (2013)). Follicular fluid from women with endometriosis caused a significantly lower oocyte maturation rate compared to controls, and the addition of transferrin to bind excess iron proved reversibility, demonstrated by an improved maturation rate (Li et al. (2020)).

Iron exposure significantly impaired murine embryo development in vitro, with rates of both apoptosis and ferroptosis positively associated with iron concentration (Chen et al. (2021)). Women with endometriosis associated infertility had significantly higher levels of iron within their endometriomas (Nagayasu et al. (2020), suggesting a role of iron in endometriosis-associated infertility. The infertile group in this study was significantly younger, and thus, this observational study may have demonstrated age-related iron levels in endometriomas as opposed to a true association with infertility.

When the effect of iron on male fertility was investigated by exposing healthy spermatozoa from men with proven fertility to the peritoneal fluid extracted from women with and without endometriosis, significantly lower rates of successful acrosome reactions alongside significantly higher concentrations of free iron in the peritoneal fluid were observed in the endometriosis group (Arumugam et al. (1994)). These findings were limited to stage III and IV endometriosis.

Although there is some evidence to suggest that iron excess may play a role in reducing fertility, overall, the studies investigating the relationship between iron levels and infertility in endometriosis have produced mixed results. .

Iron chelation

Given the key role of iron in the pathogenesis of endometriosis, it is an attractive target for potential therapeutics. Four animal studies report the action of iron chelators in endometriosis (Defrère et al., 2006, Kizilay et al., 2017, Ni et al., 2022, Chen et al., 2021). Iron chelators, like deferoxamine (DFO), bind ferric iron, forming stable inactive complexes. Injections of DFO in a murine endometriosis model found no change in the total number of endometriotic lesions but demonstrated reduced levels of iron in those lesions and a decreased proliferative index (Ki-67 immunostaining) (Defrere et al. (2006)). Furthermore, intra-peritoneally injected DFO and curcumin demonstrated implant size to significantly decrease with curcumin alone or with a combination of DFO and curcumin in a

mouse model (Kizilay et al. (2017)). Curcumin is the active molecule within the turmeric plant, which has established antioxidant and iron-binding properties.

When DFO and vitamin E were used in conjunction, iron-mediated oocyte dysmaturity was ameliorated in mice via a reduction in ferroptosis (Ni et al. (2022)). Similarly, iron chelation also partially reversed murine blastocyst dysfunction suggesting excess peritoneal iron is likely to play a role in endometriosis-associated infertility (Chen et al. (2021)).

Discussion

This review presents a summation of the current evidence regarding the role of iron in the pathophysiology of endometriosis. Localised iron excess appears to be an established feature of all ectopic endometriosis lesions. Within these lesions, oxidative stress is strongly associated with elevated iron levels, and aberrant expression of iron-transport proteins appears to be one mechanism responsible for maintaining iron excess. Iron-mediated oxidative stress is implicated in the development of a pro-inflammatory micro-environment, which is linked to subfertility, symptom severity, and, possibly, malignant transformation. The role of iron in the systemic circulation is less clear, with limited studies suggesting conflicting results. *Figure 3~~III~~* presents the pathophysiological mechanisms highlighted by this review.

The overarching viewpoint afforded through this systematic review enables a greater appreciation of the interplay between pathways and mechanisms relevant to iron, which may facilitate endometriosis establishment and progression, thus, allowing the postulation of novel theories for the pathogenesis and identification of potential therapeutic strategies.

Pathophysiological changes at the peritoneal-endometriosis interface are posited to play an important role in allowing endometriosis deposits to develop and iron appears to play a significant role in this process. We propose that the presence of retrograde menstruation and subsequent hormonally-influenced recurrent bleeding from endometriotic tissue, leads to iron excess via erythrocyte degradation. Consequential oxidative stress produces a pro-inflammatory state, associated with an abnormal resistance to ferroptosis, which encourages

577 homeostatic dysregulation and hypoxia resistance, inciting endometriotic tissue to proliferate
578 at an ectopic site. This review provides evidence for the existence of each step in this
579 pathway.

580 Iron overload is amply demonstrated in ovarian endometriomas, peritoneal endometriosis,
581 and in the peritoneal fluid of women with endometriosis. Elevated levels of erythrocytes and
582 haemoglobin are found in the peritoneal fluid of endometriosis patients and have previously
583 been ascribed to either haemorrhage from ectopic lesions or aberrant processing of
584 menstrual effluent during retrograde menstruation (D'Hooghe and Debrock, 2002, Halme et
585 al., 1984, Polak et al., 2018, Van Langendonckt et al., 2004). Bleeding, as a source of iron,
586 is supported by the findings in in this review, whereby all ovarian lesions associated with
587 haemorrhage, including endometriosis, endometrioid-type adenocarcinomas and a
588 haemorrhagic corpus luteum, demonstrated an iron-rich micro-environment.

589 The above is logical, considering that senescent erythrocytes release iron during
590 erythrophagocytosis where they are engulfed by peritoneal macrophages and undergo
591 degradation and recycling (Gupta et al., 2015). Haem is catabolised via interaction with
592 HMOX-1 to release free iron, which within normal physiological conditions, is rapidly stored
593 within the stable ferritin complex or transported extracellularly via transferrin for further
594 processing (Ganz and Gordon, 2016). However, given the excessive levels of free and
595 stable iron complexes demonstrated in the included studies, we can conclude that these
596 homeostatic processes are either overwhelmed or defective in endometriosis.

597 Altered iron transport may also have a role in the maintenance of iron excess. As outlined,
598 cellular iron importers such as DMT1 appear upregulated in endometriosis, while the iron
599 exporter ferroportin is downregulated. Increased IL-1β levels are also associated with the
600 upregulation of DMT1, leading to a pathological circular pathway of DMT1 upregulation
601 leading to cellular iron influx and induction of oxidative stress (Alvarado-Díaz et al., 2016).
602 This, in turn, leads to IL-1β-mediated inflammation and over-expression of DMT1. Coupled

with the finding of ferroportin downregulation in endometriosis (Li et al. (2021b)), abnormal iron transport does appear to play a role in iron excess. The cause of altered iron transport regulation is unclear but may suggest a genetic predisposition to endometriosis. However, studies are limited, and the suggested mechanisms remain primarily theoretical. Alternative explanations for these findings have not been investigated and may be a fruitful avenue for further research.

Oxidative stress

Iron disrupts redox homeostasis and leads to the formation of hydroxyl radicals. Hydroxyl molecules are highly toxic and, on formation, oxidise any nearby chemical group capable of reaction, including DNA, lipids and proteins, leading to cell death or DNA mutations (Galaris et al., 2019). Cellular and tissue damage is the result, and oxidative stress is implicated in malignancies, atherosclerosis and chronic inflammation (Pizzino et al., 2017). Within endometriosis, oxidation has been linked with infertility, inflammation and malignant transition (Scutiero et al., 2017, Gupta et al., 2006).

The included studies are primarily in accord with one another in describing high levels of oxidative stress in and around endometriotic lesions. The findings of equivalent TOS but deficient TAS in endometriosis suggest a deficiency in the defence against oxidation rather than an overwhelming formation of oxidative radicals (Polak et al. (2018)). Furthermore, the progressive and cumulative deterioration in the oxidative balance demonstrated with disease severity, and the volume of ectopic tissue within the peritoneum is in keeping with the cumulative snowballing effect of initial lesion establishment to facilitate disease progression reported in primate studies (Fazleabas et al., 2002, Hapangama et al., 2010).

The theory that the presence of oxidative stress alone may permit the maintenance and proliferation of endometriotic lesions (Pirdel and Pirdel, 2014) has been supported by a murine model (Defrère et al. (2006)) where iron levels were not associated with the establishment of lesions but iron excess supported their maintenance and proliferation.

Macrophages produce pro-inflammatory cytokines in response to haem and iron (Simoni et al., 1994) and stimulate carbon monoxide (CO) production. CO is a potent vasodilator and has been theorised to stimulate angiogenesis in endometriosis (Polak et al., 2018), thereby creating a hospitable environment for the development of lesions. This may partly explain why some lesions proliferate and thrive whilst others do not. The relationship between iron excess and oxidative stress is well described in the included papers and the broader literature (Donnez et al., 2016, Galaris et al., 2019, Hayashi et al., 2020, Ng et al., 2020). The evidence however seems to be incongruous suggesting that iron excess and oxidative stress could play a causative role as well as being a consequence of endometriosis. We postulate that menstrual effluent after retrograde menstruation will initiate an iron-excess and oxidative stress at the initial ectopic sites, facilitating the establishment of new lesions, while the established lesions with an iron over-load (even between menses) maintain oxidative stress and thus, cause lesion progression and contribute to symptoms. Oxidative stress and ferroptosis represent the two major pathways through which iron excess may feed into pro-inflammatory and apoptotic-resistant pathways and are worth exploring in greater detail. However, combination therapy to reduce iron overload and anti-inflammatory medications is likely to produce cumulative benefit for the patients and this is an important avenue of future research.

Beyond the pro-inflammatory effects of oxidative stress are the potential genetic mutations noted in malignancies (Hayes et al., 2020). Oxidative stress has been suggested as a direct cause of malignant transformation (Yamaguchi et al. (2008)) and EAOCs, such as endometrioid and clear cell ovarian malignancies, have been linked to oxidative stress (Dahiya et al., 2021). DNA damage from IMOS has been proposed as the likely causative factor (Taniguchi, 2017, Iwabuchi et al., 2015). The pathway from iron excess to oxidative stress is, therefore, a potential preventative target for malignant transformation. Although highly proliferative ovarian cancers contained iron laden macrophages, in contrast, EAOCs generally seem to have lower iron levels than endometriomas and the malignant

transformation of endometriosis is a relatively rare event. Until a comprehensive cellular transcriptomic, metabolomic, proteomic and mutational signature of ectopic endometriotic cells in different endometriosis sub-types are directly compared against the sub-regions of the eutopic endometrium, it is difficult to conclude the exact and specific cellular differences in endometriosis lesions. Therefore, with the current evidence it is difficult to conclude if chelation of iron could reduce the risk of the relatively rare, malignant transformation of endometriomas and further research is required to clarify this possibility.

Oxidative stress has also been implicated in endometriosis-associated subfertility by several high-quality studies (Hayashi et al., 2020, Li et al., 2020a, Singh et al., 2013). Ovarian follicles with iron-rich environments demonstrated lower-quality immature embryos, and animal models confirmed fewer viable foetuses (Hayashi et al., 2020, Li et al., 2020a, Nagayasu et al., 2020). Furthermore, there was evidence of reversibility; since oocyte maturation rates were significantly improved with the introduction of transferrin to bind and stabilise free iron (Li et al. (2020)). IMOS is, therefore, a significant factor in endometriosis-associated subfertility and a highly attractive pathway to target in future research.

Ferroptosis

Iron-mediated cell death was recognised as a novel mechanism as late as 2012 (Dixon et al., 2012), and as such, this is an evolving area of research. Iron overload is the primary driver for this form of regulated cell death, and endometriotic lesions establish and thrive in an iron-rich environment. This review highlights the original work studying the role of ferroptosis in endometriosis and the potential mechanisms leading to possible resistance.

The available data suggests aberrant resistance to ferroptosis in endometriotic tissues. The abnormal regulation or resistance to ferroptosis in endometriosis has been suggested (Ng et al. (2020)) and a link between hypercholesterolaemia and ferroptosis has been posited. Since cholesterol-derived lipophilic antioxidants is a source of protection from ferroptosis (Shimada et al., 2016), elevated cholesterol levels in peritoneal fluid of women with

endometriosis (Sharma et al., 2010) has been proposed to be a potential mechanism of abnormal ferroptosis resistance (Ng et al. (2020)). This theory was supported by the studies, demonstrating 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or Statins, in the treatment of endometriosis (Sokalska et al., 2019, Taylor et al., 2017, Villanueva et al., 2013).

The ferroptosis pathway holds promise as a potential treatment target. Inhibition of ferroptosis with ferrostatin-1 was associated with improved fertility outcomes in mice (Li et al., 2021b), but resistance to ferroptosis appears to be associated with increased viability of endometriotic cells (Wan et al., 2022a). Therefore, the relationship between ferroptosis resistance and clinically apparent symptoms remains poorly delineated and requires further research.

Hypoxic-resistance

The uterus is a highly vascular organ and eutopic endometrial physiology is finely regulated by changes in oxygen concentration (Maybin et al., 2018). Eutopic endometrial cells have high oxygen levels for normal physiological function (Reavey et al., 2021) and thus unsurprisingly, hypoxia has been proposed to play a role in abnormal iron mechanics in endometriosis (Takenaka et al. (2017)). In the peritoneal cavity, the vascularisation of endometriotic lesions via neo-angiogenesis may be sub-optimal and ectopic lesions are thus likely to be susceptible to high levels of hypoxic stress (Powell et al., 2023). In order to thrive, lesions need to develop processes such as inflammation, angiogenesis and steroidogenesis (Hsiao et al., 2015). There is an emerging link between iron physiology and hypoxic conditions (Renassia and Peyssonnaux, 2019), but only one study commented on this topic (Takenaka et al., 2017); therefore, further research is required to clarify the relationship between iron physiology and hypoxia, in the context of endometriosis.

Systemic iron

Whether localised iron excess translates into abnormal systemic iron metabolism remains unclear. The available studies present contradictory results and primary studies are generally of low quality. Within the published data, there is no convincing evidence of either systemic iron deficiency or excess, except for an association between stage IV disease and iron deficiency (Kokot et al., 2021, Hsiao et al., 2015). Considering the local haemorrhage into endometriotic lesions (particularly with endometriomas), we can postulate that women with severe endometriosis will lose iron from the circulation and heavy bleeding is also a common complaint in these women. However, it is also possible that this finding relates to excess menstrual losses or dietary insufficiency rather than any generalised metabolic changes in women with endometriosis. It is perhaps unsurprising that systemic iron levels may be unaltered in women with endometriosis but~~This~~ it is unusual for such ~~is~~ a fundamental question ~~that~~ to remain~~s~~ without a satisfactory answer. Systemic iron deficiency shares many clinical manifestations with endometriosis, including headache, dizziness or light-headedness and symptoms of restless leg syndrome (Tempest et al., 2021, Allen et al., 2013). If iron deficiency is confirmed to be prevalent in symptomatic women with endometriosis, iron replacement is a readily available treatment option to alleviate at least some of the symptoms that negatively affect quality of life. Further research is therefore required to delineate both the prevalence of abnormal systemic iron levels and the mechanisms controlling this.

Iron chelation

If iron excess is accepted to play an important role in initiating and propagating endometriosis, targeting this pathway for potential treatments is an attractive option. Thus far, this has primarily been explored via iron chelation. Iron chelation involves the introduction of an iron-binding compound into an iron-rich environment to bind toxic, free iron into stable iron complexes, rendering it inactive and suitable for recycling or storage. Deferoxamine has primarily been utilised within the included studies. DFO has an established role in the clinical management of other diseases characterised by iron excess,

including B-thalassaemia (Borgna-Pignatti and Marsella, 2015). In endometriosis, studies are limited to animal models, in which there is surgical induction of an endometriosis-like process in species which do not physiologically menstruate (Defrère et al., 2006, Kizilay et al., 2017). As such, findings are speculative but ~~using i~~intra-peritoneal injections of DFO do~~but~~ demonstrate significant reductions in iron levels, lesion size and proliferative activity. In theory, a reduction in local iron levels could decrease oxidative stress, inflammation and lesion proliferation. As such, further research is required to delineate any therapeutic role for iron chelation.

It is also important to examine how current medical therapy, primarily aimed at reducing or stopping menstrual bleeding both from the endometrium, thus reducing retrograde menstruation and locally at the lesion-site by manipulating the ovarian cycle, affects local iron overload. This is a further avenue of interest for future study.

Limitations to this review exist, despite the methodological precautions taken throughout. Namely, any review is reliant on the quality of the primary literature. In this case, a minority of the included studies were of objectively low quality with a high risk of bias and may lead to misleading conclusions. Furthermore, multiple studies failed to appropriately characterise included patients by known confounding variables such as the menstrual cycle phase, which may introduce bias to the findings. In addition, studies present significant heterogeneity in patient population, experimental techniques and research focus. It is, therefore, challenging to compare results directly. However, this review provides a contemporary summary of understanding and an overarching viewpoint allowing greater clarity when describing the pathophysiological pathways allowing endometriotic proliferation.

Weather all ectopic endometriosis lesion sub-types go through the same changes and bleed in synchrony with the eutopic endometrium is not yet fully established. The available evidence is limited and typically does not contain matched full thickness eutopic and different types of ectopic lesions from the same woman with comprehensive assessment of bleeding.

This is a further area of study, which will facilitate the understanding of the lesion-specific influence of iron in pathogenesis and thus, the therapeutic significance.

Conclusions

Degradation of erythrocytes originating from shedding endometrium via retrograde menstruation or ectopic endometriosis lesions leads to localised iron excess in endometriotic lesions. In turn, protective physiological mechanisms are either overwhelmed or primarily defective, allowing toxic iron-mediated oxidative stress to form and maintain a pro-inflammatory environment. Iron excess is associated with and directly impacts endometriotic lesion proliferation, subfertility, symptom severity and rarely, malignant transformation. Further research is required, and specific topics highlighted by this review include the role of iron chelation, ferroptosis, the relationship between iron excess and localised hypoxia, systemic iron mechanics in endometriosis and the role of IMOS in malignant transformation.

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author

Author's Roles

J.W. and D.H. conceived of the review and developed the systematic review protocol. J.W. and S.F. were responsible for database searches and data extraction. J.W. wrote the first draft of the manuscript. C.H. was responsible for creating the figures. J.W. and S.F. created the tables. All authors have provided critical review and feedback on the first draft of the manuscript with substantial input into the analysis and interpretation of the findings. All authors have subsequently reviewed and approved the final version.

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Conflicts of interest

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Figure and table legend

Figure 1 - Iron transport and homeostasis. Schematic diagram depicts major iron transport and storage proteins. Reactive iron is capable of generating hydroxyl radicals, thus iron accumulation increases the risk of oxidative stress. Abbreviations: Fe2+ (ferrous iron), Fe3+ (ferric iron), Tf (transferrin), TfR1 (transferrin receptor 1), DMT1 (divalent metal transporter 1), ZIP8/14 (ZRT/IRT-like protein 8 and 14), Fpn (ferroportin).

Figure 2 – PRISMA flow diagram. Abbreviations: WofS (Web of Science)

Figure 3 - Pathophysiology of iron in endometriosis. Schematic diagram highlighting the most established pathways involved in aberrant iron physiology in endometriotic tissues. 1. Conflicting evidence regarding systemic iron levels 2. Oxidative stress and inflammation 3.

Abnormal iron transport. Abbreviations: Fe (Iron), OS (Oxidative stress), TAS (Total antioxidant status), Fpn (Ferroportin), DMT1 (Divalent metal transporter 1), IL-1β (Interleukin 1 beta).

Table 1 – Summary table of included studies characteristics, findings and conclusions. Abbreviations: nr (not reported), PF (peritoneal fluid), MRI (magnetic-resonance imaging), LDH (lactate dehydrogenase), 8-OHdG (8-hydroxy-2'-deoxyguanosine), mRNA (messenger ribonucleic acid), RANTES (regulated upon activation, normal T cell expressed and presumably secreted), CRP (c-reactive protein), CA-125 (cancer antigen 125), ROS (reactive oxygen species), NO (nitric oxide), LPO (lipid peroxidation), TAC (total antioxidant capacity), IVF (in-vitro fertilisation), L-Ferritin (light ferritin), H-Ferritin (heavy ferritin), MDA (malondialdehyde), NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), DNA (deoxyribonucleic acid), ESC (endometrial stromal cell), DMT1 (divalent metal transporter-1), DFO (deferoxamine), IRP2 (iron-responsive element-binding protein 2), O2 (oxygen), Hb (haemoglobin), NCO4 (nuclear receptor coactivator 4), PCV (packed cell volume), ESR (erythrocyte sedimentation rate), FSH (follicle stimulating hormone), FPN (ferroportin), AOC3 (amine oxidase, copper containing 3), CF (cyst fluid), MMP-2 (matrix-metalloproteinase-2), TfR (transferrin receptor), ATP (adenosine triphosphate), OMA (ovarian endometrioma), oxyHb (oxyhaemoglobin), metHb (methaemoglobin), TAS (total antioxidant status), FRAP (ferric reducing antioxidant power), EM (endometriosis), SIRT (Sirtuin), AOPP (advanced oxidation protein products), UIBC (unsaturated iron-binding capacity), HMOX1 (haem oxygenase-1), VEGFA (vascular endothelial growth factor A), IL8 (interleukin-8), HUVEC (human umbilical vein endothelial cells), MALAT1 (metastasis associated lung adenocarcinoma 1), MUC1 (mucin-1), ADAMTS9-AS1 (ADAMTS9 antisense RNA 1), FAC (ferric ammonium citrate), Ki67 (Antigen KI-67), PARP1 (poly [ADP-ribose] polymerase 1)

Table 2 – Summary table of Newcastle-Ottawa scoring. Abbreviations: Nil

Table 1 – Summary table of included studies characteristics, findings and conclusions

First Author (year published)	Human/Animal Study	n=	Tissue types	Outcomes measured	Relevant Findings	Conclusions drawn
Akashi (2021)	Human	38		DMT-1, transferrin receptor, ferroportin, macrophages	Identified iron transporters, DMT1 upregulation, low TfR and fRM expression,	Malignant transformation of ovarian endometrioma
Alizadeh (2015)	Human	70	Serum	Iron, MDA, carbonyl	High serum iron	High serum iron indicating oxidative stress
Al-Shammaa (2020)	Human	72	Serum	Hb, PCV, ESR, Serum iron	All parameters elevated in endometriosis patients on a red meat diet	Red meat diets increase serum iron
Alvarado-Diaz (2015)	Human	10	Endometrial biopsies. Isolated endometrial stromal cells	NF-kB	Iron overload increased p65: DNA binding activity and decreased various other expressions	Iron overload has a role in endometriosis pathogenesis and development
Alvarado-Diaz (2016)	Human	55	Endometrial biopsies. Isolated endometrial stromal cells	Divalent metal transporter-1	Overexpression of DMT1 in endometriosis patients at various stages of menstrual cycle	Suggested a DMT1 modulated pathway of iron overload in endometriosis cases
Arumugam (1994)	Human	50	Peritoneal fluid, sperm incubated in PF	PF Iron conc. Acrosome reaction rates in sperm	Decrease in acrosome reaction rate was associated with an increase in iron content of PF	Endometriosis may play a role in infertility through acrosome reaction
Arumugam (1995)	Human	44	Peritoneal fluid	Iron levels, markers of free radical reactions	High iron levels seen in endometriosis. Correlated with disease severity	Raised iron in PF does not play a role in catalysing free radical reactions
Atkins (2018)	Animal: Nonhuman Primate	22	Bone marrow, liver and serum, intestinal biopsies	Iron, ferroportin	Decreased hepatic and bone marrow iron, increased ferroportin expression	Oral iron supplementation alone does not replenish iron stores in endometriosis
Bauckman (2013)	Human	nr	Ovarian ca cell lines		DFO induced apoptotic death in ovarian cells	Iron plays a role in modulating cell death in ovarian cancer cells
Benaglia (2015)	Human	39	Follicular fluid from affected and contralateral ovaru	Iron and ferritin. Oocyte retrieval rate	No difference seen in iron, ferritin higher in endometriosis	Iron does not play a role in ovarian function
Chen (2021)	Animal: Mice	nr	Murine embryos	ATP level, MMP, ROS, apoptotic and ferroptotic indices	Iron-exposure impaired embryo developmetn, increased ROS and was linked to high rates of cell death.	Iron excess in peritoneal fluid may be implicated in endometriosis-related subfertility
Chmaj-Wierzchowska (2013)	Human	86	Serum	RANTES, CRP, Iron levels, fibrinogen, leucocytes, CA-125	No difference in serum iron or inflammatory markers	No explanation for role of inflammatory factors in endometriosis
Defrere (2006)	Animal: Nude mice	24	Endo lesions, peritoneal fluid	Number of lesions, proliferation of lesions	Iron deposits mainly seen in macrophages and mesothelial cells	Possibility of iron chelation treatment in endometriosis
Fassbender (2011)	Human	40	Peritoneal biopsies	Transferrin and ferritin	Reduced ferritin mRNA expression in normal peritoneum	
Guo (2015)	Human	30	Ectopic lesions	Bilirubin, ferritin, free iron	Endometriomas of different ages varied in various features and ferritin and free iron concentration	Older endometriotic lesions have more iron content and so are wounds that undergo repeated injury and repair

Table 1 – Summary table of included studies characteristics, findings and conclusions

Hayashi (2020)	Animal: Mice	14		Offspring n, hemosiderin, oxidative stress, FSH	Iron accumulation reduced in endometriosis, causing oxidative stress.	Possible prevention mechanism of endometriosis
Iizuka (1998)	Human	nr	Ovarian lesions	Iron levels	High iron levels seen in endometriosis.	Role of iron concentration assay as a diagnostic tool for evaluation of ovarian endometriotic cyst
Imanaka (2020)	Human	83	Cyst fluid	total iron, heme iron,	Positive concentration between dysmenorrhea severity and total and heme iron	No evidence that iron indicates severity of endometriosis-related pain. May play a role in dysmenorrhea
Imanaka (2021a)	Human	307		Total iron, heme iron, free iron. Relationship with MRI	R2 value correlated with iron levels	MR relaxometry may be a better alternative to CF iron test in diagnosing OMA
Imanaka (2021b)	Human	236		Total iron, heme iron, free iron, oxuHb, 8-OHdG, metHb, antioxidants, TAC	Various iron and haem iron compounds were elevated in endometriosis	Involvement of HO-1 in regulating balance between iron-induced oxidative stress and endometriotic cyst fluid
Kizilay (2017)	Animal: Albino Wistar rats	33	Blood, endo deposits		No difference in serum iron when injected with DFO, water or curcumin. DFO and curcumin affected cell proliferation	Curcumin with/without DFO reduced cell proliferation
Kobayashi (2012)	Human	10	Eutopic endometrium, ectopic endometrium	Iron deposition, macrophage iron, oxidative stress, iron regulatory gene expression	Massive iron deposition in stroma of ovarian endometriosis, mostly in macrophages	Differential iron metabolism in ectopic endometrial stromal cells
Kokot (2021)	Human		Serum	Oxidative stress markers (TAS, FRAP, albumin, bilirubin, uric acid, iron, SIRT, AOPP)	No difference in serum iron between EM/non-EM, lower serum iron in stage IV EM compared to stage III EM	
Li (2020a)	Human	25	Follicular fluid	Transferrin, ferric ions	Reduced transferrin levels, increased follicular fluid ferric iron	Involvement of transferrin insufficiency and iron overload of follicular fluid in endometriosis related infertility
Li (2020b)	Human	32	Ectopic lesions	Ferroptosis, LPO, morphology, Ferroportin	Erastin can induce ferroptosis in ectopic endometrial stromal cells,	Role of FPN in treatment of endometriosis
Li (2021)	Human and mice	72	Peritoneal fluid	UIBC, Ferritin, Transferrin, TSAT, TIBC, ATP production, MMP, ROS, lipid peroxidation, RNA-seq	Iron overload disrupted blastocyst formation and induced lipid peroxidation via ferroptosis. Cytotoxicity was attenuated by ferroptosis inhibition . HMOX1 suppresses ferroptosis	HMOX1 suppresses ferroptosis and is upregulated in endometriosis providing a novel mechanism for treatment.
Li (2022)	Human	48	Endometrioma, eutopic endometrium	VEGFA, IL8, HUVEC	Iron overload is associated with ferroptosis in endometriomas and upregulation of VEGFA, IL8 and HUVEC	Ferroptosis in endometriomas may may trigger cytokine secretion and promote angiogenesis
Liang (2022)	Human	39	Ectopic endometrial stromal cells	Cell viability, Ferrous iron, lipid peroxidation, MDA,	MALAT1 was decreased during erastin-induced ferroptosis. MALAT 1 regulates MUC1-	Targeting of the MALAT1/MUC1 pathway could be novel therapeutic strategy.

Table 1 – Summary table of included studies characteristics, findings and conclusions

					mediated ferroptosis suppresion.	
Liu (2022)	Human	74	Serum, cyst fluid	Transferrin, Iron, ferritin, UIBC	Iron and ferritin were higher in cyst fluid than in serum.	There is limited value in serum iron metabolites as a diagnostic biomarker of endometriosis.
Lousse (2009)	Human	50	Peritoneal fluid	Macrophage ferritin, peritoneal iron, transferrin, ferritin and prohepcidin	Iron storage in peritoneal macrophages is increased in endometriosis	Relevance to targeted therapies
Milewski (2021)	Human	643	Peripheral blood	HMOX1 alleles	Endometriosis is assocaited with functional polymorphism of HMOX1	HMOX1 functional polypmorphism may play a part in endoemtriosis pathogenesis
Mori (2015)	Human	nr	Endometrium and ectopic lesions	Labile iron, catalytic iron, iron transport proteins	Higher catalytic iron in ectopic endometrial stromal cells than eutopic normal, lower ferroportin in eutopic ESCs	Ectopic ESCs play a protective role for cancer-target epithelial cells
Nagayasu (2020)	Human	77	Cyst fluid		CF iron was higher in group that was infertile due to endometriosis	Iron may play a role as a marker in predicting infertility in women with ovarian endometrioma
Ni (2022)	Human and mice	nr	Murine granulosa cells, human follicular fluid	Ferroptosis markers within granulosa cells	Follicular fluid from women with endometriosis caused iron overload-induced ferroptosis in vitro and in vivo. Iron chelation alleviated endometriosis related subfertility.	Nuclear receptor coactivator four was involved in the ferroptosis mechanism and ferroptosis further supressed oocyte maturity.
Osman (2012)	Human	38	Peritoneal fluid, serum	Iron levels	Serum iron lower in infertile endometriotic group serum but higher in peritoneal fluid	Role of oxidative stress in development and progression of endometriosis and EM-related infertility
Polak (2007)	Human	78	Peritoneal fluid	Lactoferrin levels	Lower peritoneal fluid lactoferrin in patients with minimal endometriosis than compared to controls and those with more severe endometriosis	Role of lactoferrin as a defence factor in peritoneal cavity
Polak (2018)	Human	229	Peritoneal fluid	Hb, Iron, total oxidative status, total antioxidant status	Hb, iron and oxidative status higher in endometriosis peritoneal fluid. Total antioxidant values lower.	Influence of impaired iron homeostasis on pathophysiology of peritoneal endometriosis
Rockfield (2018)	Human	nr		NCO4, H-ferritin, p21, NCO4-B	Transformed endometriotic cells had higher migratory potential	Role of NCOA4 in transition into ovarian cancer
Sanchez (2014)	Human	13	Follicular fluid	Total iron, L-ferritin, H-Ferritin, oocyte retrieval	Iron levels higher in endometriosis, ferritin expression varied depending on follicle location	Follicle aspiration at sites distant from endometrioma may increase probability of retrieving oocytes when surgical removal of endometrioma is not an option
Shigetomi (2022)	Human	235	Cyst fluid	Total, haem and free iron, oxyhaemoglobin,	All iron types, methaemoglobin/oxyhaemoglobin ration and bilirubin, were higher in endometriosis.	Iron induced oxidative stress may ecceed the bilirubin-dependent antioxidant capability

Table 1 – Summary table of included studies characteristics, findings and conclusions

				methhaemoglobin, bilirubin		
Singh (2013)	Human	340	Follicular fluid	ROS, NO, LPO, Iron, TAC,	Increased reactive oxygen species in patients who failed IVF	Possible benefits of multivitamin/mineral supplementation for patients undergoing IVF
Takahashi (1996)	Human	24	Ovarian endometriomas	MRI cyst density, iron concentration	Density correlated with iron concentration	Role of MRI and T2 signal intensity in evaluating cyst fluid characteristics of endometriomas
Takenka (2017)	Human	9		Catalytic iron, O2 levels, IRP2	High iron deposition and IRP in ESCs and cysts. Increase in IRP2 expression upregulated intracellular iron.	Insufficient oxygen in cysts may cause stabilization of IRP2 against iron-mediated degradation
Thezenas (2020)	Human		Ectopic lesions	Oxidative protein markers, LPO,	All seen in ectopic lesions	AOC3 inhibitors had analgesic effects in inflammatory pain models, possible translational applicability
Van Langendonckt (2002)	Human	70	Peritoneal fluid, serum samples, endometrium, endomtriotic lesions, normal peritoneum	Iron levels, ferritin levels	Iron and ferritin higher in endometriotic PF. Levels varied based on peritoneum adjacent to differently coloured lesions.	Relation of iron deposits to presence of endometriotic lesions
Van Langendonckt (2004)	Animal: Nude Mice	57	Endo lesions	Iron levels	No deposits found on glandular epithelium, low proliferative index in glandular epithelium	Iron conglomerates may trigger oxidative damage and chronic inflammation
Wan (2022a)	Human	18	Endometrial stromal cells	Fibulin-1	Fibulin-1 showed increased expression in both eutopic and ectopic endometrium in women with endometriosis and promoted cell viability. Inhibition of Fibulin 1 triggered ferroptosis mediated cell death	The fibulin-1/ ferroptossi pathway has an important role in endometriosis and may be a treatment target
Wan (2022b)	Human and mice	17	Endometrial stromal cells	ADAMTS9-AS1 expression, MDA, ROS, GPX4	ADAMTS9-AS1 was upregulated in ectopic endometriu, and knockdown decreased cell viability. Ferroptosis inhibition blocked the effects of ADAMTS9-AS1	ADAMTS9-AS1 acts as a competing endogenous RNAand may be a therapeutic target
Wolfler (2013)	Human	80	Pertioneal fluid	Hemopexin and heme	Heme levels not significantly different, no correlation between heme and hemopexin	Hemopexin downregulated in endometriotic PF.
Woo (2020)	Human			Ferritin, MMP-2, ROS, NFkB	Overexpression of ferritin in endometriotic tissue	Contribution of iron to migration abilities of human endometriotic cells
Yamaguchi (2008)	Human	36	Cyst fluuid	Free iron, catalutic iron, LDH, lipid peroxidase, 8-OHdG	Increased free iron and iron deposits in endometriotic cysts	Abundant free iron possibly facilitation mutation rate and therefore malignant change

Table 1 – Summary table of included studies characteristics, findings and conclusions

Yoshimoto (2015)	Human	36	Endo cyst fluid	Total iron, heme iron, free iron	Higher iron related compound levels in endometriosis	Importance of iron-related compounds as biomarkers in malignant transformation of endometriosis
Zhou (2022)	Human	103	Eutopic endometrium, ectopic endometrium	FAC, Ki67, ROS, PARP1 and SIRT1 expression	FAC inhibited cell growth, induced oxidative stress and caused apoptosis. FAC impaired PARP1 expression.	Iron overload in ESCs may be involved in the inhibition of cell proliferation

	Akashi (2021)	Alizadeh (2015)	Al-Shammaa (2020)	Alvarado-Diaz (2015)	Alvarado-Diaz (2016)	Arumugam (1994)	Arumugam (1995)	Atkins (2018)	Bauckman (2013)	Benaglia (2015)	Chen (2021)	Chmaj-Wierzchowska (2013)	Defrere (2006)	Fassbender (2011)	Guo (2015)	Hyashi (2020)	Iizuka (1998)	Imanaka (2020)	Imanaka (2021a)	Imanaka (2021b)	Kizilay (2017)	Kobayashi (2012)	Kokot (2021)	Li (2020a)	Li (2020b)	Li (2021)	Li (2022)	Liang (2022)	Liu (2022)	Lousse (2009)	Milewski (2021)	Mori (2015)	Nagayasu (2020)	Ni (2022)	Osman (2012)	Polak (2007)	Polak (2018)	Rockfield (2018)	Sanchez (2014)	Shigetomi (2022)	Singh (2013)	Takahashi (1996)	Takenka (2017)	Thezenas (2020)	Van Langendonckt (2002)	Van Langendonckt (2004)	Wan (2022a)	Wan (2022b)	Wolfler (2013)	Woo (2020)	Yamaguchi (2008)	Yoshimoto (2015)	Zhou (2022)
Is the case definition adequate? a) Yes, with independent validation* b) Yes, e.g., record linkage or based on self report c) No description	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Representativeness of cases a) Consecutive or obviously representative series of cases* b) Potential for selection bias or not stated	*	*	*		*		*	*		*	*		*		*	*		*	*	*	*		*	*	*	*	*	*	*	*	*		*	*	*	*	*		*		*	*			*	*	*	*	*			*	*
Selection of controls a) Community controls* b) Hospital controls c) No description		*			*						*		*			*					*		*			*	*	*	*		*			*												*	*	*					*
Definition of controls a) No history of disease (endpoint)* b) No description of source		*	*		*		*	*	*		*		*	*		*						*	*	*		*	*	*	*	*	*	*		*			*				*				*	*	*	*		*		*	*
Comparability of cases and controls on the basis of the design or analysis a) Study controls for phase cycle*					*		*						*	*		*					*			*	*	*				*					*	*	*		*						*	*		*					*
b) Study controls for any additional factor*		*				*		*		*	*	*	*	*		*			*		*		*	*	*	*	*	*	*	*	*			*	*		*	*	*	*	*				*	*		*					*
Ascertainment of endometriosis a) Secure record (e.g. surgical or research record)* b) Structured interview where blind to case/control status* c) Interview not blinded to case/control status d) Written self report or medical record only e) No description					*	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Same method of ascertainment for cases and controls a) Yes* b) No						*	*	*				*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*			*	*				*	*	*	*		*		*	*
Non-response rate a) Same rate for both groups* b) Non-respondent rate described c) Rate different between cases and controls with no description	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Total score	3	6	4	2	6	5	7	7	3	5	7	5	9	7	5	9	4	5	6	4	8	5	8	8	7	9	8	8	7	8	8	5	5	8	7	6	8	4	6	5	7	4	3	3	8	9	7	9	4	5	3	6	9

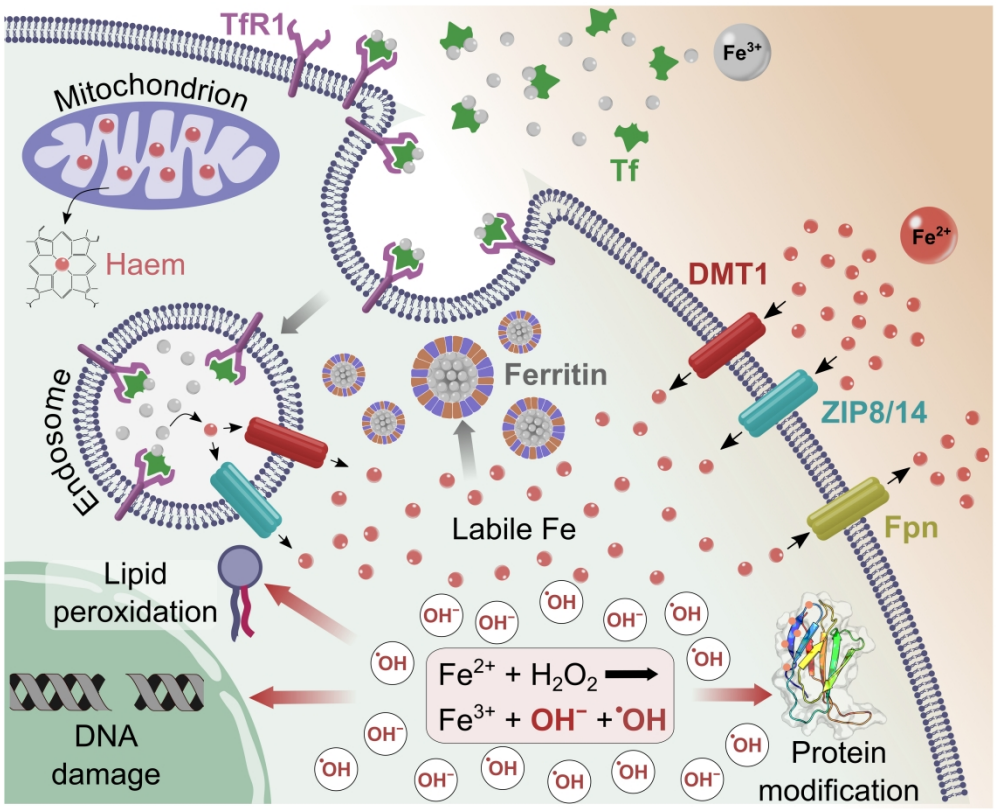

Figure 1 - Iron transport and homeostasis. Schematic diagram depicts major iron transport and storage proteins. Reactive iron is capable of generating hydroxyl radicals, thus iron accumulation increases the risk of oxidative stress. Abbreviations: Fe2+ (ferrous iron), Fe3+ (ferric iron), Tf (transferrin), TfR1 (transferrin receptor 1), DMT1 (divalent metal transporter 1), ZIP8/14 (ZRT/IRT-like protein 8 and 14), Fpn (ferroportin).

532x430mm (236 x 236 DPI)

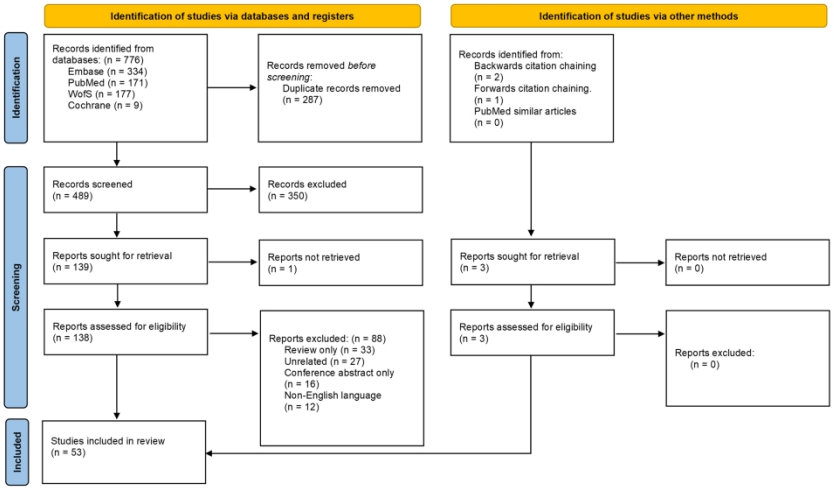

Figure 2 – PRISMA flow diagram. Abbreviations: WofS (Web of Science)

338x190mm (300 x 300 DPI)

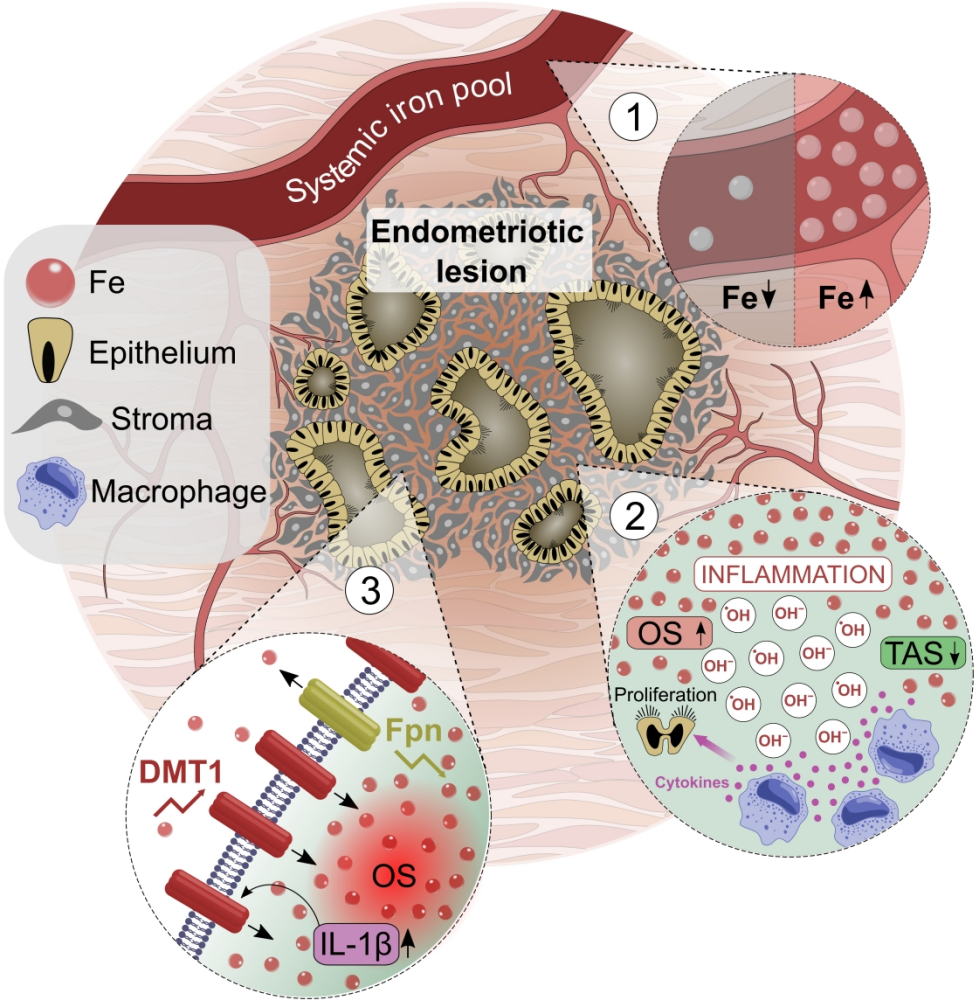

Figure 3 - Pathophysiology of iron in endometriosis. Schematic diagram highlighting the most established pathways involved in aberrant iron physiology in endometriotic tissues. 1. Conflicting evidence regarding systemic iron levels 2. Oxidative stress and inflammation 3. Abnormal iron transport. Abbreviations: Fe (Iron), OS (Oxidative stress), TAS (Total antioxidant status), Fpn (Ferroportin), DMT1 (Divalent metal transporter 1), IL-1β (Interleukin 1 beta).

507x520mm (236 x 236 DPI)

PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Lines 1-2 and 168
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Lines 25-81
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Lines 107-139
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Lines 165-166
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Lines 180-186
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Lines 174-175
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Lines 176-178
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Lines 188-198
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Lines 200-207
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Lines 200-202
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Lines 200-202
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Lines 209-210
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Lines 203-207
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Lines 203-207
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Lines 203-207
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Lines 203-207
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Lines 203-207

PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Lines 203-207
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Lines 203-207
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Lines 210-211
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Lines 210-211
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Lines 214-210
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Lines 214-220
Study characteristics	17	Cite each included study and present its characteristics.	Lines 222-225 Table I
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Table II
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Throughout results
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Throughout results
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	n/a
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	n/a
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	n/a
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Throughout results
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Throughout results
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Lines 756-938
	23b	Discuss any limitations of the evidence included in the review.	Lines 928-931
	23c	Discuss any limitations of the review processes used.	Lines 928-931

PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where item is reported
	23d	Discuss implications of the results for practice, policy, and future research.	Throughout discussion
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Lines 80-81
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Lines 80-81
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	n/a
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Lines 951-953
Competing interests	26	Declare any competing interests of review authors.	Line 955
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	n/a

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71
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