**UNIVERSIDADE FEDERAL DE MINAS GERAIS**
**Faculdade de Farmácia**
**Programa de Pós-graduação em Análises Clínicas e Toxicológicas**

Jéssica Abdo Gonçalves Tosatti

# INFLUÊNCIA DA SUPLEMENTAÇÃO DE COMPOSTOS BIOATIVOS NA RESPOSTA INFLAMATÓRIA EM DOENÇAS CRÔNICAS: revisões sistemáticas e meta-análises de ensaios clínicos randomizados

Belo Horizonte
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Orientadora: Profa. Dra. Karina Braga Gomes Borges

Coorientadora: Profa. Dra. Ana Lúcia Cândido

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**"Influência da suplementação de compostos bioativos na resposta inflamatória em doenças crônicas: revisões sistemáticas e meta-análises de ensaios clínicos randomizados"**

**JÉSSICA ABDO GONÇALVES TOSATTI**

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## RESUMO

Atualmente, há um crescente corpo de evidências indicando que inúmeros compostos bioativos, a exemplo dos ácidos graxos ômega-3 (AG ω-3) e do resveratrol, podem influenciar diretamente o estado inflamatório crônico, comumente observado em várias desordens, como na Síndrome dos Ovários Policísticos (SOP) e na Doença de Alzheimer (DA), exercendo um papel anti-inflamatório nesses casos. Considerando a importância do padrão alimentar na modulação da resposta imune, faz-se necessária uma compilação das evidências relacionadas aos mecanismos biológicos destes compostos nutricionais em doenças crônicas. Em complementação às hipóteses anteriores, a avaliação dos efeitos da metformina nos níveis de marcadores de hiperandrogenismo em mulheres com SOP surgiu como uma pergunta clínica a ser compreendida durante o manejo destas pacientes. Portanto, o presente estudo tem por objetivos (1) avaliar se a inflamação subclínica é importante na fisiopatologia da SOP; (2) investigar a influência do AG ω-3 e do resveratrol em marcadores de inflamação ou escore cognitivo, respectivamente, em pacientes com SOP ou DA, e; (3) investigar a influência do uso da metformina nos marcadores androgênicos na SOP, sendo os dois últimos itens avaliados por meio de revisão sistemática da literatura. Para tanto, foram desenvolvidos quatro estudos – um estudo primário e três revisões sistemáticas. Em relação ao estudo primário, as citocinas plasmáticas foram avaliadas por citometria de fluxo. No que se refere às revisões sistemáticas, estas foram compreendidas por pesquisas, em bases de dados eletrônicas, por artigos indexados, de acordo com a questão de pesquisa do estudo. Por fim, para o desenvolvimento das meta-análises, as estimativas agrupadas das diferenças médias ponderadas (WMD) e as diferenças médias padronizadas (SMD) foram calculadas e o modelo de efeitos aleatórios ou fixos foram adotados, de acordo com os dados primários obtidos. Em relação ao estudo primário, foram observados níveis mais baixos de fator de necrose tumoral (TNF) e razões de TNF com interleucinas (IL) 2, 4 e 6 diminuídas em pacientes com SOP em relação ao grupo controle ($p < 0,05$). Na primeira revisão sistemática, com meta-análise foi evidenciada diminuição significativa na proteína C reativa de alta sensibilidade [SMD -0,29 (IC 95% -0,56 a -0,02) mg/l] e um aumento nos níveis de adiponectina [WMD 1,42 (IC 95% 1,09 a 1,76) ng/ml)] no grupo intervenção AG ω-3 quando comparado com o grupo placebo. Na segunda revisão sistemática, pela análise de sensibilidade, os valores de *Free Androgen Index*

(FAI) [SMD: -0,42 (IC 95% -0,67 a -0,16) pontos] e os níveis de testosterona total [SMD: -0,24 (IC 95% -0,40 a -0,07) pontos] foram menores no grupo tratado com metformina, quando comparado ao grupo controle. Por fim, na terceira revisão sistemática, os achados demonstraram que ainda há poucos estudos em humanos, mas mostraram que o resveratrol atua retardando o comprometimento cognitivo em pacientes com DA, administrado isoladamente ou quando associado à dextrose e malato. Os resultados sugerem um desequilíbrio entre citocinas pró e anti-inflamatórias, com produção de citocinas contrarregulatórias na SOP. Ainda, que a suplementação de AG ω-3 poderia reduzir o estado inflamatório em mulheres com SOP, por meio da diminuição da Proteína C Reativa de Alta Sensibilidade (hs-PCR) e do aumento dos níveis de adiponectina; e que a metformina pode ser usada para melhorar o hiperandrogenismo neste grupo. Por fim, a suplementação com resveratrol parece influenciar no declínio cognitivo e funcional progressivo em pacientes com DA, quando comparado ao grupo placebo. Ademais, em relação às revisões sistemáticas, sugere-se a realização de novos estudos primários com amostras maiores, intervenções mais longas, padronização da dosagem da intervenção e melhor qualidade metodológica dos ensaios clínicos para confirmação dos resultados.

Palavras-chave: inflamação; Síndrome do Ovário Policístico; compostos fitoquímicos; Doença de Alzheimer; metformina, revisão sistemática.

## ABSTRACT

Currently, there is a growing body of evidence indicating that numerous bioactive compounds, such as omega-3 fatty acids (ω-3 AG) and resveratrol, can directly influence the chronic inflammatory state, commonly observed in several disorders, such as Polycystic Ovary Syndrome (PCOS) and in Alzheimer's Disease (AD), exerting an anti-inflammatory role in these cases. Considering the importance of the dietary pattern in modulating the immune response, it is necessary to compile evidence related to the biological mechanisms of these nutritional compounds in chronic diseases. In addition to the previous hypotheses, the evaluation of the effects of metformin on the levels of hyperandrogenism markers in women with PCOS emerged as a clinical question to be understood during the management of these patients. Therefore, the present study aims to (1) assess whether subclinical inflammation is important in the pathophysiology of PCOS; (2) to investigate the influence of ω-3 AG and resveratrol on markers of inflammation or cognitive score, respectively, in patients with PCOS or AD, and (3) to investigate the influence of metformin use on androgenic markers in PCOS, with the last two items being evaluated through a systematic review of the literature. To this end, four studies were developed – a primary study and three systematic reviews. In relation to the primary study, plasma cytokines were assessed by flow cytometry. On the other hand, systematic reviews were comprised of systematic searches, in databases, by indexed articles, according to the research question of the study. Finally, for the development of the analysis goals, the grouped estimates of the weighted average differences (WMD) and the standardized average differences (SMD) were calculated and the model of random or fixed effects was adopted, to measure the grouped results, according to the primary data obtained. Relative to the primary study, lower levels of tumor necrosis factor (TNF) and decreased ratios of TNF with interleukins (IL) 2, 4 and 6 were observed in PCOS patients compared to the control group ($p < 0.05$). In the first systematic review, with a goal, a significant change in the analysis of high-sensitivity C-reactive protein (hs-CRP) was evidenced [SMD -0.29 (95% CI -0.56 to -0.02) mg/l] and an increase in adiponectin levels [WMD 1.42 (95% CI 1.09 to 1.76) ng/ml)] in the intervention group ω-3 AG when compared with the placebo group. In the second systematic review, through sensitivity analysis, the Free Androgen Index (FAI) values [SMD: -0.42 (95% CI -0.67 to -0.16) points] and total testosterone levels [SMD: -0.24 (95% CI -0.40 to -0,07) points] were

lower in the metformin-treated group when compared to the control group. Finally, in the third systematic review, the findings showed that there are still few studies in humans, but they showed that resveratrol acts by delaying cognitive impairment in AD patients, administered alone or when associated with dextrose and malate. The results suggest an imbalance between pro- and anti-inflammatory cytokines, with production of counterregulatory cytokines in PCOS. Furthermore, that ω-3 AG supplementation could reduce the inflammatory state in women with PCOS, by decreasing hs-CRP and increasing adiponectin levels; and that metformin can be used to improve hyperandrogenism in this group. Finally, resveratrol supplementation appears to influence progressive cognitive and functional decline in AD patients when compared to the placebo group. Furthermore, in relation to systematic reviews, new primary studies with larger samples, longer interventions, standardization of intervention dosage and better methodological quality of clinical trials are suggested to validate the results.

Keywords: inflammation; Polycystic Ovary Syndrome; phytochemicals; Alzheimer Disease; metformin; systematic review.

## LISTA DE FIGURAS

## LISTA DE QUADROS

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| Ácido Alfa-Linolênico | ALA |
| Ácido Docosahexaenoico | DHA |
| Ácido Eicosapentaenoico | EPA |
| Ácidos Graxos | AG |
| Ácidos Graxos Monoinsaturados | MUFA |
| Ácidos Graxos Poliinsaturados | PUFAS |
| Capacidade Total Antioxidante | TAC |
| Cochrane Library Central Register of Controlled Trials | CENTRAL |
| Comitê de Ética e Pesquisa | COEP |
| Comprehensive Meta-Analysis | CMA |
| *Cytometric Bead Array* | CBA |
| Dehidroepiandrosterona | DHEAS |
| Descritores em Ciências da Saúde | DeCS |
| Diabetes Mellitus Gestacional | DMG |
| Diabetes Mellitus Tipo 2 | DM2 |
| Diferenças Médias Padronizadas | SMD |
| Diferenças Médias Ponderadas | WMD |
| Doença de Alzheimer | DA |
| Doenças Cardiovasculares | DCV |
| Doenças Crônicas Não Transmissíveis | DCNT |
| Emaranhados Neurofibrilares | NFT |
| Ensaios Clínicos Randomizados | ECR |
| Escala de Avaliação da Doença de Alzheimer | ADAS-cog |
| Estudo Cooperativo de Atividades de Vida Diária da Doença de Alzheimer | ADCS-ADL |
| Fator de Necrose Tumoral | TNF |
| Fator Nuclear | NF |
| Free Androgen Index | FAI |
| Genome Wide Association Study | GWAS |
| Globulina Ligadora de Hormônios Sexuais | SHBG |
| Glutationa | GSH |

| | |
|---|---|
| Grading of Recommendations, Assessment, Development and Evaluation | GRADE |
| Hipertensão Arterial Sistêmica | HAS |
| Homeostasis Model Assessment-IR | HOMA-IR |
| Índice de Massa Corporal | IMC |
| Interleucinas | IL |
| Intervalo de Confiança | IC |
| Latin American and Caribbean Health Sciences | LILACS |
| Lipopolissacarídeo | LPS |
| Malonaldeído | MDA |
| Medical Literature Analysis and Retrieve System Online | MEDLINE |
| Medical Subject Headings | MeSH |
| Meta-Análise | MA |
| Miniexame do Estado Mental | MMSE |
| National Institute of Neurological Disorders and Stroke | NINDS |
| Ômega-3 | ω-3 |
| Organização Mundial de Saúde | OMS |
| Óxido Nítrico | NO |
| Ppeptídeo β Amilóide | Aβ |
| Preferred Reporting Items for Systematic Reviews and Meta-Analyses | PRISMA |
| Proliferador de Peroxissoma | PPAR |
| Proteína C Reativa de Alta Sensibilidade | hs-PCR |
| Registro Prospectivo Internacional de Revisões Sistemáticas | PROSPERO |
| Resistência à Insulina | RI |
| Revisões Sistemáticas | RS |
| Risk of Bias 2 | RoB 2.0 |
| Síndrome dos Ovários Policísticos | SOP |
| Síndrome Metabólica | SM |
| Statistical Package for the Social Sciences | SPSS |
| Transdutor de Sinal e Ativador da Transcrição | STAT |
| United States National Library of Medicine | U.S. NLM |
| Universidade Federal de Minas Gerais | UFMG |

| | |
|---|---|
| Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico | VIGITEL |
| Cumulative Index to Nursing and Allied Health Literature | CINAHL |
| Web of Science | WOS |

**SUMÁRIO**

## 1 INTRODUÇÃO

Nas últimas décadas, um número extenso de estudos objetivou avaliar o papel da inflamação sistêmica crônica, de baixo grau, como um mecanismo em comum a várias doenças associadas, como o diabetes mellitus tipo 2 (DM2), (LONTCHI-YIMAGOU et al., 2013) as doenças cardiovasculares (DCV) (WIRTZ; von KÄNEL, 2017), a Síndrome dos Ovários Policísticos (SOP) (PATEL, 2018), cânceres (SINGH et al, 2019) e doenças demenciais, como a Doença de Alzheimer (DA) (OZBEN; OZBEN, 2019).

A partir deste interesse foi observado, também, que a inflamação sistêmica crônica, e de baixo grau, pode ser causada ou modulada por um padrão alimentar não saudável. A exemplo, tem-se o padrão alimentar ocidental, comumente associado à hiperglicemia aguda induzida pela carga oral de glicose, resposta pró-inflamatória e estresse oxidativo, gerando espécies reativas de oxigênio por meio de diferentes mecanismos, com incremento de glicose pós-prandial (BARREA et al., 2018). Há, portanto, um crescente corpo de evidências indicando que a combinação da quantidade e qualidade dos alimentos, principalmente padrões dietéticos, com alta ingestão calórica ou baixa em micronutrientes, e a susceptibilidade genética são capazes de influenciar o estado inflamatório crônico em diversas patologias (CENA; CALDER, 2020). Consequentemente, o reconhecimento do papel emergente do processo inflamatório induzido pela dieta tem sido acompanhado por esforços para identificar fatores e padrões dietéticos que possam promover ou inibir o processo inflamatório, afetando assim o risco e a gravidade da doença (BARREA et al., 2018).

Quanto à SOP, condição definida como uma doença endócrina, resultante de um desbalanço hormonal, acometendo de 5% a 18% das mulheres em idade reprodutiva, é observado um estado de inflamação crônica e de baixo grau e resistência à insulina (RI), quadro este semelhante ao de outras doenças crônicas não transmissíveis (DCNT), como a obesidade, o DM2 e as DCV (BARREA et al., 2018). Níveis aumentados de marcadores de inflamação, como a proteína C reativa de alta sensibilidade (hs-PCR), o fator de necrose tumoral (TNF) e algumas interleucinas (IL), como a IL-6 e 18, foram relatados em estudos recentes (BEDNARSKA; SIEJKA, 2017; DABRAVOLSKI et al., 2021; PATEL, 2018).

O hiperandrogenismo está presente na maioria das mulheres diagnosticadas com a síndrome, sendo definido pela elevação dos hormônios androgênicos e seus percursores (WALTERS et al., 2018). Na SOP, o hiperandrogenismo, juntamente com a inflamação subcrônica, a RI e a obesidade, criam um ciclo que tornam a síndrome ainda mais complexa. Tanto a hiperinsulinemia quanto o hiperandrogenismo atuam como promotores da inflamação na SOP, e a tríade de RI, hiperandrogenismo e inflamação de baixo grau funciona bidirecionalmente, fazendo-se necessário o uso de fármacos, como a metformina, reduzindo o quadro hiperinsulinêmico, ocorrendo a diminuição do estímulo para a esteroidogênese ovariana (VIOLLET *et al*., 2012; SHORAKAE et al., 2015).

A DA compreende entre 60 e 70% dos casos diagnosticados de demência, tendo como características fisiopatológicas mais comuns a deposição de peptídeo β amilóide (Aβ) e a síntese de emaranhados neurofibrilares (NFT) de proteína *tau* (YANG et al., 2017). Além disso, a inflamação neuronal crônica e um aumento de mediadores pró-inflamatórios, no parênquima cerebral, também podem ser identificados (KARUNAWEERA et al., 2015). Ainda, a neurodegeneração na DA está associada ao metabolismo desregulado de lipídios e carboidratos, inflamação mediada por citocinas, aumento do estresse oxidativo e celular, morte celular contínua e comprometimento vascular (SARAHIAN et al., 2021).

Dentre os fatores de risco modificáveis, vários nutrientes são conhecidos por modular a resposta inflamatória e contribuir para a proteção e tratamento de doenças crônicas. Evidências científicas demonstraram que os inúmeros constituintes bioativos de frutas e vegetais, como polifenóis, ácidos graxos poliinsaturados (PUFAS), vitaminas, minerais e fibras, podem atuar individualmente e de forma sinérgica para fornecer um alto valor nutricional e fatores anti-inflamatórios importantes, em doenças crônicas, como é o caso dos ácidos graxos ômega-3 (AG ω-3), na SOP e do resveratrol, na DA (LIU, 2013; SLAVIN; LLOYD, 2012). Ainda, durante a realização dos atendimentos às pacientes com SOP e do desenvolvimento do trabalho, surgiu a necessidade de se reunir as evidências sobre o efeito da metformina nos níveis de marcadores de hiperandrogenismo neste grupo (OHARA et al., 2021).

Considerando a importância do padrão alimentar na modulação do sistema imune, e a existência de vários nutrientes e compostos bioativos capazes de influenciar na resposta anti-inflamatória, como os polifenóis e os AG insaturados (MCGRATTAN et

al., 2019), faz-se necessário identificar, selecionar, avaliar e sintetizar as evidências relevantes disponíveis sobre os compostos nutricionais na modulação da resposta imune na SOP e na DA. Dessa forma, o presente estudo objetiva responder sobre o real benefício do tratamento com resveratrol, a partir de suplementação, na função cognitiva de pacientes diagnosticados com DA, bem como o papel do ω-3 na inflamação e no estresse oxidativo em pacientes com SOP, por meio de revisões sistemáticas, com meta-análise, o que poderá influenciar na mudança de conduta pela adoção destes compostos no tratamento dos pacientes. Ademais, a avaliação dos efeitos da metformina nos níveis de marcadores androgênicos surge com o intuito de elucidar o papel desse medicamento no controle do hiperandrogenismo no grupo com SOP.

# 2 REFERENCIAL TEÓRICO

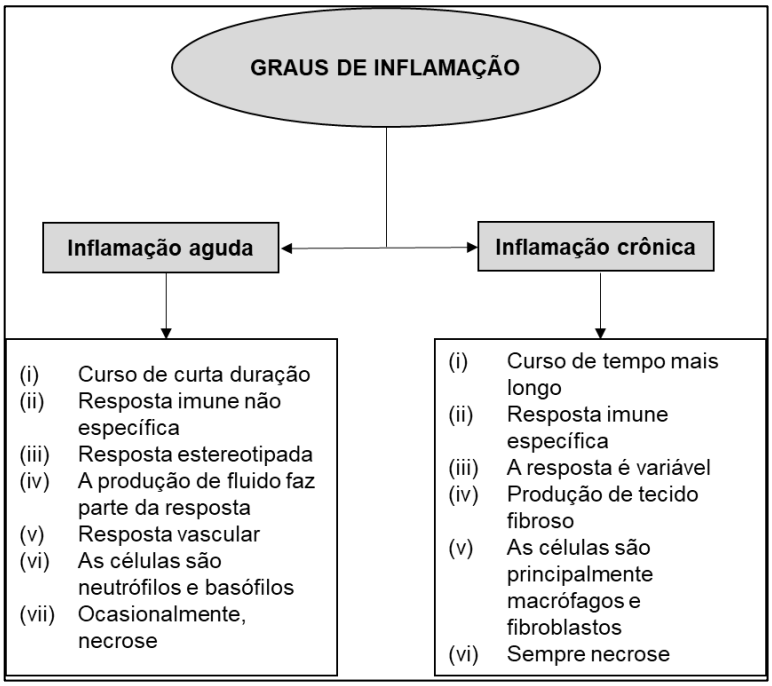
## 2.1 Inflamação

A inflamação pode ser definida como uma resposta do sistema imune a estímulos prejudiciais, como patógenos, células danificadas, presença de compostos tóxicos ou irradiação. Esta atua na remoção destes estímulos, potencializando a reparação tecidual, e promovendo a resolução do dano sendo, portanto, um mecanismo de defesa vital para a saúde (NATHAN; DING, 2010). A inflamação inclui uma longa cadeia de reações moleculares, imunológicas, bioquímicas e fisiológicas projetadas para restaurar um tecido (ARULSELVAN et al., 2016).

Um processo inflamatório, aos níveis celular e tecidual, inclui uma série de respostas como dilatação das vênulas e arteríolas, aumento da permeabilidade dos vasos e fluxo sanguíneo, recrutamento leucocitário e liberação de mediadores químicos pró-inflamatórios (ARULSELVAN et al., 2016; SCHMID-SCHÖNBEIN, 2006). Desta forma, a inflamação é um mecanismo de defesa que visa cessar a causa inicial da lesão celular e as possíveis consequências de tal lesão. No entanto, uma cascata de inflamação, que não atinge o estado de resolução, pode contribuir para desordem de órgãos e morte celular (SCHMID-SCHÖNBEIN, 2006).

A inflamação em geral consiste em fases aguda e crônica separadas, embora possa ocorrer uma sobreposição entre estas duas fases. A **Figura 1** apresenta de forma sucinta a classificação da inflamação categorizada por duração e funções imunológicas (MARKIEWSKI; LAMBRIS, 2007; SCHMID-SCHÖNBEIN, 2006). Embora os processos de resposta inflamatória dependam da natureza precisa do estímulo inicial e de sua localização no corpo, todos eles compartilham mecanismos comuns, que podem ser resumidos da seguinte forma: (1) os receptores de padrão de superfície celular reconhecem estímulos prejudiciais; (2) as vias inflamatórias são ativadas; (3) marcadores inflamatórios são liberados; e (4) células inflamatórias são recrutadas (CHEN et al., 2017).

**Figura 1 -** Classificação da inflamação categorizada por duração e funções imunológicas.




Fonte: adaptado de Arulselvan et al., 2016.

Em relação às etapas da resposta inflamatória, a inflamação aguda possui curta duração, sendo suas principais características: (1) o extravasamento de proteínas plasmáticas ou fluidos e; (2) o recrutamento de leucócitos para uma área extravascular (MARKIEWSKI; LAMBRIS, 2007). Essas reações celulares e vasculares são mediadas por fatores químicos, sendo responsáveis pelos sintomas clínicos clássicos da inflamação, como inchaço, vermelhidão, dor, calor e perda de função, associados à reação do tecido conjuntivo vascularizado (MARKIEWSKI; LAMBRIS, 2007).

Ainda na fase aguda, os leucócitos, principalmente os granulócitos, são recrutados ao longo de um gradiente quimiotático para o local da lesão, sendo o processo mediado por citocinas, quimiocinas e proteínas de fase aguda, com o intuito de neutralizar a resposta inflamatória, ou minimizar a lesão celular. Este processo de mitigação contribui para a restauração da homeostase do tecido e resolução da inflamação aguda, podendo ser suficiente para resolver o dano (GERMOLEC et al., 2018; ZHOU; HONG; HUANG, 2016).

No que diz respeito à inflamação crônica, não é possível identificar um único estímulo real. Isto significa que esta possui quadro persistente, devido à exposição prolongada

a estímulos inflamatórios, ou a uma reação inadequada a moléculas próprias (GERMOLEC et al., 2018). Geralmente ocorre por meio de infecções, que não são solucionadas por mecanismos de proteção endógena, ou a partir de algum outro mecanismo de estímulo contínuo, como ocorre na obesidade (MONTEIRO; AZEVEDO, 2010). Nesta fase, as populações de células imunes ativadas mudam para incluir um fenótipo mononuclear, podendo ocorrer danos aos tecidos e fibrose (GERMOLEC et al., 2018). É importante ressaltar que o processo molecular e celular da inflamação crônica é variado e dependente do tipo de célula e órgãos inflamados (EAVES-PYLES, 2008).

Os marcadores inflamatórios são aplicados clinicamente para indicar processos biológicos normais *versus* patogênicos e avaliar respostas a intervenções terapêuticas. Os marcadores inflamatórios podem ser preditivos de doenças inflamatórias e se correlacionar com as causas e consequências de várias doenças, como DCV, metabólicas e infecções (CARRERO et al., 2008). Os estímulos ativam células inflamatórias, como macrófagos, e induzem a produção de citocinas pró-inflamatórias, como as IL-1β e IL-6, TNF, proteínas e enzimas inflamatórias. Essas moléculas podem servir potencialmente como biomarcadores para diagnóstico de doenças, prognóstico e tomada de decisão terapêutica (GOLDSTEIN et al., 2009).

O número de doenças relacionadas com o processo inflamatório é vasto, englobando doenças infecciosas, a exemplo das hepatites virais, doenças autoimunes, como o lúpus eritematoso sistêmico e a artrite reumatóide, e crônicas, como DM2, obesidade, aterosclerose, gota, artrite reumatoide e cânceres. Ainda, têm-se observado resposta inflamatória em desordens metabólicas e endócrinas como a SOP e em processos demenciais, como na DA (CHEN et al., 2017; SARAHIAN et al., 2021).

## 2.2 Síndrome dos Ovários Policísticos

A Síndrome dos Ovários Policísticos (SOP) é definida como uma das mais importantes doenças endócrinas decorrente de um desequilíbrio hormonal que atinge 5% a 18% das mulheres em idade reprodutiva, tornando-se um importante problema de saúde pública diante das comorbidades e prevalências apresentadas (ASRM/ESHRE, 2018). A síndrome é caracterizada por sintomas como irregularidade menstrual, infertilidade, ciclos anovulatórios, hiperandrogenismo clínico e bioquímico, além de outras

manifestações metabólicas, que afetam de 30% a 70% das mulheres diagnosticadas com SOP (FANG et al, 2017).

O principal comprometimento metabólico apresentado por pacientes com SOP inclui o efeito primário da RI no músculo e tecido adiposo, com diminuição da sensibilidade à insulina e redução em até 40% da resposta insulínica nestes tecidos, quadro similar ao observado no DM2 (NORMAN et al., 2007; ROSENFIELD; EHRMANN, 2016). Este efeito é observado inclusive em pacientes não-obesas, e muitas vezes sem relação ao grau de adiposidade, sendo que a RI acomete cerca de 75% das pacientes com diagnóstico de SOP (TEED; DEEKS; MORAN, 2010). Há ainda a ocorrência de hiperinsulinemia compensatória, associada a disfunção intrínseca de células β, DM2, diabetes mellitus gestacional (DMG), hiperlipidemia, risco aumentado para DCV, obesidade, apneia do sono, doença hepática gordurosa não alcoólica e Síndrome Metabólica (SM) (TEED; DEEKS; MORAN, 2010).

O desequilíbrio hormonal, resultante da SOP, pode influenciar na saúde reprodutiva, metabólica e psicológica das pacientes. As manifestações clínicas podem incluir puberdade precoce, acne, alopecia, seborreia, ciclos menstruais irregulares, hirsutismo, infertilidade e complicações na gravidez (VANKY et al., 2004). Ansiedade, depressão e não aceitação da imagem corporal são comorbidades psicológicas frequentes (BLAY; AGUIAR; PASSOS, 2016).

Quanto à sua etiologia, a SOP é considerada uma doença complexa, na qual variantes genéticas predisponentes interagem com fortes influências ambientais para resultar em diferentes fenótipos (ESCOBAR-MORREALE; LUQUE-RAMIREZ; SAN MILLAN, 2005). Esses fatores ambientais possivelmente incluem fatores dietéticos e de estilo de vida que são fortemente influenciados pela etnia. Assim, os genes relacionados à SOP podem diferir dependendo da população estudada. Essas diferenças poderiam explicar, pelo menos em parte, as dificuldades em replicar GWAS (*Genome Wide Association Study*) em populações de origens diversas (ESCOBAR-MORREALE; LUQUE-RAMIREZ; SAN MILLAN, 2005).

O diagnóstico da SOP ainda é dificultado diante da sua heterogeneidade clínica, gerando uma série de fenótipos. Diante das dificuldades apresentadas relacionadas ao diagnóstico, em 2013, foi realizada uma revisão dos critérios diagnósticos pelas Sociedades Americana de Medicina Reprodutiva e Europeia de Embriologia Humana

e Reprodução (ASRM/ESHRE, 2018). Para tanto, foram sugeridos que os Critérios de *Rotterdam* (WANG; MOL, 2017) para o diagnóstico da SOP fossem aplicados a mulheres adultas e que não estivessem na menopausa. Dentre os critérios apresentados, a SOP seria diagnosticada quando do aparecimento de, no mínimo, dois dos três critérios propostos apresentados no **Quadro 1**.

**Quadro 1 -** Resumo dos critérios Diagnósticos propostos para SOP em adultos.

| **Categoria** | **Anormalidade específica** | **Teste recomendado** |
|---|---|---|
| Status andrógeno | Hiperandrogenismo clínico | O hiperandrogenismo clínico pode incluir o hirsutismo (definido como excesso de pelos que aparecem em um padrão masculino), acne ou alopecia androgênica. |
| | Hiperandrogenismo bioquímico | Refere-se a um nível elevado de hormônios andrógenos no soro e inclui tipicamente nível sérico de testosterona total ou livre elevados. |
| História menstrual | Oligo ou anovulação | A anovulação pode se manifestar como sangramento frequente em intervalos < 21 dias ou sangramentos infrequentes em intervalos > 35 dias. Ocasionalmente, o sangramento pode ser anovulatório, apesar de estar em um intervalo normal (25 a 35 dias). Valores intermediários de progesterona, que documentam a anovulação, podem ajudar no diagnóstico, se os intervalos de sangramento sugerirem ovulação regular. |
| Aparência ovariana | Tamanho / morfologia ovariana no ultrassom | Presença de 12 ou mais folículos de 2-9 mm de diâmetro e / ou aumento do volume ovariano > 10 ml (sem cisto ou folículo dominante) em qualquer um dos ovários. |

Adaptado de: ASRM/ESHRE, 2018

Ainda, a Organização Mundial de Saúde (OMS) e o *National Institute of Child Health and Human Development* (NICHHD) possuem diferentes critérios de diagnóstico para a SOP. Quanto aos critérios padronizados pela OMS, tem-se: (1) início peripuberal de

oligoamenorreia; (2) níveis séricos elevados de testosterona (> 80 ng/dl) e; (3) evidência ultrassonográfica de ovários policísticos. A oligomenorreia é definida como um episódio de sangramento ocorrendo menos de seis vezes por ano (YARALI et al., 2002). Os critérios do NICHHD compreendem apenas a presença de anovulação crônica hiperandrogênica após exclusão da síndrome de Cushing, de deficiência de 21-hidroxilase de início tardio, disfunção tireoidiana, hiperprolactinemia ou tumores secretores de andrógenos (MOGHETTI et al., 2000).

A SOP resulta em um desequilíbrio entre fatores pró e anticoagulantes, aumentando o risco de aterotrombose, bem como o aumento dos níveis de citocinas pró-inflamatórias, contribuindo para um estado de inflamação sistêmica crônica e de baixo grau, semelhante ao de outras DCNT, como obesidade, DM2 e DCV (BARREA et al, 2018; CARVALHO et al, 2017a). Além da RI e intolerância à glicose, esse estado é associado ao acúmulo de gordura visceral e dislipidemia (EBEJER; CALLEJA-AGIUS, 2013). Por isso, o papel da inflamação na SOP tem sido objeto de estudo, e associações foram encontradas entre níveis aumentados de marcadores de inflamação (hs-CRP, ferritina, haptoglobina, TNF, IL- 6 e IL-18) e marcadores de estresse oxidativo (malonaldeído - MDA, capacidade antioxidante total - TAC, óxido nítrico - NO e glutationa - GSH) com a SOP (BEDNARSKA; SIEJKA, 2017; CARVALHO et al., 2017b).

A resistência à ação da insulina pode estar associada a defeitos nos seus receptores, determinada geneticamente e por fatores ambientais, dificultando a utilização da glicose e podendo contribuir para o risco de distúrbios metabólicos comuns associados à SOP (SALEH; KHALIL, 2004; SÓTER et al., 2015). No entanto, na SOP, alguns órgãos como os ovários e as glândulas adrenais, são mais sensíveis à ação deste hormônio (NORMAN et al., 2007). A RI promove um quadro de hiperinsulinemia e este hormônio, em excesso, estimula enzimas envolvidas na produção de andrógenos e aumenta o efeito do hormônio luteinizante nos ovários e nas glândulas adrenais, favorecendo o hiperandrogenismo (GENAZZANI, 2016; POLAK et al., 2017; ORTIZ-FLORES et al., 2019; ROCHA et al., 2019).

### 2.2.1 Síndrome dos Ovários Policísticos e Hiperandrogenismo

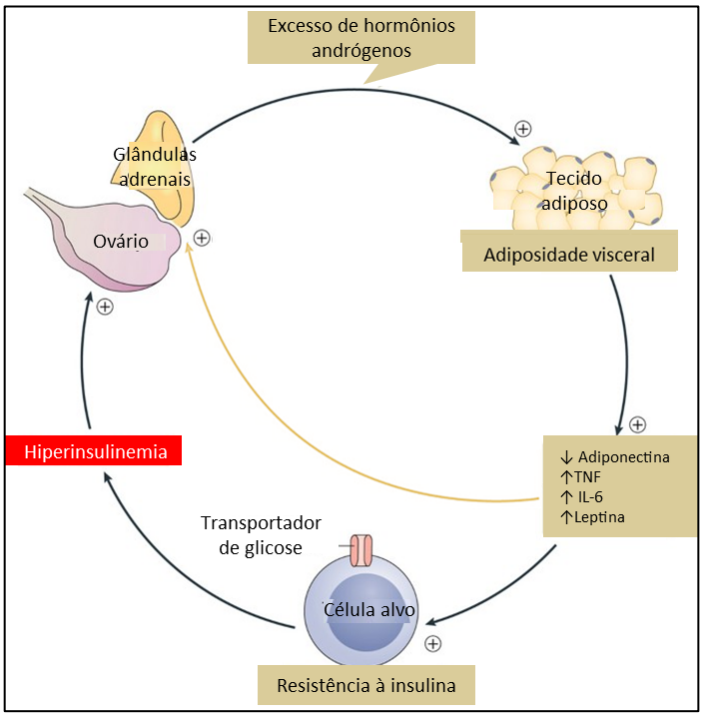
Os andrógenos são importantes no desenvolvimento fisiológico feminino, uma vez que estão associados com o desenvolvimento dos tecidos musculares, crescimento dos ossos e pelos púbicos. No entanto, seu excesso causa displasia folicular, promovendo disfunções menstruais e ovulatórias (YE et al., 2021). O hiperandrogenismo está intimamente relacionado com o desenvolvimento de comorbidades como dislipidemia, DM2 e a própria resistência à insulina (LAZÚROVÁ et al., 2019; ZENG et al., 2020). Laughlin e colaboradores (2010) associaram tanto os baixos, quanto os altos níveis de andrógenos, a um maior risco cardiovascular na população feminina, enfatizando a importância da manutenção de níveis regulares destes hormônios.

O hiperandrogenismo está presente na maioria das mulheres diagnosticadas com a SOP, sendo definido pela elevação dos hormônios androgênicos e seus percursores (WALTERS et al., 2018). Nas pacientes com SOP, se manifesta clinicamente através do hirsutismo, acne e alopecia. Os tecidos responsáveis pela síntese de andrógenos na mulher são os ovários e as glândulas adrenais, sendo que na SOP, os ovários detêm 60% da produção (BAPTISTE et al., 2010). A avaliação bioquímica do hiperandrogenismo decorre principalmente pela dosagem de testosterona total, a globulina ligadora de hormônios sexuais (SHBG), testosterona livre e pelo *Free Androgen Index* (FAI). Outros andrógenos podem se encontrar aumentados na síndrome, como a androstenediona e o dehidroepiandrosterona (DHEAS) (NORMAN et al., 2007).

Na SOP, o hiperandrogenismo, juntamente com a RI e a obesidade, criam um ciclo que torna a síndrome ainda mais complexa. A concentração elevada de andrógenos, produzidos pelos ovários e as glândulas adrenais, estimula a deposição de tecido adiposo abdominal, favorecendo o aumento da secreção destes hormônios (MA *et al*., 2021). Apesar do quadro de RI, as células tecais ovarianas exibem sensibilidade à ação deste hormônio, que juntamente com a hiperinsulinemia, favorecem o aumento da produção de andrógenos (CADAGAN et al., 2016). Porém, o excesso de hormônios androgênicos está associado ao prejuízo da ação da insulina nos tecidos periféricos (MOGHETTI et al., 1996). Portanto, a hipótese apresentada para a fisiopatologia da doença é de que esta é resultante de um “círculo vicioso” compreendido por:

- Excesso de andrógenos favorecendo a deposição de tecido adiposo abdominal e adiposidade visceral. O tecido adiposo exerce muitas dessas influências por meio de efeitos parácrinos e endócrinos mediados pela secreção aumentada ou reduzida de moléculas como leptina, adiponectina, TNF e IL-6. Além do papel bem estabelecido da RI e do hiperinsulinismo como fatores facilitadores do excesso de andrógenos, moléculas secretadas pelo tecido adiposo também podem influenciar na função adrenal e ovariana, e o próprio tecido adiposo intervém diretamente no metabolismo dos hormônios esteroides.
- Aumento da expressão de marcadores pró-inflamatórios, como a TNF e a IL-6, de leptina (peptídeo com a expressão intimamente relacionada à insulina, os glicocorticóides e as citocinas pró-inflamatórias) e a redução da adiponectina (proteína com papel principal na função metabólica regulatória e sensibilizadora da insulina no fígado e nos músculos, atuando como citocina anti-inflamatória e vasculoprotetora).
- Indução à RI e hiperinsulinismo compensatório, o que facilita ainda mais a secreção de andrógenos pelos ovários e glândulas adrenais em mulheres com SOP (ESCOBAR-MORREALE; MILLÁN, 2007) (**Figura 2**).

**Figura 2 -** Adiposidade abdominal e fisiopatologia da SOP.




Adaptado de: ESCOBAR-MORREALE, 2018.

*A interação entre a SOP e a adiposidade abdominal pode ser resultado de um círculo vicioso (representado pelas setas pretas) de excesso de andrógenios favorecendo a adiposidade visceral abdominal, que facilita o excesso de androgênios de origem ovariana e/ou adrenal pelos efeitos diretos (seta amarela) de vários mediadores autócrinos, parácrinos e endócrinos (downregulation da adiponectina e upregulation de TNF, IL-6 e leptina) ou indiretamente pela indução de RI e hiperinsulinismo (ESCOBAR-MORREALE, 2018).*

É importante observar que o excesso crônico de andrógenos de origem ovariana e/ou adrenal iniciando precocemente, ou mesmo no pré-natal, resulta em adiposidade abdominal e obesidade em mulheres com SOP. Além disso, a contribuição relativa do excesso androgênico e da adiposidade abdominal para esse círculo vicioso pode ser bastante variável entre os pacientes, contribuindo para a heterogeneidade clínica da SOP em relação às suas manifestações metabólicas (ESCOBAR-MORREALE; MILLÁN, 2007; BARREA et al., 2018).

### 2.2.2 Hiperandrogenismo e inflamação

O estado pró-inflamatório emergiu como um dos principais contribuintes para a RI e fatores de risco para DCV em mulheres com SOP. Além do papel estabelecido da adiposidade abdominal, há uma sugestão de que a disfunção metabólica e ovariana associada à SOP pode ser potencializada pelo estresse oxidativo e inflamação induzidos por nutrientes (BARREA et al., 2018). Assim como na obesidade e no DM2, também na SOP, a inflamação contribui para gerar RI e hiperinsulinemia compensatória, embora mediadores peculiares, como o hiperandrogenismo, desempenhem um papel fundamental nos desfechos metabólicos relacionados à síndrome (GONZÁLEZ, 2015).

Tanto a hiperinsulinemia, quanto o hiperandrogenismo, atuam como promotores da inflamação na SOP, e a tríade de RI, hiperandrogenismo e inflamação de baixo grau funciona bidirecionalmente (SHORAKAE et al., 2015). Estudos pioneiros de Dunai & Graf (1989) demonstraram que os andrógenos circulantes são influenciados pelos níveis de insulina independentemente das variações da liberação de gonadotrofina

apenas em mulheres com SOP, enquanto tal correlação não está presente em mulheres hígidas (BARREA et al., 2018).

Em muitos casos de SOP, são utilizados fármacos antiandrogênicos e anticoncepcionais orais com o objetivo de reduzir a acne e o hirsutismo, comuns nessas pacientes. No entanto, esta terapia medicamentosa não resolve os outros transtornos endócrinos ou metabólicos associados ao quadro hiperandrogênico (GENAZZANI, 2016). Dessa forma, surgiu a possibilidade de uma abordagem terapêutica com medicamentos que aumentem a sensibilidade dos tecidos à insulina, como a metformina. No entanto, a eficácia do tratamento com metformina no controle do hiperandrogenismo ainda não foi completamente esclarecida.

### 2.2.3 Metformina

A metformina é um hipoglicemiante oral com múltiplos mecanismos de ação no organismo devido aos seus diversos sítios de ligação (RENCBER et al., 2018; FACCHINETTI *et al*., 2019). Suas ações vão desde a diminuição da produção hepática de glicose, redução da gliconeogênese, ao limitar a disponibilidade do substrato lipídico, aumento da absorção de glicose e sensibilização dos tecidos, ambas as ações mediadas pela insulina (COSTELLO; EDEN, 2003; NADERPOOR et al., 2015; PATEL; SHAH, 2017; GUAN et al., 2020). No geral, a metformina aumenta a utilização de glicose pelo organismo, reduz a resistência à insulina e a hiperinsulinemia (COSTELLO; EDEN, 2003; KHORRAM et al., 2006). Em outras condições, a metformina também está associada à prevenção do ganho de peso e ao tratamento de DM2 e diabetes gestacional (NADERPOOR et al., 2015).

Apesar dos efeitos apresentados pela metformina, esta não causa alteração da produção pancreática de insulina, mas sim um aumento da expressão dos receptores de insulina, diminuindo os níveis plasmáticos deste hormônio, sem resultar em quadro hipoglicêmico (SALEH; KHALIL, 2004). A ação molecular da metformina consiste na inibição da respiração mitocondrial hepática, a qual promove aumento da sensibilidade à insulina e queda da expressão de enzimas envolvidas na gliconeogênese (SAM; EHRMANN, 2017; FACCHINETTI et al*,* 2019). A metformina está associada a alguns efeitos adversos, nomeadamente, gastrointestinais, tais como náusea, diarreia e flatulência. A dose diária de metformina varia de 1500 a 2000 mg,

podendo ser escalonada, de forma a amenizar possíveis transtornos indesejados (FRUZZETTI et al., 2017).

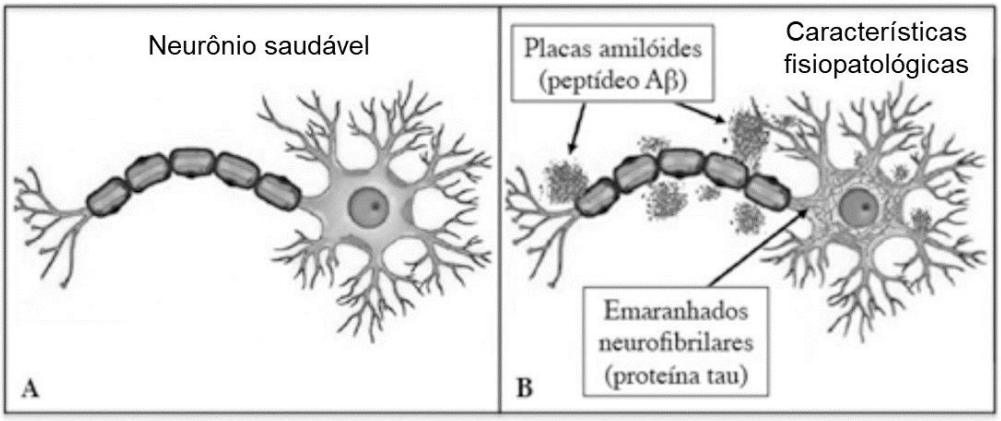
Como apresentado anteriormente, mulheres com SOP comumente apresentam quadros de RI e consequente hiperinsulinemia, similar ao observado no DM2. Diante deste quadro, desde 1994, a metformina vem sendo prescrito às pacientes com o objetivo de reduzir a resistência dos tecidos à ação deste hormônio (TANG et al., 2012; PATEL; SHAH, 2017). Alguns estudos demonstraram, com o tratamento com metformina, a atenuação da resistência à insulina em mulheres com a síndrome, ao se observar a redução do *Homeostasis Model Aassessment*-IR (HOMA de resistência à insulina), modelo de avaliação da homeostase para resistência à insulina (FRUZZETTI et al., 2017, OHARA et al., 2021).

## 2.3 Doença de Alzheimer

A demência é uma condição clínica caracterizada pelo declínio progressivo em dois ou mais domínios cognitivos, incluindo memória, linguagem, função executiva e visual-espacial, personalidade e comportamento, causando perda de habilidades para realizar atividades instrumentais e / ou básicas da vida diária, sendo a DA a causa mais comum de demência (WELLER; BUDSON, 2018). Atualmente, a DA é denominada pela Organização Mundial de Saúde (OMS) como uma condição prioritária em saúde pública, por se tratar de uma doença neurodegenerativa e por representar de 50 a 75% dos casos de demência diagnosticados (LANE; HARDY; SCHOTT, 2018). Segundo o *National Institute of Neurological Disorders and Strok*e (NINDS), a DA é uma condição associada à idade, na qual ocorre perda de memória, confusão mental e eventual declínio de habilidades cognitivas (NINDS, 2019).

A DA típica, de início tardio, é provavelmente causada por uma interação complexa entre fatores genéticos – cerca de 70% dos casos atribuível a este fator, e ambientais (VERGHESE; CASTELLANO; HOLTZMAN, 2011). Quanto à caracterização macroscópica de um cérebro de pacientes acometidos por DA, este é caracterizado por atrofia cortical, causada pela degeneração da arborização axonal colinérgica e encolhimento da árvore dendrítica. Microscopicamente, são observados depósitos de peptídeo Aβ e NFT de proteína *tau* presentes nas áreas afetadas (GIL-BEA et al., 2012) (**Figura 3**).

**Figura 3 -** Diferenças anatomopatológicas entre neurônio saudável e acometido pela DA.




Adaptado de: Falco et al., 2016.

A DA também é caracterizada por neuroinflamação crônica, ocasionada pela ativação dos astrócitos e micróglia e redução dos níveis de acetilcolina no processo simpático, diminuindo a neurotransmissão colinérgica cortical (ROSENBLUM, 2014; FRAGA et al., 2019). Além disso, os níveis de mediadores pró-inflamatórios que incluem citocinas e quimiocinas estão elevados no cérebro de pacientes com DA (LATTA; BROTHERS; WILCOCK, 2014; MAGALHÃES et al., 2018). Por fim, pode ser observada ainda a maior atividade do fator nuclear (NF) κB e do transdutor de sinal e ativador da transcrição (STAT)1α, fatores de transcrição envolvidos na expressão de genes pró-inflamatórios, indicando a presença de processo pró-inflamatório crônico (LAWRENCE, 2009).

Pacientes com suspeita clínica ou aqueles com a demência instalada devem ser avaliados e monitorados devido ao aumento do risco de progressão da doença. Para tanto, inúmeros testes de função cognitiva foram propostos para esta tarefa, diante da facilidade de aplicação e a possibilidade de avaliação de funções executivas importantes destes pacientes (AREVALO-RODRIGUEZ et al., 2015). Os instrumentos comumente aplicados para avaliar a função cognitiva de pacientes com DA são: (1) Escala de Avaliação da Doença de Alzheimer (ADAS-cog); (2) Estudo Cooperativo de Atividades de Vida Diária da Doença de Alzheimer (ADCS-ADL) e; (3) Miniexame do Estado Mental (MMSE). Considerado o padrão ouro na avaliação da eficácia dos tratamentos anti-demência, o ADAS-cog considera as mudanças no humor e no comportamento e inclui onze tarefas que avaliam tanto as questões baseadas no

observador, quanto os testes concluídos pelo sujeito. Pontuações mais altas indicam pior desempenho (KUEPER et al., 2018). O ADCS-ADL foi construído especificamente para uso com pacientes com DA, com uma escala de 23 itens que fornece uma pontuação total de 0 a 78, sendo que uma pontuação menor está relacionada à maior gravidade (KAHLE-WROBLESKI et al., 2014). O MMSE é uma avaliação de 30 questões da função cognitiva que avalia a atenção e orientação, registro, memória, cálculo, linguagem e habilidade de desenho. A presença de declínio cognitivo é decidida pelo escore total, e um escore menor está associado a uma maior gravidade (AREVALO-RODRIGUEZ et al., 2015).

Atualmente, apenas duas classes de terapia farmacológica estão disponíveis para pacientes com DA – os inibidores de colinesterase e a memantina, que tem atividade tanto como antagonista não competitivo do receptor N-metil-D-aspartato, quanto como agonista de dopamina. A dieta é conhecida por modular o sistema imunológico, e vários nutrientes e componentes bioativos podem influenciar os processos neuroinflamatórios em animais. Por exemplo, polifenóis, gorduras insaturadas e vitaminas antioxidantes inibem o estresse oxidativo e a neuroinflamação (FRAGA et al., 2017; MCGRATTRAN et al., 2019). Os polifenóis dietéticos têm sido sugeridos para auxiliar na prevenção de doenças neurodegenerativas como a DA devido às suas propriedades anti-inflamatórias e antioxidantes (CALDER et al., 2017). No entanto, não está claro se os efeitos induzidos pela dieta na neurocognição são mediados diretamente por processos neuroinflamatórios e / ou por meio de outros mecanismos imunológicos *in vivo*. Um crescente corpo de evidências sugere que a inflamação periférica e alterações na microbiota intestinal podem amplificar a neuroinflamação e acelerar a neurodegeneração (GOYAL et al., 2021; LENG; EDISON, 2021; MCGRATTRAN et al., 2019) e esses fatores externos também podem ser influenciados pela dieta (MINIHANE et al., 2015).

## 2.4 Aspectos nutricionais na inflamação crônica de baixo grau

A inflamação atua como componente essencial da imunovigilância e na defesa do hospedeiro, caracterizada por concentrações elevadas persistentes de citocinas pró-inflamatórias circulantes ao longo da vida, estando associada a uma ampla variedade de condições crônicas (MINIHANE et al., 2015). O acometimento por estas doenças,

bem como o risco de complicações associadas, são positivamente relacionadas à idade, a fatores genéticos, mas, principalmente, aos hábitos alimentares não-saudáveis (PHILLIPS et al., 2019). A pesquisa de Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico (VIGITEL) realizada em 2020, no Brasil, mostra dados importantes quanto a frequência de consumo de cinco ou mais grupos de alimentos ultraprocessados – formulações industriais feitas inteiramente ou majoritariamente de substâncias extraídas de alimentos, derivados de constituintes de alimentos ou sintetizados em laboratório com base em matérias orgânicas (BRASIL, 2014). No dia anterior à entrevista, foi relatado consumo de alimentos deste grupo por 18,5% da população adulta entrevistada. Ainda, no conjunto das 27 cidades, a frequência de diagnóstico médico de hipertensão arterial sistêmica (HAS) foi de 25,2% e de DM2 de 8,2% (BRASIL, 2021).

Há um crescente corpo de evidências indicando que a combinação da quantidade e qualidade dos alimentos, principalmente padrões dietéticos com alta ingestão calórica ou baixa em micronutrientes, associada à susceptibilidade genética, seriam capazes de influenciar o estado inflamatório crônico (CHRIST; LAUTERBACH; LATZ, 2019). Consequentemente, o reconhecimento do papel emergente do processo inflamatório induzido pela dieta no desenvolvimento de doenças tem sido intensificado (BARREA et al., 2018).

Diversos estudos sugerem que a inflamação de baixo grau é mitigada por hábitos alimentares saudáveis como a dieta mediterrânea, os quais estariam associados a menores concentrações circulantes de marcadores pró-inflamatórios (CENTRITTO et a., 2009), enquanto o tipo ocidental ou os padrões à base de carne estão positivamente associados à inflamação de baixo grau (BONACCIO et al., 2017). Em revisão sistemática desenvolvida por Barbaresko e colaboradores (2013), que investigou se padrões alimentares específicos estão associados a biomarcadores de inflamação de baixo grau, foi evidenciado que existe uma associação positiva entre dietas do tipo ocidental e à base de carne com a inflamação crônica de baixo grau, enquanto uma associação inversa existe para padrões baseados em vegetais e frutas (BARBARESKO et al., 2013). Ainda, é importante ressaltar que, entre os componentes de uma dieta saudável, grãos inteiros, vegetais, frutas e peixes estão associados com menor inflamação e um número limitado de estudos observacionais sugeriram uma ação pró - inflamatória de dietas ricas em AG saturados ou AG *trans* (CALDER et al.,

2011). Por fim, evidências científicas demonstraram que os inúmeros constituintes bioativos de frutas e vegetais, como flavonoides, carotenoides, PUFAS, vitaminas, minerais e fibras, podem atuar individualmente e de forma sinérgica para fornecer um alto valor nutricional e fatores anti-inflamatórios importantes (LIU, 2013; SLAVIN; LLOYD, 2012).

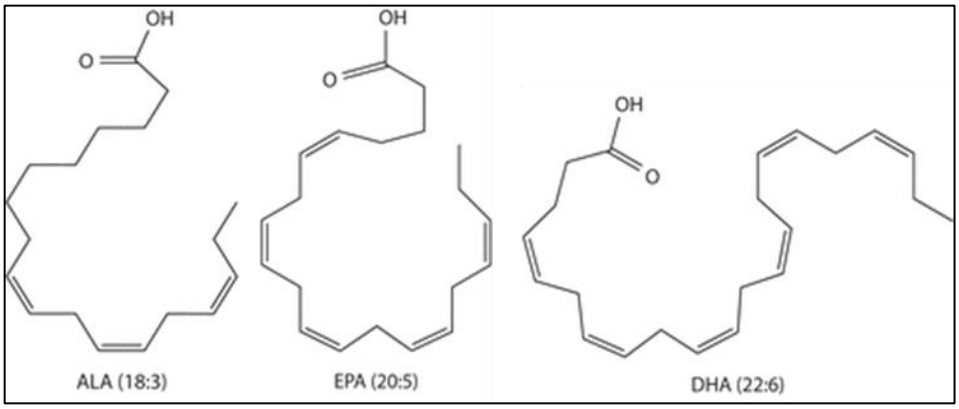
As evidências atuais sobre a influência da dieta na inflamação são baseadas em diferentes abordagens de pesquisa; isto é, com base em nutrientes, com base em grupos de alimentos ou análises de padrões de dieta completa. O exame dos padrões e índices dietéticos se tornou mais popular devido à facilidade com que essas medidas podem ser geradas a partir dos dados dietéticos existentes. Além disso, considerando que há a ingestão de combinações complexas de alimentos, ao invés de nutrientes individuais e grupos de alimentos, essas abordagens também são mais traduzíveis em termos de orientações em saúde pública (PHILLIPS et al., 2019). Sendo assim, os compostos anti-inflamatórios, que provaram ser úteis em uma doença específica, como é o caso do resveratrol e do ω-3, podem vir a ser benéficos em outras doenças inflamatórias, o que abre novas perspectivas terapêuticas (CHEN et al., 2017).

## 2.5 Ácido graxo ômega-3

Os AG ω-3 formam um grupo heterogêneo de AG com uma ligação dupla entre o terceiro e o quarto átomos de carbono na extremidade metila. Em geral, pode-se distinguir entre eles os ácidos graxos monoinsaturados (MUFA), com uma ligação dupla na cadeia de carbono, e os PUFA, com mais de uma ligação dupla na cadeia de carbono (NAGAO; YANAGITA, 2005). Os AG de ocorrência natural geralmente têm de quatro a 28 átomos de carbono. No entanto, muitos deles, especialmente aqueles encontrados no cérebro, retina e espermatozoides, possuem uma cadeia de carbono mais longa e constituem a maioria dos AG advindos da dieta (CHOLEWSKI; TOMCZYKOWA; TOMCZYK, 2018). Os AG ω-3 incluem os ácidos alfa-linolênico (ALA) (18: 3 ω-3), eicosapentaenoico (EPA) (20: 5 ω-3) e docosahexaenoico (DHA) (22: 6 ω-3) (**Figura 4**). Vale ressaltar que o ALA, é considerado AG essencial por não ser sintetizado por humanos. Os AG EPA e DHA podem ser convertidos a partir do ALA por alongamento e dessaturação da cadeia (WATANABE; TATSUNO, 2020).

Os AG ω-3 são encontrados exclusivamente em organismos aquáticos e se originam principalmente no fígado de peixes brancos magros, como o bacalhau, no corpo de peixes oleosos, como cavala e salmão, e na gordura de mamíferos marinhos (SHAHIDI; MIRALIAKBARI, 2004). Sementes de linhaça (49,2 g de ALA / 100 g de linhaça), chia e as nozes são conhecidas por serem boas fontes de ALA (SHAHIDI; AMBIGAIPALAN, 2018). Os óleos de linhaça, noz, canola e soja possuem quantidades significativas de ALA, enquanto os óleos de salmão, sardinha e arenque contêm quantidades relativamente altas de EPA e DHA (SHAHIDI; AMBIGAIPALAN, 2018).

**Figura 4** - Estrutura química dos ácidos graxos poliinsaturados ômega-3.




ALA: ácido α-linolênico; EPA: ácido eicosapentaenóico; DHA: ácido docosahexaenóico.

Adaptado de: Shahidi; Ambigaipalan, 2018.

Evidências sugerem que a suplementação com AG ω-3 aumenta a sensibilidade à insulina e os níveis de adiponectina plasmática, reduz a hiperinsulinemia, triglicerídeos plasmáticos, gordura hepática e atenua a inflamação e a resposta ao estresse oxidativo em adultos (CARPENTIER; PORTOIS; MALAISSE, 2006; CUSSONS et al., 2009).

A suplementação de ácidos graxos ômega-3 pode estar associada a uma diminuição na inflamação e nos níveis de citocinas pró-inflamatórias, ao estimular a secreção de adipocinas anti-inflamatórias, a exemplo da adiponectina (CALDER, 2017). Além disso, esses efeitos podem estar relacionados à sua influência nas vias de sinalização que regulam a expressão de genes que codificam citocinas pró-inflamatórias (KANY; VOLLRATH; RELJ, 2019). Essa regulação pode estar associada ao NF-κB, um dos

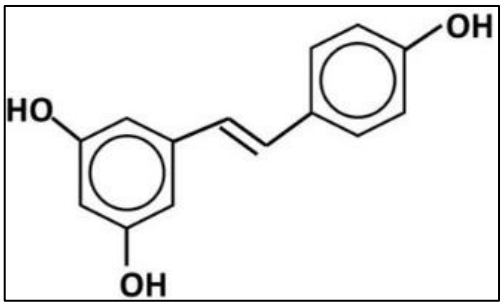
principais fatores de transcrição envolvidos na supra regulação dos genes que codificam citocinas pró-inflamatórias, conforme dito anteriormente, além de moléculas de adesão e ciclooxigenase-2 (COX-2) (BABCOCK et al., 2002). O EPA diminui a ativação de NF-κB induzida por lipopolissacarídeo (LPS) em monócitos, e o DHA reduz a ativação de NF-κB em resposta a LPS em macrófagos e células dendríticas (NOVAK et al., 2003). A influência de EPA e DHA na ativação de NF-κB envolve o receptor ativado por proliferador de peroxissoma (PPAR)-γ, resultando na inibição da ativação de NF-κB e redução da produção das citocinas pró- inflamatórias TNF e IL-6 (CALDER, 2017).

## 2.6 Resveratrol

O resveratrol (trans-3,4,5-triidroxistilbeno) pertencente à família dos estilbienos. É um polifenol que desempenha papel benéfico na prevenção e progressão de doenças crônicas relacionadas à inflamação, como DM2, obesidade, DCV, na neurodegeneração e no câncer (PIOTROWSKA; KUCINSKA; MURIAS, 2012). O resveratrol foi isolado, pela primeira vez, da raiz do heléboro-branco (*Veratum grandiflorum*) em 1940 sendo, posteriormente, extraído da raiz da *Polygonum cupsidatum*, uma espécie de erva nativa da Ásia, em 1963, sendo ambas usadas amplamente nas medicinas tradicionais chinesa e japonesa (TIMMERS; AUMERX; SCHRAUWEN, 2012). O resveratrol começou a ganhar notoriedade na área farmacológica apenas em 1997, quando um estudo *in vivo* investigou suas propriedades como agente quimiopreventivo no câncer em ensaios que representavam estágios principais da carcinogênese (JANG et al., 1997).

A estrutura química do resveratrol é composta por um grupo P-hidroxila no anel A e por um sistema de dupla ligação conjugada, responsável por sua propriedade antioxidante (**Figura 5**). O resveratrol pode se apresentar como dois isômeros geométricos: *cis* e *trans*-resveratrol. A forma trans é sintetizada em plantas, como produto da via dos fenilproponóides e, quando exposta a raios ultravioletas, pode ser isomerizado em *cis* (AHMED et al., 2017).

**Figura 5** - Estrutura química do *trans*-resveratrol.




Adaptado de: Rao et al., 2020.

O resveratrol foi encontrado em mais de 70 espécies de plantas, sendo a principal fonte alimentar o vinho tinto. No entanto, o composto pode ser encontrado em outras fontes, como demonstrado por Reinisalo e colaboradores (2018) (**Quadro 2**).

**Quadro 2** - Principais fontes de resveratrol.

| | |
|---|---|
| Cacau (*Theobroma cacao L*) | Boldo (*V. myrtillus*) |
| Uva (*Vitis vinifera L.)* | Cranberry (*V. macrocarpon*) |
| Lúpulo (*Humulus lupulus L.)* | Tomate (*Lycopersicon esculentum Mill.*) |
| Amendoim (*Arachis hypogaea L.*) | Mirtilo alto (*V. corymbosum*) |
| Morango (*Fragaria x ananassa Duch.*) | Vinho tinto |
| Cana-de-açúcar (*Saccharum spp.*) | Vinho branco |

Adaptado de: Reinisalo et al., 2018.

Embora o resveratrol apresente boa absorção, sua biodisponibilidade é menor devido à baixa solubilidade em água. Esta baixa biodisponibilidade resulta em meia-vida biológica mais curta, levando a um metabolismo rápido e depuração rápida, limitando assim a capacidade de se acumular no tecido alvo, podendo a estabilidade e biotransformação influenciar nas suas propriedades antioxidantes (REGE et al., 2014). Uma dose oral de 25 mg de resveratrol resulta em menos de 5 µg/mL de concentração do princípio ativo no soro, e uma dose intravenosa de 0,2 mg resulta em 7 ng/mL de concentração. Sendo assim, a modificação na estrutura química e no metabolismo podem aumentar a sua eficácia ao aumentar sua biodisponibilidade (RAO et al., 2020).

Estudos *in vitro* e *in vivo* revelaram os efeitos neuroprotetores do resveratrol, relacionados à sua capacidade de atravessar a barreira hematoencefálica e exercer seu efeito antioxidante aumentando as enzimas antioxidantes (PALLE; NEERATI, 2018). O resveratrol foi considerado benéfico contra a morte celular de neurônios e

disfunção celular em doenças como epilepsia, Doença de Huntington e DA (RAO et al., 2020).

## 2.7 Revisão sistemática

As revisões sistemáticas (RS) representam um tipo específico de pesquisa em que as unidades de análise são os estudos primários originais. Trata-se, portanto, de um estudo secundário cujo objetivo é agrupar estudos primários semelhantes, publicados ou não, avaliando criticamente sua metodologia e o grau de evidência destes estudos, sumarizando os resultados de forma narrativa ou por meio de análise estatística, a meta-análise (MA) (GONZÁLEZ; URRUTIA; ALONSO-COELLO, 2011). As RS são ferramentas essenciais para sintetizar as informações científicas disponíveis e incrementar o tamanho amostral, com consequente aumento do poder das hipóteses, se considerarmos que a RS apresenta o melhor nível de evidência para responder questões de pesquisa e para embasar a prática clínica, pela validade das conclusões dos estudos primários e identificando áreas para pesquisas futuras (COOK; MULROW; HAYNES, 1997).

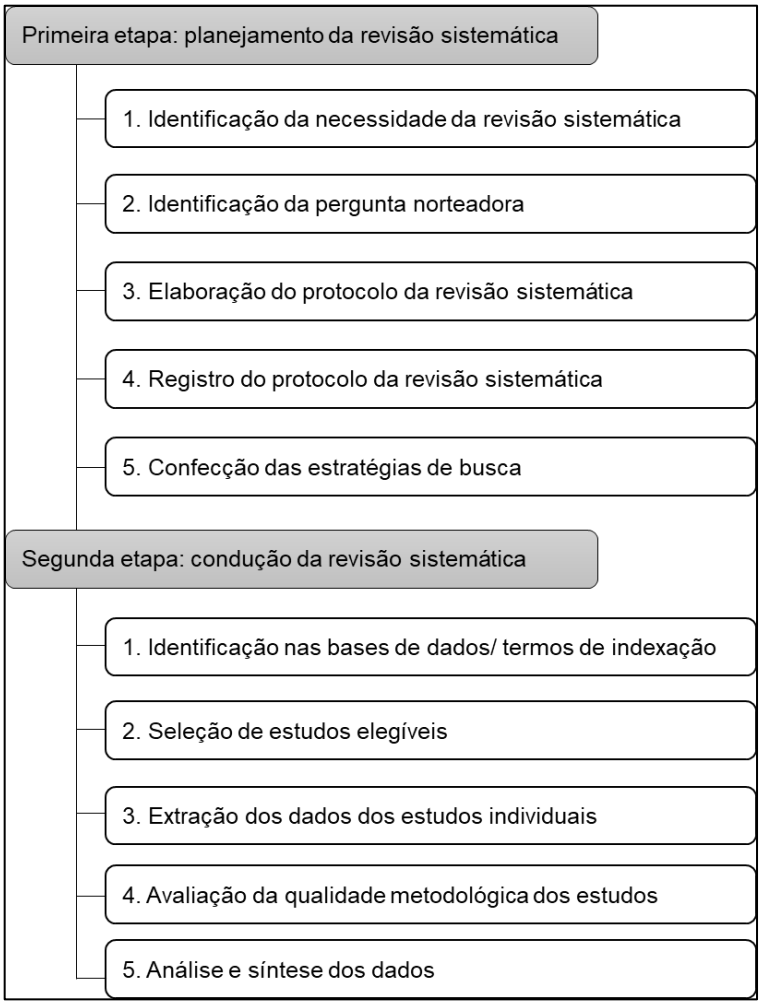
Uma RS tenta reunir todas as evidências científicas que se enquadram nos critérios de elegibilidade, pré-estabelecidos no registro da revisão, para responder a uma pergunta de pesquisa específica. A RS usa métodos explícitos e sistemáticos, que são selecionados com o objetivo de minimizar possíveis vieses, fornecendo resultados mais confiáveis a partir dos quais conclusões podem ser tiradas e decisões tomadas (ANTMAN et al., 1992). A metodologia de RS, pioneira e desenvolvida pela *Cochrane*, atualmente em sua sexta edição (HIGGINS et al., 2021), apresenta uma metodologia altamente estruturada, transparente e reprodutível (CHANDLER; HOPEWELL, 2013). Isso envolve: a especificação *a priori* de uma questão de pesquisa; clareza sobre o escopo da revisão e quais estudos são elegíveis para inclusão; envidar todos os esforços para encontrar todas as pesquisas relevantes, a partir de uma busca abrangente na literatura científica e; garantir que as questões de parcialidade nos estudos incluídos sejam levadas em consideração (LASSERSON; THOMAS; HIGGINS, 2021).

A qualidade de uma RS depende muito da extensão em que os métodos são seguidos para minimizar o risco de erro e de viés durante o processo de revisão. Esses métodos

rigorosos distinguem as RS das revisões tradicionais da literatura. Como tal, o relato explícito e detalhado dos métodos usados na síntese é uma necessidade e uma marca registrada de qualquer RS bem conduzida. A tipologia de revisões é vasta, incluindo: revisões de intervenção, revisões de estudos primários experimentais, revisões de custo ou análises de avaliação econômica, revisões de prevalência ou incidência, revisões de precisão do teste de diagnóstico, revisões de prognóstico e metodológicas e revisões de efetividade (MUNN et al., 2018).

Há um consenso sobre as etapas exigidas para a condução de uma RS de qualquer tipo de evidência. As etapas de planejamento e condução de uma RS estão evidenciadas na **Figura 6**.

**Figura 6 -** Etapas de planejamento e condução de uma revisão sistemática.




Fonte: adaptado de OLIVEIRA; GOTTSCHALL; SILVA, 2017.

### 2.7.1 Questão de pesquisa

As RS devem ter, por essência, uma pergunta científica a qual o trabalho irá responder. Esta deve ser clara, e sua resposta fornecerá informações significativas que poderão ser usadas para orientar a tomada de decisão, devendo ser apresentada de forma clara e precisa no protocolo. As perguntas podem ser extremamente específicas ou muito amplas, embora, se amplas, o mais apropriado é dividi-las em uma série de questões relacionadas mais específicas (CRD, 2009). As perguntas da revisão devem especificar o foco do trabalho, os tipos de participantes, tipos de intervenções e comparadores e os tipos de desfechos considerados. O ideal é que os revisores apliquem o acrônimo PICO como uma estratégia para tornar a questão de pesquisa prática para a construção da estratégia de busca de revisões sistemáticas de intervenção (RICHARDSON et al., 1995). O acrônimo é definido por:

| | |
|---|---|
| **P** | População |
| **I** | Intervenção |
| **C** | Controle |
| **O** | Desfechos (*Outcomes*) |

Esses elementos da questão da revisão, juntamente com o desenho do estudo, serão então refinados para determinar os critérios de inclusão específicos que serão usados na seleção dos estudos para a RS. Em algumas situações, nem todos os elementos serão relevantes, por exemplo, nem todas as perguntas da revisão requerem o tipo de desenho do estudo a ser incluído ou o controle avaliado (CRD, 2009).

### 2.7.2 Protocolo da revisão sistemática

O protocolo da RS, *a priori,* define os objetivos e métodos a serem aplicados no desenvolvimento da revisão, além de fornecer um plano ou proposta para a RS. O protocolo deve descrever (CRD, 2009; TUFANARU et al., 2020):

- O contexto e a justificativa para a revisão, incluindo as hipóteses e as incertezas;
- Os critérios de seleção do estudo (critérios de inclusão e exclusão);

- As medidas de resultados, intervenções e comparações consideradas;
- A estratégia de busca proposta para identificar estudos relevantes;
- As bases de dados nas quais serão realizadas as buscas;
- Os procedimentos de seleção do estudo;
- O processo de avaliação crítica e instrumentos;
- O processo de extração de dados e instrumentos;
- Os métodos aplicados à avaliação da qualidade dos estudos incluídos e da evidência;
- O processo para resolver divergências entre os revisores na seleção do estudo, extração de dados e decisões de avaliação crítica;
- As abordagens propostas para a síntese, e;
- Se será realizada meta-análise.

Na elaboração do protocolo, deve-se especificar os métodos com antecedência para reduzir o risco de introdução de viés na revisão. Por exemplo, critérios de inclusão claros evitam selecionar estudos apenas se seus resultados refletem uma conclusão favorável. O protocolo deve apresentar ainda um título claro e descritivo que permita aos leitores e pesquisadores identificar prontamente o escopo e a relevância da revisão (CRD, 2009; TUFANARU et al., 2020). A publicação de um protocolo de RS, antes do início dos trabalhos, pode reduzir o risco de viés do estudo desenvolvido, promove a transparência dos métodos e processos, reduz o potencial de duplicação de pesquisa, permite a revisão por pares dos métodos planejados antes de serem concluídos e oferece uma oportunidade para a equipe de revisão planejar recursos e logística para realizar a própria revisão.

### 2.7.3 Identificação nas bases de dados / termos de indexação

A realização de uma pesquisa completa para identificar estudos relevantes é um fator chave para minimizar o viés no processo de revisão. O processo de pesquisa deve ser o mais transparente possível e documentado de forma a permitir sua avaliação e reprodução. Os estudos podem ser localizados usando uma combinação de pesquisas em bases de dados eletrônicas, busca manual em listas de referências de estudos relevantes, busca manual de jornais e resumos de conferências, contato com autores do estudo, especialistas, fabricantes e outras organizações e pesquisas por

citações relevantes. Quaisquer restrições relacionadas ao tempo de pesquisa, bem como ao idioma dos estudos devem ser declaradas (CRD, 2009; LEFEBVRE et al., 2021).

De acordo com a Recomendação Cochrane (2021), as bases de dados mínimas a serem avaliadas são: (1) da *Cochrane Library Central Register of Controlled Trials* (CENTRAL); (2) o *Medical Literature Analysis and Retrieve System Online* (MEDLINE) via *Pubmed* e; (3) *Embase*. Além da busca nestas bases, recomenda-se a identificação de resultados de pesquisa divulgados como relatórios ou documentos de discussão (LEFEBVRE et al., 2021). A identificação de literatura cinzenta, como artigos não publicados, é um processo difícil, mas alguns estão incluídos em bases de dados específicas, como o *OpenGrey*. Bibliotecas de organizações de pesquisa especializadas e sociedades profissionais também podem fornecer acesso a coleções de literatura cinzenta (TUFANARU et al., 2020).

A estratégia de pesquisa direcionada a cada uma das bases deve ser aplicada tanto a pesquisas por termos que remetam ao tema, ao título de assunto, quanto por palavras de texto. As estratégias de busca são explicitamente projetadas para serem altamente sensíveis, de forma que tantos estudos potencialmente relevantes, quanto possíveis, sejam recuperados. Para tanto, aplica-se os Descritores em Ciências da Saúde (DeCS), referentes aos objetos de estudo, sendo estes definidos como uma linguagem única na indexação de artigos de revistas científicas, livros, anais de congressos, relatórios técnicos, e outros tipos de materiais. Os DeCS foram desenvolvidos a partir do *Medical Subject Headings* (MeSH) da *United States National Library of Medicine* (NLM) com o objetivo de permitir o uso de terminologia comum para pesquisa em múltiplos idiomas, proporcionando um meio consistente e único para a recuperação da informação. São aplicados também os “termos sinônimos” (Emtree terms) ou derivados do descritor principal, combinados a operadores booleanos (“AND” e “OR). Consequentemente, as buscas tendem a recuperar inúmeros registros que não atendem aos critérios de inclusão (LEFEBVRE et al., 2021; TUFANARU et al., 2020).

### 2.7.4 Seleção dos estudos elegíveis

Esta seção da RS deve descrever, de forma detalhada, o processo de inclusão dos estudos avaliados, com base na avaliação dos títulos e resumos dos trabalhos que resultaram da busca na literatura, (compreendendo a primeira etapa de seleção), e com base na leitura do texto completo (compreendendo a segunda etapa de seleção dos estudos). Tanto a seleção dos estudos, quanto a extração dos dados, próxima etapa, devem ser realizadas, de forma independente, por dois autores da equipe responsável pelo desenvolvimento da RS. O processo em duplicata é indicado com o objetivo de reduz o risco de cometer erros e a possibilidade de a seleção de dados ser influenciada pelo discernimento de uma única pessoa (LI; HIGGINS; DEEKS, 2021). Ainda, deve ser apresentado pelos autores os procedimentos para resolver possíveis divergências entre os revisores, como a discussão entre eles ou a resolução por um terceiro revisor, também de forma independente (PORRITT; GOMERSALL; LOCKWOOD, 2014).

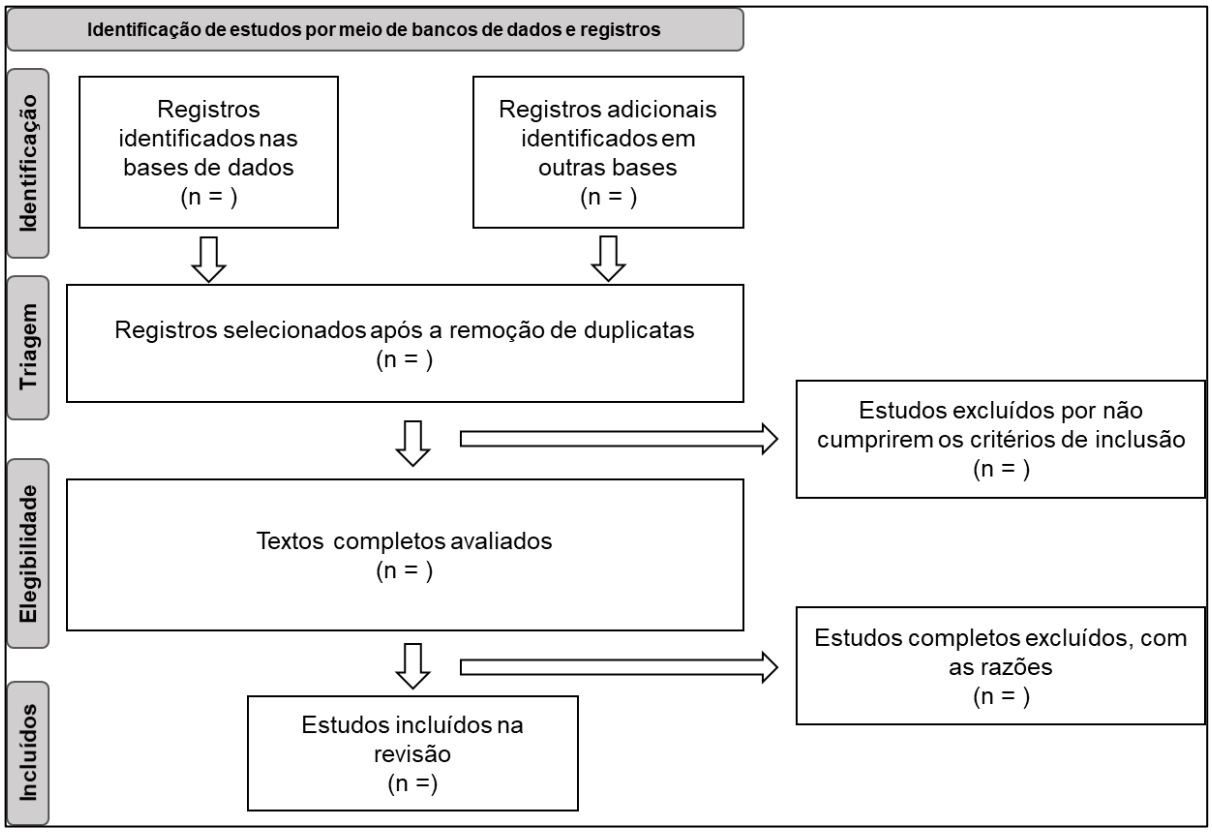
A avaliação de elegibilidade dos estudos deve levar em consideração também os critérios de inclusão estabelecidos em protocolo, devendo ser explícitos e inequívocos. Esses critérios serão utilizados no processo de seleção, quando for decidido se um estudo será incluído ou não na avaliação. Normalmente, é suficiente fornecer critérios de inclusão explícitos sem especificar critérios de exclusão explícitos; presume-se que a exclusão seja baseada em critérios que são opostos aos de inclusão. No entanto, às vezes, para maior clareza, a fim de evitar qualquer ambiguidade potencial, é recomendado fornecer critérios de exclusão explícitos (PORRITT; GOMERSALL; LOCKWOOD, 2014; TUFANARU et al., 2020).

Devem ser consideradas duas categorias de critérios de inclusão: (1) critérios de inclusão baseados nas características do estudo e; (2) critérios de inclusão baseados nas características da publicação. Os critérios de inclusão baseados nas características do estudo são aqueles relacionados aos tipos de participantes e ambientes, tipos de intervenções, comparadores, tipos e medidas de resultados e tipos de estudos. Os critérios de inclusão com base nas características da publicação são aqueles associados ao tipo de publicação (publicado em bases de dados científicas comerciais; documentos não publicados em bases de dados

comerciais, por exemplo, documentos de ensaios) (PORRITT; GOMERSALL; LOCKWOOD, 2014; CRD, 2009).

Toda a etapa de seleção dos estudos deve ser feita com o auxílio de um *software* de gerenciamento de referências como Mendeley® (Versão 2.61.1. Londres, Reino Unido) ou EndNote® (Versão X5, Londres, Reino Unido), o que otimiza o trabalho da equipe por permitir o compartilhamento dos trabalhos para avaliação, retirada de duplicatas pelo programa e exportação dos dados para o formato de planilhas para melhor controle das etapas e documentação dos artigos incluídos e excluídos e suas razões (TUFANARU et al., 2020). Ao final do processo de seleção, um fluxograma mostrando o número de estudos restantes em cada etapa da seleção deve ser apresentado (**Figura 7**), sendo uma forma simples e útil de documentar o processo de seleção dos estudos.

**Figura 7 -** Exemplo de fluxograma de seleção de artigos para revisão sistemática.




Fonte: Adaptado de PAGE et al, 2021

As recomendações para relatar RS de intervenção, com ou sem MA, e por consequência a apresentação do fluxograma de seleção, foram desenvolvidas pelo

grupo *Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (PRISMA). O PRISMA é um conjunto mínimo de itens com base em evidências para relatar em RS e MA. O relatório concentra-se principalmente nas revisões que avaliam os efeitos de intervenções, mas também pode ser usado como base para reportar RS com objetivos diferentes como por exemplo, avaliação de etiologia, diagnóstico ou prognóstico (PAGE et al., 2021).

### 2.7.5 Extração de dados

A extração de dados é o processo pelo qual os pesquisadores obtêm as informações necessárias sobre as características do estudo e os resultados dos estudos incluídos como: detalhes de métodos, participantes, ambiente, intervenções, desfechos, resultados, publicações e investigadores (LI; HIGGINS; DEEKS, 2021). Os requisitos de extração de dados variam de revisão para revisão, e os formulários de extração devem ser adaptados à questão da revisão.

Os dados extraídos devem incluir detalhes específicos sobre os participantes, exposição de interesse e resultados obtidos pelos autores. Independentemente do foco da RS, dados adicionais devem ser extraídos como: métodos de estudo, covariáveis e o tamanho da amostra de cada estudo incluído na revisão. Os métodos de coleta de dados de exposição e resultados, que comumente incluem questionários, registros ou entrevistas, também devem ser indicados (CRD, 2009).

### 2.7.6 Avaliação da qualidade metodológica

A avaliação da qualidade metodológica, ou avaliação crítica, é um processo conduzido em RS para estabelecer a validade interna e o risco de viés dos estudos que atendem aos critérios de inclusão da revisão (TUFANARU et al., 2020). O viés refere-se a erros sistemáticos, em qualquer tipo de estudo, que resultem em uma estimativa incorreta da associação entre risco putativo ou fatores preditivos e o resultado do estudo. É importante tentar detectar a direção do viés, isto é, se é no sentido de uma mudança na estimativa do efeito do risco ou não (HIGGINS et al., 2021).

Quando ensaios clínicos randomizados (ECR) são incluídos, a ferramenta recomendada para avaliação de risco de viés dos estudos é a versão revisada da

ferramenta *Cochrane*, *Risk of Bias 2* (RoB 2) (STERNE et al., 2011). A ferramenta é estruturada em cinco domínios: (1) viés decorrente do processo de randomização; (2) enviesamento devido a desvios das intervenções pretendidas; (3) viés devido à falta de dados de resultado; (4) viés na medição do resultado e (5) viés na seleção do resultado relatado. Dentro de cada domínio, uma série de perguntas ('perguntas de sinalização') visa obter informações sobre as características do estudo que são relevantes para o risco de viés. As opções de resposta para as perguntas de sinalização são: (1) Sim; (2) Provavelmente sim; (3) Provavelmente não; (4) Não e (5) Não há informações. As respostas às perguntas de sinalização e julgamentos sobre o risco de parcialidade devem ser apoiadas por justificativas por escrito. Os julgamentos podem ser de risco ‘baixo’, ‘com considerações’ ou ‘alto’ a todos os domínios, a partir de um algoritmo da ferramenta, por desfecho avaliado. O risco geral de viés para o resultado é a avaliação menos favorável em todos os domínios de viés. Tanto os julgamentos associados a cada um dos cinco domínios, quanto os julgamentos de risco de viés geral, propostos pelo algoritmo, podem ser substituídos pelos autores da revisão, com justificativa (HIGGINS et al., 2021; STERNE et al., 2011).

### 2.7.7 Síntese de dados

A síntese dos dados obtidos a partir das análises dos estudos primários deve ser combinada e relatada na RS. Essencialmente, em uma RS de eficácia, há duas opções de síntese: a síntese estatística, proveniente de uma MA, ou o resumo narrativo (síntese narrativa). Os autores devem garantir que as estimativas de efeito que serão calculadas, a partir da MA, correspondam ao tipo de dado (dicotômicos e / ou contínuos) que eles sugeriram e que serão coletados em seu protocolo. (TUFANARU et al., 2020).

## 2.8 Meta-análise

A MA pode ser definida como a revisão quantitativa e síntese dos resultados de estudos relacionados, mas independentes. Refere-se a um tratamento estatístico de resultados quantitativos, de dois ou mais estudos separados que trata uma medida sumária produzindo uma estatística geral, junto com seu intervalo de confiança. A MA mensura efeitos, ou seja, o contraste entre os desfechos de dois grupos tratados de

forma distinta (TUFANARU et al., 2020). O resumo narrativo sempre deve ser incluído para complementar os detalhes técnicos fornecidos sobre o processo e os resultados, mesmo que uma MA seja realizada, podendo fornecer uma síntese de dados que podem não ter sido identificados na análise estatística (NORMAND, 1999).

As propostas da MA devem ser pré-especificadas no protocolo da RS a ser desenvolvida. Para tanto, a síntese de informações, aplicando-se a MA, pode fornecer estimativas do grau de benefício de uma terapia particular e se o benefício depende de características específicas dos estudos com base as diferenças entre os estudos primários (NORMAND, 1999). A MA deve ser reservada para os resultados de estudos considerados homogêneos, isto é, considerados suficientemente semelhantes do ponto de vista metodológico, relacionado ao desenho e à qualidade de execução dos estudos incluídos; e clínico, associado aos participantes incluídos, intervenções, comparadores, configurações e resultados dos estudos primários (HAIDICH, 2010). Quando os resultados apresentarem heterogeneidade significativa e, diante de uma justificativa clínica plausível, realiza-se então a análise de sensibilidade (QIAN; MAHDI, 2020).

### 2.8.1 Desenvolvimento da meta-análise a partir de ensaios clínicos randomizados de dados contínuos

A MA é normalmente um processo compreendido por duas etapas. No primeiro estágio, uma estatística resumida é calculada para cada estudo e, no segundo estágio, uma estimativa de efeito de intervenção resumida (combinada) é calculada como uma média ponderada dos efeitos de intervenção estimados nos estudos individuais (DEEKS; HIGGINS; ALTMAN, 2021).

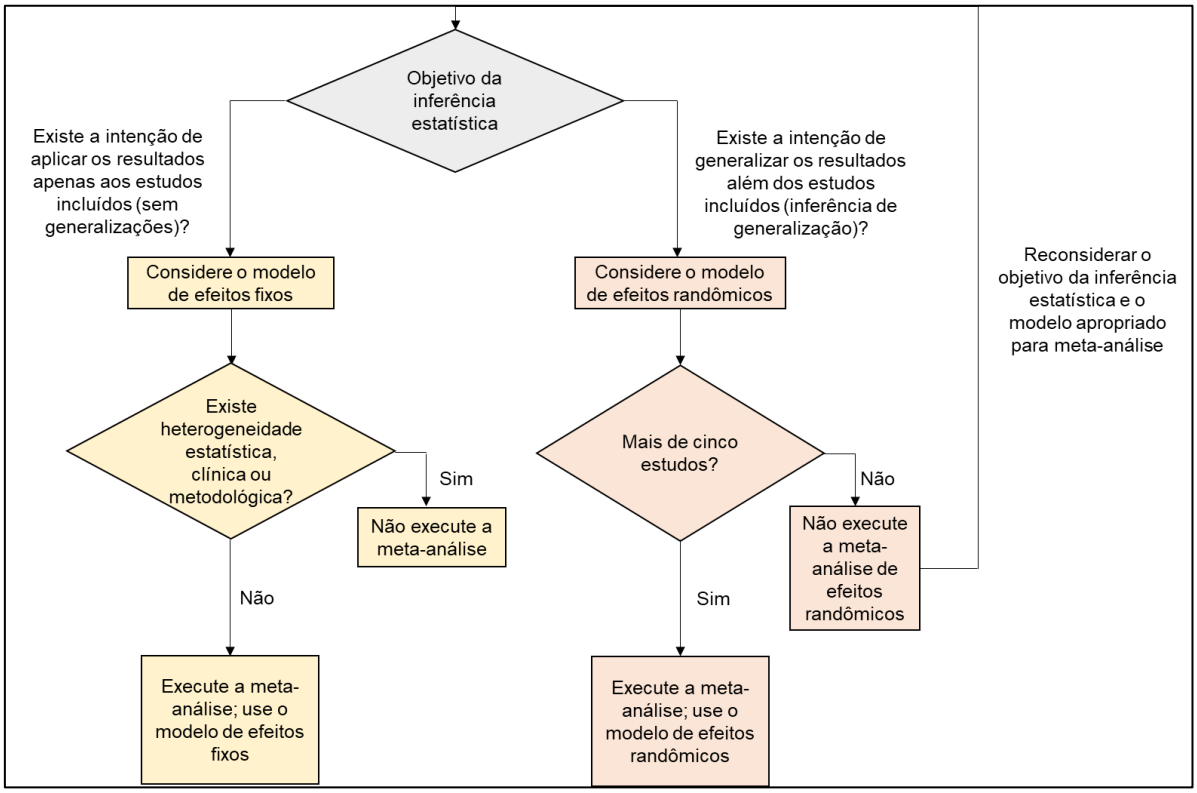
Quanto ao modelo estatístico empregado, existem duas categorias que são aplicadas à MA:

a. Modelo de efeitos fixos: assume-se que o tamanho do efeito verdadeiro para todos os estudos é idêntico e os tamanhos dos efeitos estimados nos estudos são diferentes apenas devido a erros na estimativa do tamanho do efeito.

b. Modelo de efeitos aleatórios: assume-se uma ampla distribuição de efeitos e que o tamanho do efeito de resumo da MA é uma estimativa da média de uma distribuição de efeitos verdadeiros (BORENSTEIN et al., 2010).

A decisão de usar um modelo estatístico ou outro é complexa e frequentemente subjetiva; no entanto, existem critérios que podem orientar as decisões sobre qual modelo usar. A **Figura 8** apresenta uma proposta de fluxograma de decisão para a seleção do modelo estatístico para a MA (TUFANARU et al., 2015).

**Figura 8 -** Proposta de fluxograma de decisão para seleção do modelo estatístico para meta-análise.



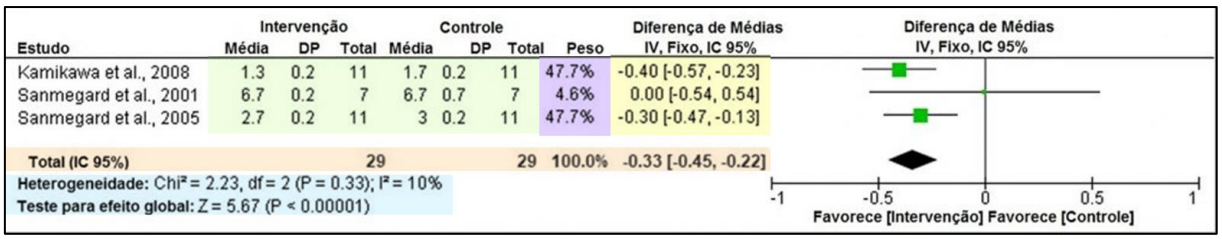

Fonte: Adaptado de TUFANARU et al., 2015.

Dados contínuos, obtidos a partir da extração de dados dos estudos primários, são inseridos em um software de análise. Existem quatro softwares comumente utilizados para a realização da MA. São estes: *Review Manager*®, *R*®, ambos gratuitos; *Comprehensive Meta-Analysis* (CMA) e *Stata*, pagos. Neles, após a inserção dos resultados, estes serão combinados e, em seguida, apresentados visualmente em um gráfico de floresta, ou *forest plot* (CROWTHER; LIM; CROWTHER, 2010).

Para os tamanhos de efeito relacionados a diferenças em dados contínuos, são usados os dados sobre a resposta média, o desvio padrão e o número de participantes em cada grupo – intervenção e controle. A diferença nas médias é a diferença entre a resposta média no grupo de intervenção e a resposta média no grupo de controle, sendo denominada como diferença da média ponderada (WMD). A WMD é usada na MA de dados contínuos se todos os estudos incluídos mediram o resultado usando o mesmo instrumento de medição ou a mesma unidade de medida, sendo os resultados expressos nas unidades naturais (clínicas) usadas para o instrumento de medição comum. A diferença média padronizada (SMD) é uma diferença de médias usada quando há variabilidade na medida dos dados (TUFANARU et al., 2020).

Conforme mencionado, as MA são geralmente ilustradas usando um gráfico de floresta que exibe estimativas de efeito e intervalos de confiança para estudos individuais e MA (LEWIS; CLARKE, 2001). A **Figura 9** exemplifica os principais elementos de um *forest plot*.

**Figura 9 -** Exemplo de gráfico de floresta ou “forest plot”.




Fonte: Adaptado de CROWTHER; LIM; CROWRHER, 2010.

Como demonstrado na **Figura 9**, os nomes dos estudos primários incluídos na análise apresentam-se à esquerda, estando seus resultados sinalizados em verde. Em amarelo, os valores de estatística da MA, por estudo incluído, e o combinado geral sinalizado em alaranjado. A sinalização em lilás mostra o peso dado a cada estudo, que é baseado no número de participantes (estudos maiores recebem mais peso). A sinalização em azul exibe as estatísticas para a MA, incluindo se o resultado geral é estatisticamente significativo (teste para efeito geral), e duas medidas de heterogeneidade (testes $\chi^2$ e $I^2$) (CROWTHER; LIM; CROWRHER, 2010; DEEKS; HIGGINS; ALTMAN, 2021).

Na extrema direita está a representação gráfica dos resultados, conhecida como *forest plot*. Os estudos são apresentados horizontalmente, sendo que o eixo horizontal representa a magnitude da diferença entre o grupo intervenção e controle. Cada estudo é representado por uma caixa verde e uma linha horizontal preta. A caixa verde representa o resultado do estudo - quanto maior a caixa, maior o peso do estudo no resultado geral. A linha horizontal preta representa os intervalos de confiança de 95% para esse estudo. Se tanto a caixa quanto a linha horizontal estiverem à esquerda da linha vertical, então esse estudo mostra que a intervenção é significativamente melhor do que o controle para o desfecho medido, ao passo que, se a caixa e a linha horizontal estiverem todas à direita da linha vertical, então o controle é significativamente melhor que o grupo intervenção. Se a caixa ou linha horizontal cruzar a linha vertical, então o estudo individual não é significativo (CROWTHER; LIM; CROWRHER, 2010).

O resultado geral é representado por um diamante, com o seu tamanho sendo determinado pelos intervalos de confiança de 95% para o resultado combinado geral. Se o losango não tocar a linha vertical, o resultado geral é significativo, sendo que à esquerda a intervenção é melhor do que o grupo de controle em relação o desfecho medido, e à direita indica que o grupo controle é melhor que o grupo intervenção. Se o diamante tocar a linha, não há diferença significativa entre os dois grupos (DEEKS; HIGGINS; ALTMAN, 2021).

### 2.8.2 Heterogeneidade

Uma das principais dificuldades na realização de uma MA é que os estudos combinados são diferentes, resultando em heterogeneidade. Sempre haverá alguma heterogeneidade entre os estudos devido ao acaso, mas ao realizar uma MA, isso precisa ser investigado para determinar se os dados podem ser combinados de forma confiável (CROWTHER; LIM; CROWTHER, 2010). A avaliação é realizada a partir da interpretação do teste Q de Cochran ($\chi^2$) e do $I^2$ de Higgins. O $\chi^2$ é o teste tradicional de heterogeneidade sendo realizado de forma semelhante ao teste de hipótese em uma estatística tradicional, com uma hipótese nula ($H_0$) e uma hipótese alternativa ($H_i$). A $H_0$ afirma que existe homogeneidade entre as estimativas da amostra do

parâmetro da população entre os diferentes estudos. A $H_i$ afirma que existe heterogeneidade entre as estimativas da amostra (SEDGWICK, 2015).

O $I^2$ representa magnitude da variabilidade nas estimativas de efeito devido à heterogeneidade ou variabilidade entre os estudos, tendo seus valores variando de 0 a 100%. Um guia aproximado para interpretação no contexto de meta-análises de ensaios clínicos randomizados seria (LI; HIGGINS; DEEKS, 2021):

- 0% a 40%: pode não ser importante;
- 30% a 60%: pode representar heterogeneidade moderada;
- 50% a 90%: pode representar heterogeneidade substancial;
- 75% a 100%: considerável heterogeneidade.

Vale ressaltar que a importância do valor observado de $I^2$ depende da: (1) magnitude e direção do efeito e; (2) força da evidência para heterogeneidade, por exemplo, valor p do teste de $\chi^2$, ou um intervalo de confiança para $I^2$ (LI; HIGGINS; DEEKS, 2021). Ainda, com um número de estudos reduzidos (<20) tanto o $I^2$, quanto o intervalo de confiança e o teste $\chi^2$, devem ser interpretados com muita cautela (HUEDO-MEDINA et al., 2006).

### 2.8.3 Análise de Subgrupo

A análise de subgrupo refere-se a diversos agrupamentos de estudos com base em características específicas, como o desenho do estudo. Essas características podem incluir os tipos de participantes, tipos de comparadores e os resultados. Para esses subgrupos, é possível realizar uma MA e comparar os efeitos de resumo calculados dentro dos subgrupos. Recomenda-se que, se forem realizadas análises de subgrupos, estas sejam limitadas em número, sejam pré-planejadas no protocolo de revisão e que explicações e justificativas sejam fornecidas explicitamente, devendo os resultados serem interpretados cuidadosamente (TUFANARU et al., 2020).

### 2.8.4 Análise de Sensibilidade

A análise de sensibilidade avalia para quais decisões alternativas ou intervalos de valores, baseados em decisões que eram arbitrárias ou pouco claras, deve-se repetir a análise primária ou MA. A análise de sensibilidade pode envolver a realização da MA duas vezes: a primeira vez incluindo todos os estudos e, segundo, incluindo apenas aqueles que são definitivamente conhecidos como elegíveis (DEEKS; HIGGINS; ALTMAN, 2021). Se os resultados diferirem significativamente nas análises de sensibilidade, isso é uma indicação de que o resultado deve ser interpretado com prudência (TUFANARU et al., 2020).

### 2.8.5 Viés de Publicação

O viés de publicação ocorre quando os estudos publicados diferem sistematicamente de todos os estudos realizados sobre um tópico. Este surge quando estudos com resultados significativos ou resultados positivos em uma direção específica têm maior probabilidade de serem publicados em comparação com estudos sem resultados significativos ou resultados negativos, podendo ter um efeito prejudicial sobre a validade das revisões sistemáticas (MCKENZIE et al., 2021). A melhor maneira de minimizar o impacto do viés de publicação em uma RS é a inclusão de registros de ensaios e estudos não publicados ou literatura cinzenta (LAU et al., 2006; STERNE et al., 2011).

Os métodos aplicados à avaliação de viés de publicação são: (1) avaliação do gráfico de funil e; (2) testes estatísticos de Egger e Begg. Os gráficos de funil são gráficos de dispersão nos quais uma estimativa do efeito de cada estudo é plotada contra uma medida de tamanho ou precisão (ou erro padrão). Os testes estatísticos para assimetria de gráfico de funil (também conhecidos como testes de viés de publicação) investigam a associação entre a estimativa do tamanho do efeito e a medida do tamanho ou precisão do estudo. As hipóteses estatísticas nulas para esses testes refletem a hipótese de simetria do gráfico, isto é, a hipótese de não viés de publicação. Mas um achado de valor p não significativo para o teste de assimetria não exclui viés, uma vez que esses testes são conhecidos por terem baixo poder estatístico (TUFANARU et al., 2020). É importante observar que, quando a revisão

sistemática é composta por estudos primários com alto risco de viés, a meta-análise não deve ser realizada (LANGAN et al., 2019).

### 2.8.6 Avaliação da certeza da evidência

A avaliação da qualidade da evidência produzida pela RS deve levar em consideração as características dos estudos individuais que contribuíram para o desfecho, assim como dos seus resultados combinados, cujo efeito pode ser calculado com a aplicação da MA (TUFANARU et al., 2020). A Cochrane adota a abordagem *Grading of Recommendations, Assessment, Development and Evaluation* (GRADE) para avaliar a certeza de um corpo de evidências (SCHÜNEMANN et al., 2021). Avaliar a certeza de um corpo de evidências envolve a consideração do risco de viés dentro e entre os estudos, inconsistência, evidência indireta, imprecisão das estimativas de efeito e risco de viés de publicação, bem como os domínios que podem aumentar a confiança na estimativa do efeito, implicando em uma avaliação da certeza de um corpo de evidências para cada desfecho (SANTESSO et al., 2020).

No GRADE, um corpo de evidências de estudos randomizados começa com uma classificação de alta certeza, enquanto um corpo de evidências de estudos não randomizados começa com uma classificação de baixa certeza. A classificação mais baixa para estudos não randomizados é o resultado do viés potencial induzido pela falta de randomização (SCHÜNEMANN et al., 2021).

Na avaliação GRADE há domínios que podem levar à diminuição do nível de certeza de um corpo de evidência, como: (1) Risco de parcialidade ou limitações no projeto detalhado e implementação; (2) Evidência indireta; (3) Heterogeneidade inexplicável ou inconsistência dos resultados; (4) Imprecisão de resultados e; (5) Alta probabilidade de viés de publicação. Por outro lado, a presença de gradiente dose-resposta pode aumentar o nível de certeza da evidência produzida (SCHÜNEMANN et al., 2021).

## 3 JUSTIFICATIVA

Sabe-se que a SOP resulta em um comprometimento metabólico e um processo inflamatório subclínico e crônico, tendo como efeito primário, a RI. Esse efeito possui papel significativo em sua patogênese, levando à ocorrência de hiperinsulinemia compensatória, associada a disfunção intrínseca de células β, DM2 e DMG, hiperlipidemia, DCV, obesidade e SM (TEED; DEEKS; MORAN, 2010).

Ainda, a DA é também caracterizada por um estado inflamatório crônico e de baixo grau, resultante da neuro inflamação crônica, expressão de mediadores pró-inflamatórios, indicando a presença de processo pró-inflamatório crônico (LAWRENCE, 2009). Todo este contexto metabólico, concomitante a um estado pró-inflamatório crônico e de baixo grau, pode ser minimizado a partir de hábitos alimentares saudáveis, associados ao aumento do consumo de compostos bioativos, a exemplo do AG ω-3 (CALDER, 2017) e do resveratrol (RAO et al., 2020).

Sendo assim, a hipótese do presente estudo é de que (1) a suplementação com AG ω-3, composto bioativo presente em peixes (salmão, arenque, sardinha e atum) e em oleaginosas (linhaça e chia) seja capaz de influenciar positivamente nos níveis de marcadores inflamatórios e de estresse oxidativo, em mulheres diagnosticadas com SOP, quando comparado ao grupo placebo; (2) o tratamento com metformina seja benéfico no controle dos níveis de marcadores de hiperandrogenismo, em mulheres diagnosticadas com SOP, quando comparadas ao grupo placebo e; (3) a suplementação com resveratrol, que atua em vias de inflamação, possa melhorar os escores obtidos a partir de instrumentos de avaliação de função cognitiva de pacientes com a DA, quando comparado a um grupo placebo. Os resultados obtidos poderão oferecer novas perspectivas terapêuticas nas duas condições clínicas com possível mudança nos protocolos de acompanhamento desses pacientes.

# 4 OBJETIVOS

## 4.1 Objetivo geral

Investigar a influência do AG ω-3 e do resveratrol em marcadores de inflamação ou escore cognitivo, respectivamente, em pacientes com a SOP ou DA e a influência da metformina nos níveis de marcadores androgênicos em pacientes com SOP por meio de uma revisão sistemática da literatura.

## 4.2 Objetivos específicos

- Avaliar se a suplementação com AG ω-3em pacientes com SOP pode influenciar nos níveis de marcadores inflamatórios e de estresse oxidativo a partir de revisão sistemática da literatura e meta-análise.
- Avaliar se o tratamento com metformina em pacientes com SOP pode influenciar nos níveis de marcadores androgênicos a partir de revisão sistemática da literatura e meta-análise.
- Avaliar se a suplementação com resveratrol em pacientes com DA pode influenciar os escores de avaliação de função cognitiva a partir de revisão sistemática da literatura e síntese narrativa.

# 5 MÉTODOS

Para responder aos objetivos propostos, foram desenvolvidos quatro estudos – um estudo primário e três revisões sistemáticas, duas com meta-análise, e uma com síntese narrativa, sendo todas de ensaios clínicos randomizados. A seguir, serão detalhados os métodos aplicados para o desenvolvimento dos quatro estudos.

## 5.1 Métodos – estudo primário

Este estudo subsidiou nossa hipótese de que a inflamação subclínica é importante na fisiopatologia da SOP, o que motivou o desenvolvimento da RS seguinte. O estudo foi aprovado pelo Comitê de Ética e Pesquisa (COEP) da Universidade Federal de Minas Gerais (UFMG), sob identificação CAAE 0379.0.203.000–11. O Termo de Consentimento Livre e Esclarecido (TCLE), por escrito, foi obtido de todas as participantes antes da inclusão no estudo (**Apêndice A**). O estudo, de desenho caso-controle, incluiu 97 mulheres com SOP (idade entre 20 e 44 anos) e 99 mulheres saudáveis como grupo controle (idade entre 18 e 45 anos). O grupo SOP foi selecionado no Hospital Borges da Costa – UFMG, no período de 2011 a 2013. Os critérios da Sociedades Americana de Medicina Reprodutiva e Europeia de Reprodução Humana/Embriologia (ASRM/ESHRE) foram usados para o diagnóstico de SOP. O grupo controle foi composto por funcionários e alunos da UFMG selecionados no mesmo período, sem o diagnóstico de SOP.

O grupo controle hígido foi caracterizado por ciclos ovulatórios com menstruação regular durando 25–35 dias e fase lútea com níveis de progesterona sérica superiores a 5 ng/mL. Os controles apresentaram níveis androgênicos normais, ausência de manifestações cutâneas, relacionadas ao excesso de androgênio, e ausência de ovários policísticos à ultrassonografia. Todas as voluntárias suspenderam as atividades físicas 24 horas antes da participação no estudo. Foram excluídas as voluntárias que apresentavam as seguintes condições: doença inflamatória aguda; diabetes mellitus; tireoide, adrenal, rim, fígado ou doenças autoimunes; câncer; hipogonadismo; gravidez; ou hiperprolactinemia. Mulheres em uso de medicamentos anti-inflamatórios, insulina, metformina, isotretinoína, antirretroviral, ciclosporina ou anticoncepcionais orais também foram excluídas.

A coleta das amostras e a quantificação das citocinas plasmáticas, a qual foi realizada por citometria de fluxo utilizando o *Cytometric Bead Array* (CBA) da BD Biosciences®, foram realizadas em trabalho anterior desenvolvido pela Dra. Mirelle Oliveira Sóter. As análises estatísticas foram realizadas no *software Statistical Package for the Social Sciences* (SPSS), versão 21.0. A normalidade foi avaliada pelo teste de Kolmogorov-Smirnov, sendo as variáveis paramétricas apresentadas como média e desvio padrão e variáveis não paramétricos expressas como mediana e intervalo interquartil. Para comparação dos dados paramétricos, foi utilizado o teste t de Student e, para variáveis não-paramétricas, foi aplicado o teste de Mann-Whitney. O valor de $p < 0,05$ foi considerado significativo.

## 5.2 Métodos – revisões sistemáticas de ensaios clínicos randomizados

### 5.2.1 Métodos aplicados à revisão sistemática – “Influence of n-3 fatty acid supplementation on inflammatory and oxidative stress markers in patients with polycystic ovary syndrome: a systematic review and meta-analysis”.

A condução e o desenho desta revisão sistemática e meta-análise seguiram o protocolo predeterminado de acordo com as recomendações do Manual Cochrane. Os resultados foram relatados de acordo com a declaração de itens do PRISMA. O protocolo do presente estudo foi registrado no Registro Prospectivo Internacional de Revisões Sistemáticas (PROSPERO) (CRD42019129199).

#### 5.2.1.1 Estratégia de busca

A questão de pesquisa foi estruturada de acordo com o acrônimo PICO composta por:

| | |
|---|---|
| **P** | Mulheres diagnosticadas com SOP |
| **I** | Ácidos graxos ômega-3 |
| **C** | Placebo |
| **O** | Marcadores inflamatórios e de estresse oxidativo |
| **S** | Ensaios clínicos randomizados |

Para tanto, foram realizadas buscas em bases de dados eletrônicas por literatura abrangente. As buscas foram realizadas nas bases de dados: MEDLINE, via PubMed), CENTRAL, *Scopus* e *Latin American and Caribbean Health Sciences* (LILACS) até novembro de 2019 com o objetivo de identificar ECR que relataram o efeito da suplementação de AG ω-3 em marcadores inflamatórios e de estresse oxidativo em pacientes com SOP, com idade superior a 18 anos. A pesquisa inicial incluiu os termos *Medical Subject Headings* (MeSH): "Polycystic Ovary Syndrome" e "Fatty Acids, Omega-3". Também incluiu os termos de entrada associados a uma estratégia de alta sensibilidade para a busca por ECR desenvolvida pela Colaboração Cochrane. O **quadro 3** apresenta a estratégia de busca aplicada à base eletrônica MEDLINE via PubMed.

**Quadro 3 -** Estratégia de busca aplicada à base eletrônica MEDLINE via PubMed.

| |
|---|
| ("Polycystic Ovary Syndrome"[Mesh]) OR (Polycystic Ovary Syndrome[Text Word] OR Ovary Syndrome, Polycystic[Text Word] OR Syndrome, Polycystic Ovary[Text Word] OR Stein-Leventhal Syndrome[Text Word] OR Stein Leventhal Syndrome[Text Word] OR Syndrome, Stein-Leventhal[Text Word] OR Sclerocystic Ovarian Degeneration[Text Word] OR Ovarian Degeneration, Sclerocystic[Text Word] OR Sclerocystic Ovary Syndrome[Text Word] OR Polycystic Ovarian Syndrome[Text Word] OR Ovarian Syndrome, Polycystic[Text Word] OR Polycystic Ovary Syndrome 1[Text Word] OR Sclerocystic Ovaries[Text Word] OR Ovary, Sclerocystic[Text Word] OR Sclerocystic Ovary[Text Word]) AND ("Fatty Acids, Omega-3"[Mesh]) OR (Fatty Acids, Omega-3[Text Word] OR n-3 Fatty Acids[Text Word] OR n 3 Fatty Acids[Text Word] OR n-3 Polyunsaturated Fatty Acid[Text Word] OR n 3 Polyunsaturated Fatty Acid[Text Word] OR n-3 PUFA[Text Word] OR PUFA, n-3[Text Word] OR n 3 PUFA[Text Word] OR Omega 3 Fatty Acids[Text Word] OR n3 PUFA[Text Word] OR PUFA, n3[Text Word] OR n3 Polyunsaturated Fatty Acid[Text Word] OR n3 Oils[Text Word] OR n-3 Oils[Text Word] OR n 3 Oils[Text Word] OR Omega-3 Fatty Acids[Text Word] OR n3 Fatty Acid[Text Word] OR Fatty Acid, n3[Text Word]) AND (((((((((randomized controlled trial[Publication Type]) OR controlled clinical trial[Publication Type]) OR randomized[Title/Abstract]) OR placebo[Title/Abstract]) OR drug therapy[ MeSH Subheading]) OR randomly[Title/Abstract]) OR trial[Title/Abstract]) OR groups[Title/Abstract])) NOT ((animals[ MeSH Terms]) NOT humans[ MeSH Terms]) |

Os mesmos termos foram usados na pesquisa por estudos clínicos e literatura cinzenta nas bases eletrônicas: *National Institutes of Health* (www.clinicaltrials.gov), Registro Brasileiro de Ensaios Clínicos (www.ensaiosclinicos.gov.br) e *Turning Research into Practice* (www.tripdatabase.com). Todos os estudos potencialmente elegíveis foram considerados para revisão, independentemente do idioma e data de publicação. Uma busca manual também foi realizada nas listas de referência de revisões relevantes.

5.2.1.2 Critérios de inclusão e exclusão

Foram incluídos ECR que analisaram o efeito da suplementação de AG ω-3 em marcadores inflamatórios e de estresse oxidativo em pacientes diagnosticadas com SOP. O desfecho foi considerado como mudanças na concentração ou atividade desses marcadores ao fim do *follow-up*. Foram excluídos os estudos que não relataram os desfechos de interesse, estudos não randomizados, estudos observacionais, revisões, estudos experimentais e aqueles que incluíram crianças, adolescentes (menores de 18 anos) ou mulheres grávidas. Estudos que não apresentaram como desfechos marcadores de estresse oxidativo ou inflamatório também foram excluídos.

5.2.1.3 Seleção de estudos e extração de dados

Inicialmente, os estudos recuperados das bases de dados foram inseridos em uma única biblioteca eletrônica e as duplicatas excluídas utilizando-se o *software* EndNote® (Versão X5, Londres, Reino Unido). Dois revisores (J.A.G.T. e M.T.A.) analisaram independentemente os títulos e resumos dos artigos recuperados na pesquisa bibliográfica, revisaram o texto completo dos artigos publicados e extraíram os dados usando uma ferramenta de extração de dados padronizada. Quaisquer discordâncias entre os revisores, em relação aos dados dos estudos primários, foram elucidadas por um terceiro investigador (K.B.G. ou V.E.A).

Os dados extraídos incluíram o número de participantes, desenho do estudo, duração do ensaio e características demográficas e antropométricas dos pacientes (idade e Índice de Massa Corporal - IMC). Dados de suplementação de ácido graxo ômega-3, dos grupos intervenção e controle, foram coletados. Dados sobre marcadores inflamatórios e de estresse oxidativo, determinados pelos estudos primários, no início e no final do estudo foram extraídos. As alterações percentuais nas concentrações dos biomarcadores foram calculadas para os estudos que apresentaram valores basais e finais.

5.2.1.4 Avaliação do risco de viés entre os estudos e da certeza da evidência

O risco de viés dos estudos e a qualidade da evidência foram avaliados independentemente por dois revisores (J.A.G.T. e M.T.A.) seguindo as diretrizes da Colaboração Cochrane, e um terceiro revisor (K.B.G. ou V.E.A), resolveu quaisquer disparidades. A ferramenta Cochrane RoB 2.0 foi aplicada para avaliar o risco de viés nos estudos individuais.

A abordagem GRADE foi usada para avaliar a qualidade das evidências para cada desfecho: hs-PCR, adiponectina, visfatina, óxido nítrico (NO), glutationa (GSH), malonaldeído (MDA) e capacidade total antioxidante (TAC).

5.2.1.5 Análise estatística

As diferenças entre os valores médios e desvios-padrão, no início e no final dos estudos, foram usadas para relatar as mudanças nas concentrações dos marcadores inflamatórios e de estresse oxidativo investigados. A heterogeneidade entre os estudos foi avaliada pelo teste Q de Cochran, e o valor $p \leq 0,10$ foi considerado significativo. O valor de $I^2$ foi avaliado, levando-se em conta a magnitude da heterogeneidade, considerada alta se $I^2 \geq 50,0\%$. As estimativas combinadas das diferenças médias ponderadas (WMD) para NO, GSH, TAC e MDA, e as estimativas das diferenças médias padronizadas (SMD) para hs-PCR, adiponectina e visfatina entre a suplementação de ácido graxo ômega-3 e grupos de controle foram calculados por meio do modelo de efeitos randômicos. O viés de publicação não foi acessado por meio de assimetria de gráfico de funil, uma vez que, de acordo com a Colaboração Cochrane, ele deve ser realizado quando houver pelo menos 10 estudos incluídos na meta-análise. No presente estudo, para todos os desfechos foram incluídos, no máximo, sete estudos primários. As análises estatísticas foram realizadas no software Review Manager 5®. Valores significativos foram considerados com valor $p < 0,05$ e apresentados com intervalo de confiança (IC) de 95%.

5.2.2 Métodos aplicados à revisão sistemática – “Influence of metformin on hyperandrogenism in women with Polycystic Ovary Syndrome: a systematic review and meta-analysis of randomized clinical trials.”

O desenho deste estudo seguiu as recomendações do *Cochrane Manual for Systematic Reviews of Interventions*, sendo relatado de acordo com o PRISMA, tendo seu protocolo registrado no PROSPERO, sob a identificação CRD42021235761.

5.2.2.1 Estratégia de busca

As estratégias de busca aplicadas às bases de dados eletrônicas foram construídas com base na sigla PICO, de acordo com a questão de pesquisa proposta:

| | |
|---|---|
| **P** | Mulheres diagnosticadas com SOP |
| **I** | Tratamento com metformina |
| **C** | Placebo |
| **O** | Níveis dos marcadores androgênicos |
| **S** | Ensaios clínicos randomizados |

A revisão da literatura foi realizada com base em buscas nas bases de dados eletrônicas: MEDLINE via PubMed, CENTRAL, Embase, CINAHL, Web of Science e Scopus até novembro de 2021, com o objetivo de identificar ECR que relataram os efeitos do tratamento com metformina nos níveis de marcadores androgênicos em mulheres adultas com SOP. A busca incluiu os termos MeSH “Polycystic Ovary Syndrome”, “Metformin” e “Hyperandrogenism” ou “Androgenism” e seus respectivos termos de entrada. Para considerar apenas ECR, foi usada a estratégia de busca de alta sensibilidade desenvolvida pela Cochrane Collaboration. A estratégia de busca completa, realizada na base de dados MEDLINE, é apresentada no **Quadro 4**.

**Quadro 4 -** Estratégia de busca aplicada à base eletrônica MEDLINE via PubMed.

| |
|---|
| ((("Polycystic Ovary Syndrome"[Mesh]) OR (Polycystic Ovary Syndrome[Text Word] OR Ovary Syndrome, Polycystic[Text Word] OR Syndrome, Polycystic Ovary[Text Word] OR Stein-Leventhal Syndrome[Text Word] OR Stein Leventhal Syndrome[Text Word] OR Syndrome, Stein-Leventhal[Text Word] OR Sclerocystic Ovarian Degeneration[Text Word] OR Ovarian Degeneration, Sclerocystic[Text Word] OR Sclerocystic Ovary Syndrome[Text Word] OR Polycystic Ovarian Syndrome[Text Word] OR Ovarian Syndrome, Polycystic[Text Word] OR Polycystic Ovary Syndrome 1[Text Word] OR Sclerocystic Ovaries[Text Word] OR Ovary, Sclerocystic[Text Word] OR Sclerocystic Ovary[Text Word])) AND (("Metformin"[Mesh]) OR (Metformin[Text Word] OR Dimethylbiguanidine[Text Word] OR Dimethylguanylguanidine[Text Word] OR Glucophage[Text Word] OR Metformin Hydrochloride[Text Word] OR Hydrochloride, Metformin[Text Word] OR Metformin HCl[Text Word] OR HCl, Metformin[Text Word]))) AND ((("Hyperandrogenism"[Mesh]) OR (Hyperandrogenism[Text Word])) OR (("Androgens"[Mesh]) OR (Androgens[Text Word] OR Androgen Receptor Agonist[Text Word] OR Agonist, Androgen Receptor[Text Word] OR Receptor Agonist, Androgen[Text Word] OR Androgenic Compounds[Text Word] OR Compounds, Androgenic[Text Word] OR Androgen Receptor Agonists[Text Word] OR Agonists, Androgen Receptor[Text Word] OR Receptor Agonists, Androgen[Text Word] OR Androgenic Agents[Text Word] OR Agents, Androgenic[Text Word] OR Androgen[Text Word] OR Androgen Effect[Text Word] OR Effect, Androgen[Text Word] OR Androgen Effects[Text Word] OR Effects, Androgen[Text Word]))) |

Os termos aplicados à busca principal foram utilizados para pesquisa de literatura cinza nas bases de dados Open Gray (http://www.opengrey.eu/search/) e Open Access Theses and Dissertations (https://oatd.org/). Também foram realizadas buscas de estudos clínicos e protocolos nas bases de dados do National Institutes of Health (www.clinicaltrials.gov), no ReBEC (www.ensaiosclinicos.gov.br) e no Turning Research in Practice (www.tripdatabase.com), e uma busca manual também foi realizada nas listas de referências de revisões relevantes. Todos os estudos potencialmente elegíveis foram considerados para revisão, independentemente do idioma e data de publicação.

5.2.2.2 Critérios de inclusão e exclusão

Apenas ECR que avaliaram os efeitos do tratamento com metformina sobre os níveis de marcadores androgênicos em mulheres maiores de 18 anos com SOP foram incluídos. O desfecho primário foi considerado como alterações nos valores iniciais e finais dos biomarcadores androgênicos avaliados pelos estudos primários, a saber: (1) androstenediona; (2) DHEAS; (3) FAI; (4) SHBG; (5) testosterona livre e; (6) testosterona total. Os estudos foram excluídos se utilizassem metformina apenas como intervenção adjuvante (associada a intervenções primárias como restrição calórica ou anticoncepcional oral), se não relatassem pelo menos um dos desfechos

de interesse ou se não fossem randomizados ou estudos placebo controlados. Artigos de revisão, relatos de caso e estudos comentados também foram excluídos.

5.2.2.3 Seleção de estudos e extração de dados

Essa etapa incluiu a recuperação dos estudos obtidos na pesquisa nas bases de dados e sua inserção em uma única biblioteca eletrônica. As duplicatas foram excluídas usando o software Endnote®, (Versão X5, Londres, Reino Unido). Após a retirada das duplicatas, dois autores (A.F.S.F. e J.A.G.T.) analisaram independentemente os títulos e resumos dos demais artigos e, após a exclusão dos estudos que não atenderam aos critérios de inclusão, os mesmos revisores procederam à leitura completa dos artigos e extraíram os dados usando uma ferramenta padronizada de extração de dados hospedada no software Microsoft Excel® (Versão 365, Washington, Estados Unidos). Qualquer discordância entre os revisores foi resolvida por um terceiro investigador (K.B.G.).

Os dados extraídos dos artigos incluídos foram número de participantes alocados em cada grupo (intervenção e placebo), duração do acompanhamento do ECR, características demográficas da população estudada, dosagem de metformina administrada ao grupo intervenção e características do placebo grupo. As informações também foram extraídas dos níveis de biomarcadores androgênicos, realizados no início e no final do acompanhamento do ECR, ou da diferença entre as médias, dependendo do formato de apresentação dos dados pelos estudos primários.

5.2.2.4 Avaliação do risco de viés entre os estudos e da certeza da evidência

A avaliação do risco de viés dos estudos incluídos foi avaliada independentemente por dois revisores (A.F.S.F. e J.A.G.T.), de acordo com as recomendações do *Cochrane Manual for Systematic Reviews of Interventions*, e um terceiro revisor (K.B.G.) resolveu qualquer disparidade. A ferramenta *Cochrane* Rob 2.0 (Versão 9 beta, Londres, Reino Unido), hospedada no software Microsoft Excel® (Versão 365, Washington, Estados Unidos), foi aplicada para avaliar a qualidade metodológica dos estudos individuais, sendo estruturada em cinco domínios: (1) viés resultante do processo de randomização; (2) viés devido a desvios das intervenções pretendidas;

(3) viés devido à falta de dados de desfecho; (4) viés na medição de resultados e (5) viés na seleção de resultados relatados. As opções de resposta para as perguntas são: (1) sim; (2) provavelmente sim; (3) provavelmente não; (4) não e; (5) não há informações. Ao final, notas – altas, com considerações e baixas – foram atribuídas a todos os domínios, com base em um algoritmo da ferramenta, para cada um dos estudos incluídos.

5.2.2.5 Síntese e análise de dados

Para avaliar as mudanças nos níveis de marcadores androgênicos, os valores obtidos no início e no final de cada estudo foram extraídos e incluídos na meta-análise. O modelo de efeitos aleatórios foi aplicado porque todos os desfechos avaliados tiveram mais de cinco estudos incluídos. Os resultados foram apresentados como SMD com IC de 95% como medida de efeito, dadas as possíveis diferenças nos métodos de mensuração dos marcadores entre os estudos incluídos.

A heterogeneidade nos estudos primários foi avaliada pelo teste Cochran Q, e o valor de p ≤0,10 foi considerado estatisticamente significativo. O $I^2$ de Higgins também foi realizado para avaliar a magnitude da heterogeneidade, que foi considerada alta se $I^2$ ≥ 50,0%. A heterogeneidade dos resultados avaliados pela meta-análise foi explorada com análise de sensibilidade, excluindo cada estudo em série. Para tanto, a meta-análise foi realizada duas vezes: a primeira incluiu todos os estudos e a segunda incluiu apenas aqueles definitivamente reconhecidos como elegíveis, dentro dos fatores avaliados.

O viés de publicação foi avaliado por meio da assimetria do gráfico de funil e do índice do teste de Egger para todos os desfechos, exceto FAI, pois, de acordo com as recomendações do *Cochrane Manual for Systematic Reviews of Interventions*, o método deve ser utilizado quando houver pelo menos 10 estudos incluídos no meta-análise. Para o teste de Egger, valores de p<0,05 foram considerados significativos. As análises estatísticas foram realizadas usando o *software* Review Manager® (versão 5, Londres, Reino Unido), exceto para viés de publicação, que foi avaliado usando o *software* CMA® (versão 3, Englewood, EUA).

5.2.2.6 Avaliação da certeza da evidência

A abordagem GRADE forneceu os métodos para avaliar a certeza da evidência para cada um dos resultados avaliados: (1) androstenediona; (2) DHEAS; (3) FAI; (4) SHBG; (5) testosterona total e; (6) testosterona livre. Para tanto, os dados compilados no Review Manager® (versão 5, Londres, Reino Unido) foram importados para a ferramenta de desenvolvimento de diretrizes (www.gradepro.org) e as evidências foram classificadas em alta, moderada, baixa ou muito baixa qualidade, de acordo com critérios de avaliação de certeza (risco de viés, inconsistência, evidência indireta e imprecisão) e outras considerações (viés de publicação e possíveis fatores de confusão). Ao final, a certeza da evidência foi classificada como certeza alta, moderada, baixa ou muito baixa.

5.2.3 Métodos aplicados à revisão sistemática – “Effects of resveratrol supplementation on the cognitive function of patients with Alzheimer's disease: a systematic review of randomized controlled trials”.

Esta revisão sistemática de estudos de intervenção foi desenvolvida de acordo com os critérios determinados pelo *Cochrane Handbook for Systematic Reviews of Interventions* (versão 6.2) e relatada de acordo com o PRISMA, tendo seu protocolo registrado no PROSPERO, sob a identificação CRD42021229234.

5.2.3.1 Estratégia de busca

O acrônimo PICOS foi aplicado para estruturar as estratégias de busca construídas, com base na questão de pesquisa proposta pelo estudo. Portanto, a sigla foi composta por:

| | |
|---|---|
| **P** | Adultos com diagnóstico de DA |
| **I** | suplementação de resveratrol |
| **C** | Placebo |
| **O** | Avaliação do desempenho cognitivo e funcional |
| **S** | Ensaios clínicos randomizados |

As pesquisas foram realizadas nas bases de dados eletrônicas CENTRAL, *Cumulative Index to Nursing and Allied Health Literature* (CINAHL), Embase, MEDLINE via PubMed, *Scopus* e *Web of Science* (WOS), até agosto de 2021, com o objetivo de identificar apenas ECR controlados por placebo que relataram o efeito da suplementação de resveratrol na função cognitiva de adultos com diagnóstico de DA. A pesquisa incluiu os termos MeSH 'doença de alzheimer' e 'resveratrol' e seus respectivos termos de entrada usando os operadores booleanos. Para direcionar a pesquisa ao desenho do estudo proposto, a estratégia de pesquisa de alta sensibilidade, desenvolvido pela Colaboração Cochrane Collaboration para ECR, foi adicionada. O **Quadro 5** apresenta a estratégia de busca aplicada à base eletrônica MEDLINE via PubMed.

**Quadro 5 -** Estratégia de busca aplicada à base eletrônica MEDLINE via PubMed.

| |
|---|
| (("Alzheimer Disease"[Mesh]) OR (Alzheimer Disease[Text Word] OR Alzheimer's Disease[Text Word] OR Dementia, Senile[Text Word] OR Senile Dementia[Text Word] OR Dementia, Alzheimer Type[Text Word] OR Alzheimer Type Dementia[Text Word] OR Alzheimer-Type Dementia (ATD)[Text Word] OR Alzheimer Type Dementia (ATD)[Text Word] OR Dementia, Alzheimer-Type (ATD)[Text Word] OR Alzheimer Type Senile Dementia[Text Word] OR Primary Senile Degenerative Dementia[Text Word] OR Dementia, Primary Senile Degenerative[Text Word] OR Alzheimer Sclerosis[Text Word] OR Sclerosis, Alzheimer[Text Word] OR Alzheimer Syndrome[Text Word] OR Alzheimer Dementia[Text Word] OR Alzheimer Dementias[Text Word] OR Dementia, Alzheimer[Text Word] OR Dementias, Alzheimer[Text Word] OR Senile Dementia, Alzheimer Type[Text Word] OR Acute Confusional Senile Dementia[Text Word] OR Senile Dementia, Acute Confusional[Text Word] OR Dementia, Presenile[Text Word] OR Presenile Dementia[Text Word] OR Alzheimer Disease, Late Onset[Text Word] OR Late Onset Alzheimer Disease[Text Word] OR Alzheimer's Disease, Focal Onset[Text Word] OR Focal Onset Alzheimer's Disease[Text Word] OR Familial Alzheimer Disease (FAD)[Text Word] OR Alzheimer Disease, Familial (FAD)[Text Word] OR Alzheimer Diseases, Familial (FAD)[Text Word] OR Familial Alzheimer Diseases (FAD)[Text Word] OR Alzheimer Disease, Early Onset[Text Word] OR Early Onset Alzheimer Disease[Text Word] OR Presenile Alzheimer Dementia[Text Word])) AND (("Resveratrol"[Mesh]) OR (Resveratrol[Text Word] OR 3,5,4'-Trihydroxystilbene[Text Word] OR 3,4',5-Trihydroxystilbene[Text Word] OR 3,4',5-Stilbenetriol[Text Word] OR trans-Resveratrol-3-O-sulfate[Text Word] OR trans Resveratrol 3 O sulfate[Text Word] OR SRT 501[Text Word] OR SRT501[Text Word] OR SRT-501[Text Word] OR cis-Resveratrol[Text Word] OR cis Resveratrol[Text Word] OR Resveratrol, (Z)-[Text Word] OR trans-Resveratrol[Text Word] OR trans Resveratrol[Text Word] OR Resveratrol-3-sulfate[Text Word] OR Resveratrol 3 sulfate[Text Word])) |

Os mesmos termos MeSH aplicados à pesquisa principal foram usados na busca por literatura cinzenta na base de dados *Open Gray* (http://www.opengrey.eu/search/), por estudos clínicos e protocolos no *National Institutes of Health* (www.clinicaltrials.gov), no *Turning Research into Practice* (www.tripdatabase.com) e no Registro Brasileiro de Ensaios Clínicos (ReBEC) (www.ensaiosclinicos.gov.br) e por teses e dissertações no *Open Access Theses and Dissertations* (https://oatd.org/). Além disso, uma busca manual também foi realizada nas listas de referências de revisões relevantes. Todos

os estudos potencialmente elegíveis foram considerados para revisão, independentemente da data de publicação e idioma.

5.2.3.2 Critérios de Inclusão e Exclusão

Foram incluídos apenas ECR controlados por placebo, sem limite de duração de acompanhamento. Foram analisados estudos que avaliaram os efeitos da suplementação de resveratrol, isolada ou em combinação com outros compostos, no desempenho cognitivo e funcional, medido por instrumentos de avaliação da função cognitiva, em pacientes com diagnóstico de DA. Os instrumentos aplicados para avaliar a função cognitiva de pacientes com DA, pelos estudos incluídos, deveriam ser (1) ADAS-cog; (2) ADCS-ADL; ou (3) MMSE.

Quanto aos critérios de exclusão, foram considerados estudos que não relataram os desfechos de interesse, estudos não controlados por placebo e não randomizados, bem como artigos de revisão, relatos de casos e estudos de comentários. Estudos que incluíram pacientes não diagnosticados com DA, estudos experimentais, bem como estudos de desenho observacional também foram excluídos.

5.2.3.3 Seleção de estudos e processo de extração de dados

A etapa de seleção dos estudos incluiu a união de todos os arquivos exportados, resultantes das buscas nas bases de dados eletrônicas, literatura cinzenta e busca manual, em um único arquivo eletrônico e importados para o gerenciador de referência Mendeley®, (Versão 2.61.1. Londres, Reino Unido) para remover duplicatas. Dois pesquisadores (J.A.G.T. e A.F.S.F.) avaliaram primeiro os títulos e resumos de todos os estudos primários e depois o texto completo foi lido. Um terceiro pesquisador (K.B.G.) resolveu divergências em ambas as etapas. Toda a etapa de seleção dos estudos foi realizada por meio de ferramenta de avaliação padronizada, hospedada no software Microsoft Excel® (Versão 365, Washington, Estados Unidos), para facilitar o acesso de todos os pesquisadores envolvidos no desenvolvimento da revisão sistemática.

A extração dos dados dos estudos primários foi realizada por dois pesquisadores (J.A.G.T. e A.F.S.F.) a fim de solucionar eventuais erros de coleta, obtendo dados

sobre os autores dos estudos, país e ano de publicação, tempo de seguimento, número total de participantes incluídos, e pelo grupo - intervenção e placebo. Também foram coletados dados sobre a suplementação com resveratrol, isoladamente ou em combinação, relacionados à dosagem e frequência da suplementação e os escores dos desfechos analisados - ADAS-cog, ADCS-ADL e MMSE, no início e no final do seguimento. Para dois estudos incluídos, foi necessário entrar em contato com os autores para maiores informações sobre os escores avaliados, mas não obtivemos resposta. Portanto, foi necessário estimar valores gráficos a partir de um dos estudos usando o software *Web Plot Digitizer*®, versão 4.5 (https://apps.automeris.io/wpd/).

Os dados extraídos de cada um dos estudos primários foram resumidos em tabelas e a revisão narrativa, por desfecho, foi realizada de forma a fornecer uma síntese dos resultados obtidos, de acordo com a intervenção realizada.

#### 5.2.3.4 Avaliação do risco de viés entre os estudos

Dois investigadores independentes (J.A.G.T. e A.F.S.F.) avaliaram o risco de viés dos estudos incluídos na revisão sistemática com base no indicado pela Colaboração Cochrane e um terceiro revisor (K.B.G.) resolveu as disparidades. Para tanto, foi aplicada a ferramenta de avaliação de viés Rob 2.0.

## 6 RESULTADOS

Os resultados da presente tese de doutorado compreenderam quatro manuscritos originais cuja metodologia foi descrita previamente na seção “Métodos”. Todos os manuscritos serão apresentados a seguir na formatação original publicada de acordo com as normas dos periódicos.

6.1 Estudo I. *The hallmark of pro- and anti-inflammatory cytokine ratios in women with polycystic ovary syndrome.*
Artigo publicado no periódico *Cytokine.*
Fator de impacto: 2.952, equivalente ao Qualis CAPES B1.

6.2 Estudo II. *Influence of n-3 fatty acid supplementation on inflammatory and oxidative stress markers in patients with polycystic ovary syndrome: a systematic review and meta-analysis.*
Artigo publicado no periódico *British Journal of Nutrition.*
Fator de impacto: 3.334, equivalente ao Qualis CAPES A1.

6.3 Estudo III. *Influence of metformin on hyperandrogenism in women with Polycystic Ovary Syndrome: a systematic review and meta-analysis of randomized clinical trials.*
Artigo submetido ao periódico *BJOG: An International Journal of Obstetrics & Gynaecology.*
Fator de impacto: 6.531, equivalente ao Qualis CAPES A1.

6.4 Estudo IV. *Effects of resveratrol supplementation on the cognitive function of patients diagnosed with Alzheimer's Disease: a systematic review of randomized controlled trials.*
Artigo publicado no periódico *Drugs & Aging.*
Fator de impacto: 3.923, equivalente ao Qualis CAPES A1.

## 6.1 Estudo I. The hallmark of pro- and anti-inflammatory cytokine ratios in women with polycystic ovary syndrome.

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Short communication

# The hallmark of pro- and anti-inflammatory cytokine ratios in women with polycystic ovary syndrome

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ABSTRACT

Polycystic Ovary Syndrome (PCOS) is a heterogeneous endocrinopathy considered to be the most common metabolic disorder in women of reproductive age. Women with PCOS present with an increased risk of non-communicable diseases (NCDs), especially low-grade chronic inflammation mediated by proinflammatory cytokines, and insulin resistance. This study aimed to investigate cytokine levels and their ratios in PCOS women compared to a healthy control group. This study evaluated 97 women with PCOS and 99 healthy women as controls. The PCOS diagnosis was performed according to ESHRE/ASRM. Plasma cytokines were evaluated by flow cytometry. We observed lower TNF levels, and decreased TNF/IL-6, TNF/IL-2, and TNF/IL-4 ratios in PCOS patients compared to the control group ($p < 0.05$). These results indicate an imbalance between pro- and anti-inflammatory cytokines, with prominent counter-regulatory cytokine production. These changes may be important in explaining the phenotypes present in PCOS and to direct better interventions for patients with this syndrome.

## 1. Introduction

Polycystic Ovary Syndrome (PCOS) is a multi-symptom endocrinopathy that results from androgen excess, particularly testosterone, and ovarian dysfunction [1]. The prevalence of PCOS can range from 6% to 20%, depending on the diagnostic criteria. PCOS is one of the most common metabolic/endocrine disorders in women of reproductive age [2]. The syndrome has heterogeneous clinical manifestations, and women with hyperandrogenic phenotypes are at a higher risk of developing dyslipidemia, hypertension, and type 2 diabetes mellitus (T2DM) at a younger age when compared with healthy women of the same age [3].

Adipose tissue is an active organ that secretes adipokines, hormones, and cytokines; it is associated with endocrine processes that regulate immunity and inflammatory response, glucose, and fatty metabolism, and reproductive capacity [4]. The pathophysiology of PCOS is not yet well known, but it has been suggested that abdominal obesity, hyperandrogenism, and insulin resistance are associated with its development [5]. A low-grade chronic inflammation mediated by proinflammatory cytokines may be induced by excess central fat in women with PCOS [6]. The aim of this research was to investigate plasma cytokine levels and their ratio in PCOS women compared with a healthy control group.

## 2. Methods

This study was approved by the local Ethics Committee (COEP) of the Federal University of Minas Gerais (UFMG) (n. CAAE 0379.0.203.000–11). Written informed consent was obtained from all participants before inclusion in the study. This case-control study included 97 women with PCOS (aged from 20 to 44 years) and 99 healthy women as a control group (18 to 45 years). The PCOS group was selected at Hospital Borges da Costa - UFMG, Brazil, from 2011 to 2013. The control group was composed of employees and students from UFMG selected in the same period. The European Society of Human Reproduction/Embryology and the American Society for Reproductive Medicine criteria (ESHRE/ASRM) was used for diagnosis of PCOS [7]. The healthy control group was characterized by ovulatory cycles with regular menses lasting 25–35 days and a luteal phase with a serum progesterone level greater than 5 ng/mL. The controls showed normal

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androgen levels, absence of skin manifestations related to androgen excess, and absence of polycystic ovaries on ultrasound. All subjects suspended physical activities 24 h prior to study participation. Individuals that presented with the following conditions were excluded: acute inflammatory disease; diabetes mellitus; thyroid, adrenal, kidney, liver, or autoimmune diseases; cancer; hypogonadism; pregnancy; or hyperprolactinemia. Women using anti-inflammatory medications, insulin, metformin, isotretinoin, antiretroviral, cyclosporine, or oral contraceptives were also excluded. Venous blood samples were collected after fasting for 12 h. The samples were centrifuged at 2500g for 20 min at 4 °C to obtain the plasma (EDTA) or serum. Aliquots were stored at −80 °C until use. The determination of plasma cytokines was performed by flow cytometry using the Cytometric Bead Array (CBA) (BD Biosciences), following the manufacturer's instructions. Statistical analyses were performed using the software SPSS version 21.0. Normality was assessed using the Kolmogorov-Smirnov test. Parametric variables were presented as mean and standard deviation; non-parametric were expressed as median and interquartile range. For comparison of parametric data, we used the Student *t*-test. For non-parametric variables, we used the Mann-Whitney. $P < 0.05$ value was considered significant.

## 3. Results and discussion

The PCOS and control groups had similar age (30.63 ± 4.99 years for PCOS and 29.53 ± 7.06 years for controls) and body mass index (BMI) [28.52 (7.20) kg/m2 for PCOS and 23.69 (5.22) kg/m2 for controls]. No difference in fasting glucose levels was observed between the groups (4.8 ± 9.1 mmol/L for PCOS, 4.7 ± 8.2 for controls, $p = 0.265$), but higher insulin levels were observed in PCOS patients [12.3 (17.4) uUI/mL] when compared to control group [7.7 (3.4) uUI/mL, $p < 0.001$]. The levels of tumor necrosis factor (TNF), transforming growth factor-beta (TGF-β), interleukins (IL) IL-10, IL-6, IL-2, IL-4 and interferon-gamma (IFN-γ) in both groups, along with statistically significant ratios of these cytokines are presented in Table 1. We observed lower TNF levels (Fig. 1**a**), as well as TNF/IL-2 (Fig. 1**b**), TNF/IL-4 (Fig. 1**c**), and TNF/IL-6 (Fig. 1**d**) ratios in women with PCOS compared to the control group.

The understanding of inflammation-related disorders in women with PCOS is an essential component for any measure of prevention of associated diseases, such as cardiovascular disease and T2DM [8].

The TGF-β and INF-γ levels did not show significant differences between the PCOS and control groups. These data corroborate the findings of a randomized clinical trial conducted with 32 volunteers (PCOS: n = 20; control: n = 12) that assessed the impact of PCOS on the levels of circulating cytokines and the effects of metformin on insulin action and cytokine levels [9]. The study suggested that the association between inflammation in skeletal muscle and insulin resistance in PCOS differed from other insulin-resistant states such as obesity and T2DM, where the circulating levels of TGF-β and INF-γ are not routinely altered by PCOS [9].

**Table 1**
Concentrations and ratio of biomarkers on PCOS and control groups.

| Markers | PCOS group | Control group | p Value |
|---|---|---|---|
| TNF | 1.71 (1.25) | 2.21 (1.49) | 0.011* |
| TGF-β | 7.43 (3.23) | 8.43 (6.24) | 0.335 |
| IL-10 | 4.00 (2.29) | 3.78 (2.25) | 0.779 |
| IL-6 | 2.60 (3.04) | 2.83 (3.38) | 0.233 |
| IFN-γ | 2.31 (1.13) | 2.36 (1.32) | 0.111 |
| IL-2 | 4.38 (0.73) | 4.35 (0.64) | 0.426 |
| IL-4 | 5.49 (1.96) | 5.12 (1.66) | 0.574 |
| TNF/IL-6 | 0.57 (0.19) | 0.61 (0.20) | 0.023* |
| TNF/IL-2 | 0.40 (0.35) | 0.49 (0.39) | 0.037* |
| TNF/IL-4 | 0.28 (0.41) | 0.38 (0.43) | 0.010* |

Data are median (interquartile range). * p Value < 0.05 was considered statistically significant.

It is known that pro-inflammatory cytokines TNF and IL-6 are closely related to obesity and insulin resistance. Adipose tissue is responsible for producing both cytokines, and many PCOS women present accumulations of visceral fat and alterations in insulin sensitivity [10]. However, in the present study, higher levels of TNF were observed in the control group and no significant difference was observed for IL-6 levels. A possible explanation would be that TNF levels have an inverse correlation with endogenous hyperinsulinemia [11], which was observed in women with PCOS in our study, although this correlation was not significant ($p = 0.279$, $r = -0.043$). Inflammation and glucose are interrelated with mutual causation. Insulin, being the only glucose-lowering hormone in the body, prevents the detrimental effects of hyperglycemia through metabolic regulation. Insulin acts as an anti-inflammatory factor through suppression of proinflammatory cytokines and immune mediators [12]. Curiously, we observed a lower TNF/IL-6 ratio in PCOS women, indicating higher IL-6 production in this group, although its measurement was not sensitive enough to detect this variation between the groups.

No differences in IL-10 were observed between the groups. Although IL-10 is positively regulated in the adipose tissue to limit the systemic pro-inflammatory response commonly observed in obesity [13], a case-control study comparing lean or overweight PCOS women to overweight controls showed no significant differences in levels of IL-10, corroborating our findings in the present study [13].

We found lower TNF/IL-2 and TNF/IL-4 ratios in the PCOS group compared to controls. Although IL-2 and IL-4 are pleiotropic cytokines, their anti-inflammatory effects are commonly seen in several diseases [14,15]. Our results suggest that these cytokines are released as counter-regulators of the sub-clinical and systemic inflammatory process commonly observed in PCOS women, although individually their levels did not differ between the groups.

We assessed the ratios of pro-inflammatory to anti-inflammatory cytokines in subjects with PCOS compared to controls. The overall results indicated that PCOS is related to an imbalance between pro- and anti-inflammatory cytokines, with prominent counter-regulatory cytokine production. The interplay between PCOS, inflammation, abdominal adiposity, hyperandrogenism, and hyperinsulinism could be key in explaining the phenotypes presented by PCOS women and suggest possible interventions for the treatment of PCOS and its complications.

## CRediT authorship contribution statement

**Jéssica A.G. Tosatti:** Writing - original draft, Data curation, Formal analysis. **Mirelle O. Sóter:** Conceptualization, Data curation. **Cláudia N. Ferreira:** Data curation, Writing - review & editing. **Ieda de F.O. Silva:** Data curation. **Ana L. Cândido:** Data curation, Writing - review & editing. **Marinez O. Sousa:** Conceptualization, Project administration. **Fernando M. Reis:** Data curation, Writing - review & editing. **Karina B. Gomes:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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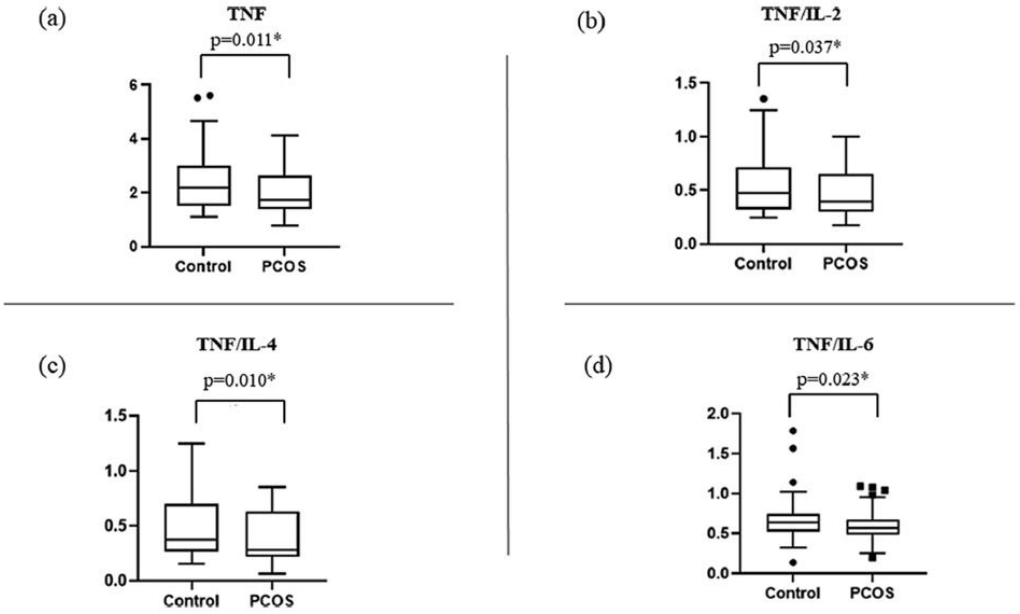


Fig. 1. Comparation of TNF concentration (a) and ratios of TNF/IL-2 (b), TNF/IL-4 (c) and TNF/IL-6 (d) on PCOS and control groups.

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6.2 Estudo II. Influence of n-3 fatty acid supplementation on inflammatory and oxidative stress markers in patients with polycystic ovary syndrome: a systematic review and meta-analysis.

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# Influence of *n*-3 fatty acid supplementation on inflammatory and oxidative stress markers in patients with polycystic ovary syndrome: a systematic review and meta-analysis

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**Abstract**

Polycystic ovary syndrome (PCOS) is defined as a reproductive endocrine disease that results in a low-grade inflammatory and pro-oxidant state. Dietary factors, including *n*-3 fatty acids, may have a key role in improving metabolic disorders in PCOS patients. The present study aimed to investigate the influence of *n*-3 fatty acid supplementation on inflammatory and oxidative stress (OS) markers in patients with PCOS. A systematic literature search of Medline/PubMed, Cochrane Central Register of Controlled Trials, Scopus and Lilacs, until November 2019, was conducted. Randomised clinical trials that reported inflammatory and OS markers as endpoints in women with PCOS receiving *n*-3 fatty acid supplementation were included. The pooled estimates of the weighted mean differences (WMD) and the standard mean differences (SMD) were calculated. Random effects models were adopted to measure the pooled outcomes. Among the 323 studies retrieved, ten fulfilled the inclusion criteria for a meta-analysis. We founded a significant decrease in high-sensitivity C-reactive protein (hs-CRP) (SMD −0·29 (95 % CI −0·56, −0·02) mg/l) and an increase in adiponectin (WMD 1·42 (95 % CI 1·09, 1·76) ng/ml) concentrations in the intervention group when compared with the placebo group. No statistically significant results were found in the meta-analysis for visfatin, nitric oxide, GSH or malondialdehyde levels or total antioxidant capacity. The data suggest that supplementation of *n*-3 fatty acids could reduce the inflammatory state in women with PCOS, through a decrease in hs-CRP and an increase in adiponectin levels.

**Key words: Polycystic ovary syndrome: *n*-3 Fatty acid supplementation: Inflammation: Oxidative stress markers: Meta-analyses**

Polycystic ovary syndrome (PCOS) is defined as an endocrine disease resulting from a hormonal imbalance that affects 5–18 % of women of reproductive age, making it an important public health problem in view of the co-morbidities and prevalence currently presented[1]. The syndrome is characterised by symptoms such as menstrual irregularity, anovulatory infertility, clinical and biochemical hyperandrogenism, as well as other metabolic manifestations, which affect from 30 to 70 % of women with PCOS[2–4].

The hormonal imbalance that occurs with PCOS can influence the reproductive, metabolic and psychological health of patients. Clinical manifestations may include precocious puberty, acne, alopecia, seborrhoea, irregular menstrual cycles, hirsutism, infertility and complications in pregnancy[5]. Anxiety, depression and non-acceptance of body image are frequent psychological co-morbidities[6]. Metabolic impairment includes the primary effect of insulin resistance (IR) on muscle and adipose tissue, with compensatory hyperinsulinaemia, associated with intrinsic *β*-cell dysfunction, type 2 diabetes mellitus and gestational diabetes, hyperlipidaemia, increased risk of CVD, obesity, sleep apnoea, non-alcoholic fatty liver disease and the metabolic syndrome (MetS)[7].

**Abbreviations:** hs-CRP, high-sensitivity C-reactive protein; IR, insulin resistance; MDA, malondialdehyde; MetS, metabolic syndrome; NO, nitric oxide; OS, oxidative stress; PCOS, polycystic ovary syndrome; RCT, randomised controlled trial; ROS, reactive oxygen species; SMD, standard mean difference; TAC, total antioxidant capacity.

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In 2018, a guideline for the assessment and management of PCOS was published by the American Society for Reproductive Medicine/European Society of Human Reproduction and Embryology, which endorsed the Rotterdam criteria for the diagnosis of PCOS in adult women who were not in menopause[8]. Among these criteria, PCOS can be diagnosed when at least two of the three proposed criteria are present, categorised as follows: (1) androgen status – clinical or biochemical hyperandrogenism; (2) menstrual history – oligo- or anovulation or (3) ovarian appearance – polycystic morphology on ultrasound[8].

The PCOS state of low-grade inflammation is similar to that of other non-communicable diseases, such as obesity, type 2 diabetes mellitus and CVD[9]. The role of inflammation in PCOS has been the subject of several studies, and associations have been found between increased levels of inflammation markers (high-sensitivity C-reactive protein (hs-CRP), ferritin, TNF and IL-6 and IL-18) and oxidative stress (OS) markers (malondialdehyde (MDA), total antioxidant capacity – TAC, nitric oxide (NO) and GSH) with PCOS[10–12].

In fact, a case–control study[12] that aimed to evaluate the relationship between polymorphisms in genes encoding inflammation-associated cytokines and the metabolic profiles of Brazilian women with PCOS ($n$ 97) *v.* a control group ($n$ 99) observed that fasting glucose levels varied according to IL-6 genotype, while the hirsutism score, 2-h glucose tolerance test, total cholesterol and TAG levels varied according to the IL-10 genotype. Serum lipid levels were also related to interferon-$\gamma$ and transforming growth factor-$\beta$ genotypes, suggesting that cytokine gene polymorphisms may promote abnormal metabolic features in PCOS[12].

Another case–control study[13] aimed to determine the relationship between OS markers and lipid profiles in patients with PCOS. This study included fifty PCOS patients and fifty healthy controls and revealed that serum MDA levels were significantly higher in PCOS patients than in controls and that TAC was significantly lower in the PCOS group[13]. It is known that TAC is also reduced in many diseases such as hypertension, type 2 diabetes mellitus, obesity and the MetS[14].

Among the environmental factors, numerous nutrients are known to modulate the inflammatory response and contribute to the protection and treatment of non-communicable diseases, such as MUFA and PUFA[14–16]. The long-chain $n$-3 fatty acids, namely $\alpha$-linolenic acid (ALA), EPA and DHA, are commonly considered 'essential' fatty acids, since they are not synthesised in the human body and are mostly obtained from the diet[17]. EPA and DHA are found naturally in marine sources, including cold water fish, shellfish and seaweed. ALA can be converted to DHA or EPA after ingestion and is found in seeds such as chia, flaxseed and pumpkin seeds, as well as in vegetable and oilseeds like nut oils. In addition, it is also present in small amounts in other vegetable sources, such as spinach and kale[18].

Evidence suggests that $n$-3 fatty acid supplementation increases insulin sensitivity and plasma adiponectin levels, reduces hyperinsulinaemia, plasma TAG and liver fat and attenuates inflammation and the OS response in adults[19,20]. However, the role of $n$-3 fatty acid supplementation in controlling OS and chronic low-grade inflammation in women with PCOS is still uncertain. Therefore, the present study aimed to systematically review and meta-analyse randomised controlled trials (RCT) investigating the influence of $n$-3 fatty acid supplementation on inflammatory and OS markers in patients with PCOS.

## Methods

The conduct and design of this systematic review and meta-analysis followed the predetermined protocol according to the Cochrane Handbook's recommendations[21]. Results were reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement[22]. The protocol of the current study was published on the International Prospective Register of Systematic Reviews (PROSPERO) (CRD42019129199).

### *Search strategy*

The PICO acronym search question was composed of: P (participants) = Women with Polycystic Ovary Syndrome; I (intervention) = $n$-3 fatty acid supplementation; C (control) = Placebo and O (outcomes) = Inflammatory and OS markers levels. A literature review was performed by searching the electronic databases Medline/PubMed (Medical Literature Analysis and Retrieve System Online), Cochrane Central Register of Controlled Trials (CENTRAL), Scopus and Lilacs (Latin American and Caribbean Health Sciences) until November 2019 to identify RCT, which reported the effect of $n$-3 fatty acid supplementation on inflammatory and OS markers in PCOS patients over 18 years of age. The initial search included the Medical Subject Headings terms 'Polycystic Ovary Syndrome' and 'Fatty Acids, Omega-3'. It also included the entry terms associated with a high-sensitivity strategy for the search of RCT developed by The Cochrane Collaboration[21]. Online Supplementary material 1 describes the search strategy used on the PubMed database.

The same terms were used to search for clinical studies in the National Institutes of Health (www.clinicaltrials.gov), the Brazilian Registry of Clinical Trials (www.ensaiosclinicos.gov.br) and the Turning Research into Practice (www.tripdatabase.com) databases. All potentially eligible studies were considered for review, regardless of the language and date of publication. A manual search was also implemented in the reference lists of relevant reviews[23–27].

### *Inclusion and exclusion criteria*

We included only RCT that analysed the effect of $n$-3 fatty acid supplementation on inflammatory and OS markers in women with PCOS. The outcome was considered as changes in the concentration or activity of these markers from baseline until the end of the study. Studies that did not report the outcomes of interest, non-randomised studies and those that included children, adolescents (under 18 years of age) or pregnant women were excluded. Studies that did not present as endpoints inflammatory or OS markers were also excluded.

### *Study selection and data extraction*

Initially, the studies retrieved from the databases were input into a single electronic library, and duplicates were excluded

using the EndNote® software. Two reviewers (J. A. G. T. and M. T. A.) independently analysed the titles and abstracts of the articles retrieved from the literature search, reviewed the full text of the published articles and extracted the data using a standardised data extraction tool. Any disagreements between the reviewers regarding the study data were resolved by a third investigator (K. B. G. or V. E. A.).

The extracted data included the number of participants, study design, trial duration and patients' demographic and anthropometric characteristics (age and BMI). *n*-3 Fatty acid supplementation data from the intervention and control groups were collected. Informative data about inflammatory and OS markers collected at baseline and the end of the study were extracted. Percentage changes in biomarker concentrations were calculated for the studies that presented baseline values.

### *Assessment of bias across studies and quality of evidence*

The risk of bias of the studies and the quality of evidence were assessed independently by two reviewers (J. A. G. T. and M. T. A.) following the Cochrane guidelines[28], and a third reviewer (K. B. G. or V. E. A.) resolved any disparity. The Cochrane Risk of Bias Tool for Randomized Trials – Rob 2.0 was applied to assess the risk of bias in individual studies according to the recommendations of the Cochrane Collaboration[28,29]. The Rob 2.0 is structured into five domains: (1) bias arising from the randomisation process; (2) bias due to deviations from intended interventions; (3) bias due to missing outcome data; (4) bias in measurement of the outcome and (5) bias in selection of the reported result. The response options for the signalling questions are: (1) yes; (2) probably yes; (3) probably no; (4) no and (5) no information[29].

The Grading of Recommendations, Assessment, Development and Evaluation approach[30] was used to assess the quality of the evidence for each outcome: hs-CRP, adiponectin, visfatin, NO, GSH, MDA and TAC. This approach assesses the strength of the evidence quality by including factors that can decrease quality (e.g. methodological quality, directness of evidence, heterogeneity, precision of effect estimates and risk of publication bias) or increase it (e.g. large magnitude of effect, reduction or spurious effect due to plausible confounding factors, dose–response gradient). Each evaluated factor was rated as high, moderate, low or very low[28,30].

### *Statistical analyses*

Differences between the mean values and standard deviations at baseline and at the end of the study were used to report the changes in inflammatory and OS marker concentrations[31]. Heterogeneity between studies was assessed by Cochran's Q test, and $P \leq 0{\cdot}10$ was considered statistically significant. The $I^2$ test was also performed to evaluate the magnitude of heterogeneity, which was considered high if $I^2 \geq 50{\cdot}0\%$. The pooled estimates of the weighted mean differences (WMD) for NO, GSH, TAC and MDA and the estimates of the standard mean differences (SMD) for hs-CRP, adiponectin and visfatin between *n*-3 fatty acid supplementation and control groups were calculated using the random effects model[32], since significant heterogeneity among studies was identified in preliminary models. This approach also provided a more conservative assessment of the average effect size. Subsequently, sensitivity (subgroup) analyses were conducted by including variables to determine how much of the between-study difference could be explained by these variables. Publication bias was not accessed through funnel plot asymmetry, since according to the Cochrane Handbook[21], it should be used when there are at least ten studies included in the meta-analysis, and all outcomes evaluated in the present meta-analysis had, at maximum, seven studies included. The statistical analyses were performed using Review Manager 5® software. Significant values were considered as $P < 0{\cdot}05$ with the 95 % CI.

## Results

### *Study characteristics*

Fig. 1 shows the flow chart of the study selection process. The electronic database search identified 323 studies. After analysis of the titles, 210 abstracts were maintained and, subsequently, 178 were excluded after abstract reading. In this stage, the Kappa coefficient of agreement between the two investigators was 0·933. Eighteen RCT were selected for full-text reading after analysis of the abstracts; eight studies were excluded for not fulfilling the inclusion criteria, and thus, ten[33–42] randomised clinical trials were included in this systematic review with meta-analysis.

One study[42] was included as two independent reports because the findings were described by different interventions of interest (flaxseed and fish oils). The total sample size of all studies comprised 381 patients diagnosed with PCOS (nine studies[33–41] diagnosed by the Rotterdam criteria and one study[42] by the National Institutes of Health criteria) with a mean age of 27·05 years and 384 controls with a mean age of 27·10 years. All the included studies were parallel RCT that comprised 6–12 weeks of follow-up. Subgroup analyses based on sources of *n*-3 fatty acids (fish oil *v.* flaxseed and *n*-3 fatty acids *v.* *n*-3 fatty acids plus vitamins D and E) were performed to check the sources of heterogeneity between the studies. However, no significant differences were found between subgroups after the tests.

The included studies were grouped according to the assessed outcome: (1) inflammatory[33–39,41,42] and (2) OS[33,34,36,40,41] marker changes, when supplemented with just *n*-3 fatty acids[32,35–39,42] or co-supplemented with vitamin D[34] or E[40,41] in patients with PCOS. Table 1 presents the characteristics of the studies included. Online Supplementary material 2 shows the changes in biomarkers of the included studies. Online Supplementary material 3 presents the laboratory characteristics of the participants before and after *n*-3 fatty acid intervention.

### *Inflammatory markers – high-sensitivity C-reactive protein*

Of the ten selected studies, six[33,34,36,37,41,42] investigated the effects of *n*-3 fatty acid supplementation on the circulating concentrations of hs-CRP compared with a placebo group. The mean follow-up time of the studies was 9 weeks (6–12 weeks), and they included thirty-four to sixty participants (mean age

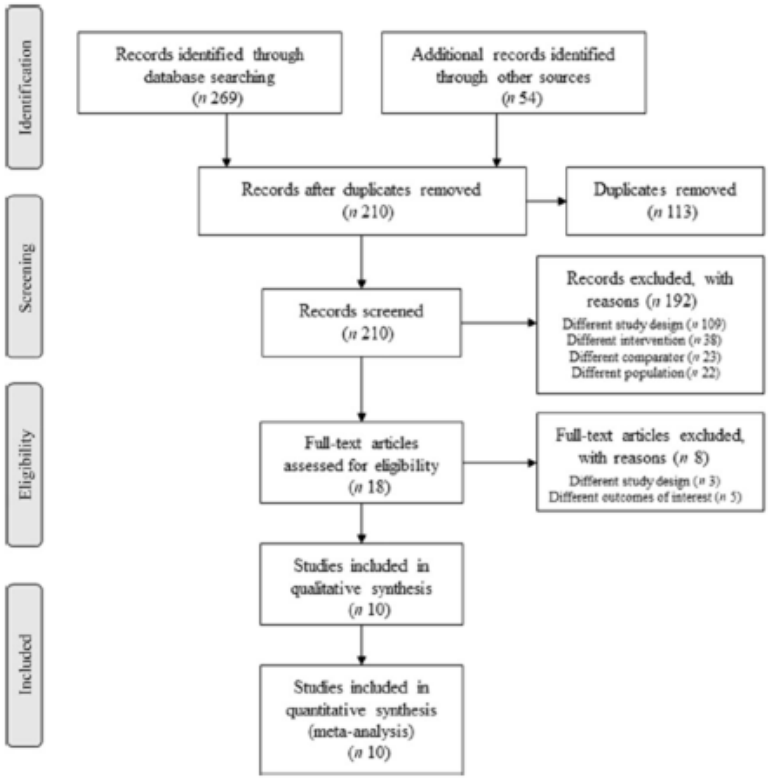


**Fig. 1.** Flow chart of the literature search and the study selection process.

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28 years). The mean BMI was 29·57 kg/m$^2$, and one study[42] used National Institutes of Health criteria to diagnose PCOS. Two studies[33,42] had fish oil as the intervention, three[36,37,42] with flaxseed oil and two with *n*-3 fatty acid co-supplementation with D[34] and E[41] vitamins. One study[34] did not report the placebo used, four studies[33,36,37,41] used paraffin oil and one[42] soya oil as the placebo.

Overall, *n*-3 fatty acid intervention decreased significantly hs-CRP concentrations when compared with the control group (SMD −0·29 (95 % CI −0·56, −0·02) mg/l; $I^2 = 38$ %, $P_{\text{for heterogeneity}}$: 0·14; Fig. 2(a)).

### *Inflammatory markers – adiponectin*

A total of four studies[35,37,38,42] were included in meta-analysis that evaluated the effects of *n*-3 fatty acid supplementation on the circulating concentrations of adiponectin compared with a placebo group. The studies included 34–195 participants (mean age 27 years), and one study[42] used National Institutes of Health criteria to diagnose PCOS. The mean follow-up time of the studies was 8 weeks (6–12 weeks), and the mean BMI was 27·76 kg/m$^2$. Four studies[35,37,38,42] had fish oil as the intervention and one[42] used flaxseed oil as *n*-3 fatty acid supplementation.

The pooled data from four studies showed a significant effect of *n*-3 fatty acid supplementation on increasing adiponectin concentrations (weighted mean difference 1·42 (95 % CI 1·09, 1·76) ng/ml; $I^2 = 5$ %, $P_{\text{for heterogeneity}}$: 0·38; Fig. 2(b)).

### *Inflammatory markers – visfatin*

The meta-analysis that evaluated the concentrations of visfatin, under *n*-3 fatty acid supplementation when compared with a placebo group, included two studies[38,39]. Both studies used the Rotterdam criteria for PCOS diagnosis, and the intervention was composed of 180 mg of EPA and 120 mg of DHA. The mean follow-up time was 8 weeks. In addition, the mean age and BMI were 27·2 and 30·12 kg/m$^2$, respectively.

In the meta-analysis, we did not observe a decrease of visfatin concentrations in the *n*-3 fatty acid supplementation group when compared with the control group. (weighted mean difference −0·00 (95 % CI −0·05, 0·05) ng/ml; $I^2 = 0$ %, $P_{\text{for heterogeneity}}$: 0·86; Fig. 2(c)).

### *Oxidative stress markers – nitric oxide*

Four studies[33,34,36,41] were included in the meta-analysis that evaluated serum levels of NO in the *n*-3 fatty acid group

**Table 1.** Characteristics of the studies investigating inflammation and oxidative stress markers concentrations from *n*-3 fatty acid supplementation (Mean values and standard deviations)

| Author and country | Study duration (weeks) | Mean age (years) | | | | | | Group characteristics | | Baseline and at end-of-trial BMI (kg/m²) | | | | Diagnosis criteria of PCOS | Interest outcomes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Intervention | | | Control | | | | | Intervention | | Control | | | |
| | | No. of patients | Mean | SD | No. of patients | Mean | SD | Intervention | Control | Mean | SD | Mean | SD | | |
| Amini *et al.* (2018)[33]; Iran | 12 | 27 | 27·2 | 6·2 | 27 | 28·9 | 4·2 | 2000 mg/d of fish oil per d | 100 mg of paraffin oil per d | 25·8<br>25·6 | 4·8<br>4·7 | 25·9<br>25·8 | 4·3<br>4·4 | Rotterdam criteria | hs-CRP, NO, TAC, GSH and MDA |
| Jamilian *et al.* (2018)[34]; Iran | 12 | 30 | 26·8 | 4·4 | 30 | 25·1 | 3·7 | 2000 mg/d of fish oil plus 50 000 IU of vitamin D every 2 weeks | Not reported | 27·4<br>27·1 | 3·9<br>3·8 | 27·1<br>27·0 | 7·0<br>7·1 | Rotterdam criteria | hs-CRP, NO, TAC, GSH and MDA |
| Mejia-Montilla *et al.* (2017)[35]; Venezuela | 12 | 97 | 23·6 | 3·4 | 98 | 23·3 | 3·9 | 180 mg of EPA and 120 mg of DHA per d | 1000 mg of paraffin oil per d | 26·4<br>25·7 | 3·0<br>3·1 | 26·0<br>26·2 | 2·7<br>2·8 | Rotterdam criteria | Adiponectin |
| Mirmasoumi *et al.* (2017)[36]; Iran | 12 | 30 | 28·4 | 6·4 | 30 | 27·0 | 3·2 | 1000 mg/d of flaxseed oil | 500 mg of paraffin oil per d | 26·9<br>26·9 | 5·1<br>5·0 | 26·7<br>26·6 | 5·3<br>5·4 | Rotterdam criteria | hs-CRP and NO |
| Mohammadi *et al.* (2012)[37]; Iran | 8 | 30 | 27·3 | 4·27 | 31 | 27·7 | 4·53 | 720 mg EPA and 480 mg DHA per d | Four capsule contained 500 mg paraffin oil | 28·7<br>28·6 | 3·21<br>3·30 | 28·8<br>28·8 | 2·90<br>2·94 | Rotterdam criteria | hs-CRP and adiponectin |
| Nadjarzadeh *et al.* (2015)[38]; Iran | 8 | 39 | 26·9 | 5·9 | 39 | 26·9 | 5·0 | 540 mg EPA and 360 mg DHA per d | 1000 mg of paraffin oil per d | 31·5<br>31·1 | 5·7<br>5·9 | 31·8<br>31·8 | 3·9<br>3·7 | Rotterdam criteria | Visfatin and adiponectin |
| Rafraf *et al.* (2013)[39]; Iran | 8 | 30 | 27·3 | 4·3 | 31 | 27·7 | 4·5 | 720 mg EPA and 480 mg DHA per d | Paraffin oil | 28·7<br>28·6 | 3·2<br>3·3 | 28·7<br>28·8 | 2·9<br>2·9 | Rotterdam criteria | Visfatin |
| Rahmani *et al.* (2016)[40]; Iran | 12 | 34 | 24·9 | 5·5 | 34 | 26·6 | 5·6 | 400 mg of ALA plus 400 IU of vitamin E per d | Not reported | 28·4<br>28·2 | 4·4<br>4·6 | 29·0<br>29·0 | 6·5<br>6·5 | Rotterdam criteria | TAC, GSH and MDA |
| Talari *et al.* (2018)[41]; Iran | 12 | 30 | Not reported | | 30 | Not reported | | 400 mg of ALA plus 400 IU of vitamin E per d | Paraffin oil | Not reported | | Not reported | | Rotterdam criteria | hs-CRP and NO |
| Vargas *et al.* (2011)[42]; USA | 6 | 17 | 29·4 | 1·6 | 17 | 28·9 | 1·0 | 545 mg ALA per d | Soya oil | 35·0<br>35·1 | 2·5<br>2·6 | 33·2<br>33·3 | 1·8<br>1·7 | National Institutes of Health criteria | hs-CRP and adiponectin |
| Vargas *et al.* (2011)[42]; USA | 6 | 17 | 31·7 | 1·9 | 17 | 28·9 | 1·0 | 358 mg EPA plus 242 mg DHA per d | Soya oil | 36·3<br>36·6 | 1·9<br>1·8 | 33·2<br>33·3 | 1·8<br>1·7 | National Institutes of Health criteria | hs-CRP and adiponectin |

PCOS, polycystic ovary syndrome; hs-CRP, high-sensitivity C-reactive protein; NO, nitric oxide; TAC, total antioxidant capacity; MDA, malondialdehyde; IU, international units; ALA, α-linolenic acid.

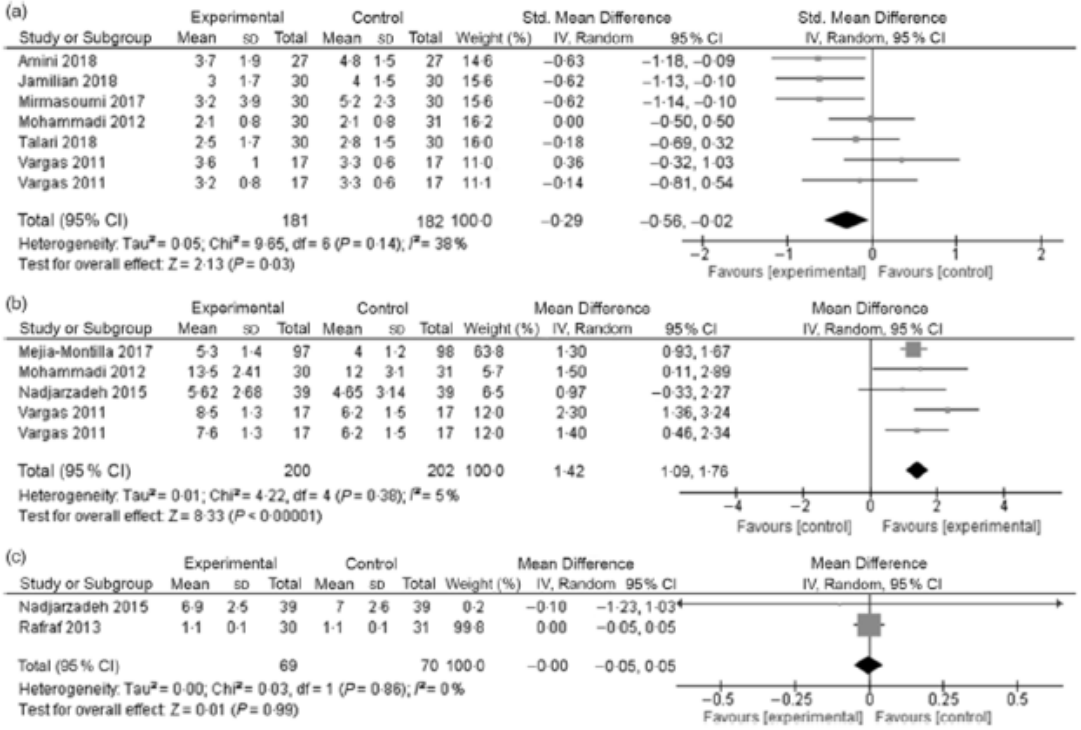


**Fig. 2.** (a) Change in high-sensitivity C-reactive protein (mg/l) concentrations due to *n*-3 fatty acid supplementation. (b) Change in adiponectin (ng/ml) concentrations due to *n*-3 fatty acid supplementation. (c) Change in vistatin (ng/ml) concentrations due to *n*-3 fatty acid supplementation.



compared with a placebo. The mean follow-up time of the studies was 12 weeks, and they included 54–68 participants (mean age 27 years). The mean BMI was 26·57 kg/m², and all studies used the Rotterdam criteria for PCOS diagnosis. Two studies[36,41] used flaxseed oil in the intervention group, and the Talari *et al.* study co-supplemented the group with vitamin E[41]. Another two studies used fish oil as *n*-3 fatty acid supplementation, and Jamilian *et al.*[34] co-supplemented with vitamin D. Three studies[33,36,41] used paraffin oil as the placebo.

*n*-3 fatty acid supplementation did not decrease serum levels of NO in the intervention group, when compared with the placebo group (SMD −0·01 (95% CI −0·69, 0·67) (mmol/l); $I^2 = 85\%$, $P_{\text{for heterogeneity}}$: 0·0002; Fig. 3(a)).

### *Oxidative stress markers – GSH, malondialdehyde and total antioxidant capacity*

For meta-analysis of these OS markers, three studies[33,34,40] were included that evaluated the influence of *n*-3 fatty acid supplementation on serum levels of GSH, MDA and TAC when compared with the placebo group. The mean follow-up of the studies was 12 weeks, and they included 54–68 participants (mean age 27 years). The mean BMI was equal to 27·19 kg/m², and all studies used Rotterdam criteria for PCOS diagnosis. Two studies used fish oil as *n*-3 fatty acid supplementation, and the study by Jamilian *et al.*[34] co-supplemented with vitamin D. Another study[40] included used flaxseed oil plus vitamin E as the intervention. One study[33] reported using paraffin oil as the placebo.

We did not observe a significant decrease in serum levels of GSH, MDA or TAC in the group *n*-3 fatty acids when compared with the placebo group (GSH – SMD 0·24 (95% CI −0·42, 0·90) (mmol/l); $I^2 = 80\%$, $P_{\text{for heterogeneity}}$: 0·007; Fig. 3(b); MDA – SMD −0·17 (95% CI −1·01, 0·66) (mmol/l); $I^2 = 87\%$, $P_{\text{for heterogeneity}}$: 0·0004; Fig. 3(c) and TAC – SMD 0·27 (95% CI −0·45, 1·00) (mmol/l); $I^2 = 83\%$, $P_{\text{for heterogeneity}}$: 0·003; Fig. 3(d)).

### *Risk of bias and quality of the body of evidence*

The risk of bias in the included studies is summarised in Fig. 4. In domain 1, regarding the process of randomisation, one study presented a high risk of bias because it did not report a randomisation process on methods. Domain 2, the risk of bias due to deviations from the intended interventions, was classified as low because no studies presented a possible negative effect of assignment to intervention. Domain 3, regarding the risk of bias due to missing outcome data, was classified as low risk related to the availability of data from 95% of the participants in all included studies. In the risk of measuring the result – domain 4, we did not identify a likely directional bias, that is,

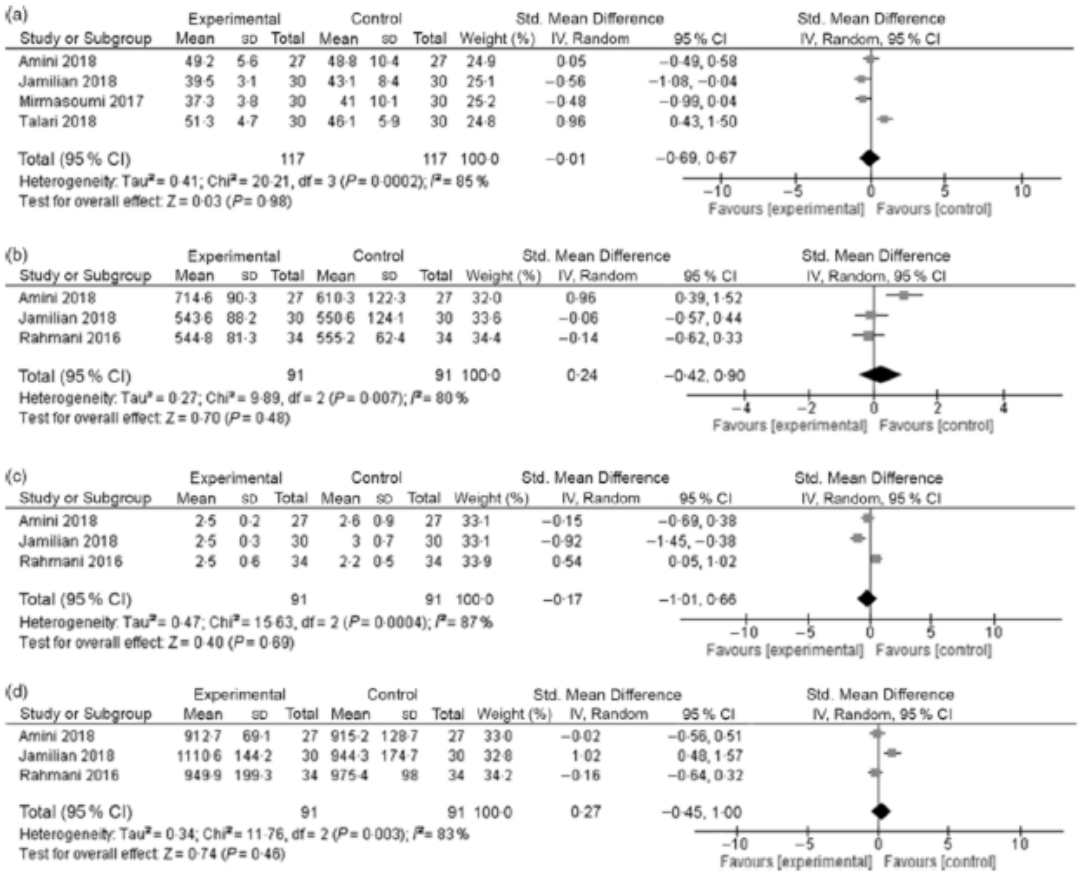


**Fig. 3.** (a) Change in nitric oxide (NO) (mmol/l) levels due to *n*-3 fatty acid supplementation. (b) Change in GSH (mmol/l) levels due to *n*-3 fatty acid supplementation. (c) Change in malondialdehyde (mmol/l) levels due to *n*-3 fatty acid supplementation. (d) Change in total antioxidant capacity (mmol/l) levels due to *n*-3 fatty acid supplementation.

measuring results that are not suitable for the outcomes that the authors planned to evaluate – directed to one of the interventions. For domain 5 – risk of selection of the reported result, we evaluated this domain as low risk because no individual study presented any evidence of selective reporting bias of results. Overall, eight[34–36,38–42] studies were evaluated as some concerns related to the intervention, one study[33] was evaluated as low risk and another study[37] as high risk, associated with evaluation of domains 1 and 2.

The quality of the body of evidence for each outcome of the current systematic review is described in online Supplementary material 4. The directness of evidence was classified as high considering the precision of the main effect estimates of interest. The results were highly heterogeneous, and no dose–response effect could be established. For one of the outcomes evaluated (inflammation biomarker changes), a clinically relevant effect of great magnitude was demonstrated. In summary, the quality of the body of evidence of this systematic review was classified as moderate.

## Discussion

The present systematic review with meta-analysis of RCT analysed the role of *n*-3 fatty acid supplementation in PCOS patients considering plasma concentrations of inflammatory and OS markers. Intervention studies that compared *n*-3 fatty acid supplementation with a placebo were associated with no difference in visfatin levels nor in OS markers. However, it was observed that *n*-3 fatty acid supplementation significantly increased the circulating concentrations of adiponectin and decreased hs-CRP levels in PCOS patients when compared with placebo.

PCOS results in a pro-inflammatory state, and the development of metabolic dysfunction is supported by chronic low-grade inflammation. This state may be associated with the accumulation of visceral fat, glucose intolerance, IR and dyslipidaemia, all of which are presented in patients diagnosed with PCOS[43]. We observed in the present systematic review that *n*-3 fatty acid supplementation can reduce the levels of hs-CRP, an inflammatory maker known to be increased in

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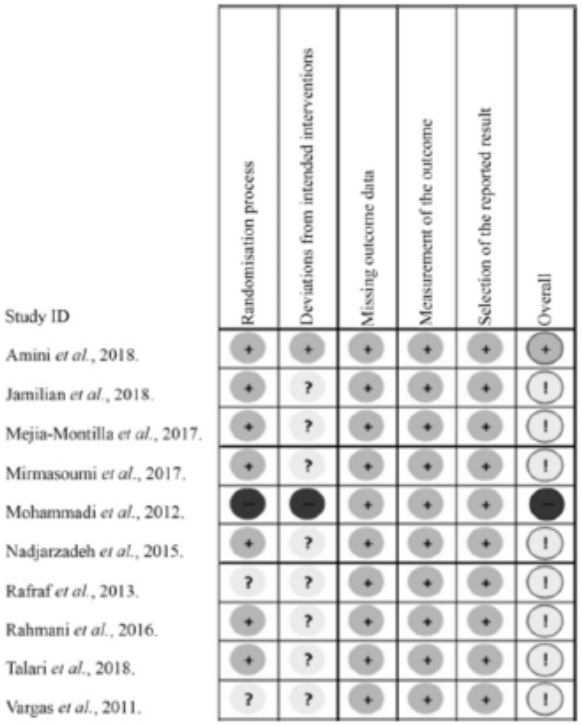


**Fig. 4.** Cochrane Collaboration bias risk graph. +, Low risk; ?, some concerns; −, high risk.

women with PCOS[43], which can predict a cardiovascular event[44]. hs-CRP is an acute-phase protein produced by the liver following stimulation by IL-6, the endocrine cytokine originating from adipocytes[45,46].

The mononuclear cells of women with PCOS are in an activated state, as evidenced by increased plasma hs-CRP, which may play a role in obesity[44]. A meta-analysis of thirty-one articles ($n$ 3648 women) that aimed to evaluate the status of serum inflammatory markers in women with PCOS showed that circulating hs-CRP was 96 % higher in women with PCOS compared with controls (95 % CI 71 %, 122 %, $P < 0·0001$) and it was associated with a high prevalence of obesity in the women with PCOS in the studies analysed[47]. $n$-3 Fatty-acid-derived mediators have been implicated in the resolution of inflammation, with a significant protective effect by inhibition of NF-κB activity, which rises in many inflammatory diseases[48]. In fact, our results corroborate the findings of a systematic review and meta-analysis that aimed to evaluate the effect of $n$-3 fatty acid supplementation on serum levels of inflammatory biomarkers in patients on haemodialysis. This study showed a significant decrease in serum levels of hs-CRP in the $n$-3 fatty acid supplementation group when compared with placebo (SMD −2·09; 95 % CI −3·62, −0·56, $P < 0·05$)[49].

The state of low-grade inflammation is the result of the accumulation of visceral fat because this tissue is capable of producing cytokines, chemokines and other adipokines, such as adiponectin, which act, directly or indirectly, as mediators of systemic inflammation[50]. $n$-3 Fatty acid supplementation may be associated with a decrease in inflammation and in pro-inflammatory cytokine levels by stimulating the secretion of anti-inflammatory adipokines (such as adiponectin)[51]. Moreover, these effects of $n$-3 fatty acids could be related to their influence on signalling pathways that regulate the expression of genes encoding pro-inflammatory cytokines[52–54]. This regulation could be associated with NF-κB, one of the main transcription factors involved in up-regulation of the genes encoding pro-inflammatory cytokines, adhesion molecules and cyclo-oxygenase-2[55]. In fact, EPA decreases lipopolysaccharide-induced NF-κB activation in monocytes, and DHA reduces NF-κB activation in response to lipopolysaccharide in macrophages and dendritic cells[52–54]. Besides, the influence of EPA and DHA on NF-κB activation involves PPAR-γ, resulting in inhibition of NF-κB activation and reduced production of the pro-inflammatory cytokines TNF and IL-6 due to lipopolysaccharide stimulation[51].

In women, adiponectin controls steroidogenesis of ovarian granulosa and theca cells, oocyte maturation and embryo development beyond insulin sensitising and known anti-inflammatory effects, which also modulates folliculogenesis and androgen synthesis in ovaries[55]. Its receptor was also found in endometrial and placental cells, suggesting that this adipokine might play a crucial role in fetal growth, trophoblast invasion and

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embryo implantation[55]. *n*-3 Fatty acids may improve insulin sensitivity by enhancing the production and secretion of anti-inflammatory adipokines, such as adiponectin, consequently reducing inflammation and proinflammatory cytokines[56]. A previous meta-analysis that assessed the effectiveness and safety of *n*-3 fatty acids for patients with PCOS included nine RCT (*n* 591 patients) and observed that, compared with the control group, *n*-3 fatty acids may improve adiponectin levels (weighted mean difference 1·34; 95 % CI 0·51, 2·17; $P = 0·002$)[25]. Plasma adiponectin concentrations correlate negatively with body weight and BMI in women with or without PCOS. Moreover, hypoadiponectinaemia is associated with higher degrees of hyperinsulinaemia and IR; hence, it could be related not only to obesity but also to the metabolic alterations that characterise PCOS[57].

It has been reported that circulating levels of visfatin and its gene expression were increased in women with PCOS, compared with controls matched by BMI and age[58]. Visfatin has been identified as a protein of 52 kDa produced by the bone marrow, liver and muscle and, during pregnancy, by the epithelium of the amniotic membrane, chorionic trophoblast and decidua[58]. Years later, it was proven that visfatin is produced by adipocytes and has insulin-mimetic action[59]. Visfatin has the ability to stimulate proinflammatory activity by enhancing TNF and IL-6 secretion, which further increases IR. On the other hand, the insulin-like effect of visfatin is not sufficient to counteract IR in conditions such as type 2 diabetes mellitus and PCOS, despite its high serum levels[59]. In fact, a meta-analysis with seventeen studies (1341 subjects – 695 cases and 646 controls) showed that visfatin levels are higher in women with PCOS compared with non-PCOS controls; furthermore, the study did not indicate a correlation between high visfatin levels and BMI, homoeostatic model assessment for IR or total testosterone levels in PCOS patients when compared with controls[59]. The changes in visfatin levels induced by *n*-3 fatty acids vary according to the type of dietary fat, supporting the hypothesis that visfatin up-regulation by EPA could be another mechanism by which *n*-3 fatty acids may improve insulin sensitivity[60].

OS is characterised by the imbalance between the capacity of the body to neutralise free radical molecules, using antioxidant enzymes, and their production. Excessive reactive oxygen species (ROS) generation promotes inflammation by activation of redox and inflammatory signalling pathways such the NF-κB pathway[57]. PCOS patients demonstrate OS due to hyperglycaemia, IR and chronic inflammation. Moreover, higher levels of NEFA lead to excess production of ROS[61]. A cross-sectional study suggested that excess androgen increases the generation of ROS from leucocytes, p47phox gene expression and the formation of MDA. OS increases chronic inflammation and vice versa[62]. Furthermore, OS and inflammation in the ovaries play an important role in the pathogenesis of PCOS and cause the development of atherosclerotic lesions in the ovary[63].

The mechanism by which OS could be reduced following *n*-3 fatty acid supplementation is still unclear, but it has been assumed that these effects may occur through immunomodulation and decreased leucocyte activation[64,65]. In this context, it is known that activated immune cells produce cytokines that consequently promote ROS generation. Moreover, EPA and DHA are effective as superoxide scavengers in an unsaturation-dependent manner, given the high unsaturation level of *n*-3 fatty acids[66].

PCOS patients have an increased risk of developing the MetS, which may be related to OS and cardiovascular events. While obesity can be a putative factor leading to the MetS, the relationship between the MetS and PCOS is attributed mainly to IR[67]. A prospective controlled study evaluated whether the presence of the MetS in PCOS patients could influence endoplasmic reticulum stress markers, OS and leucocyte endothelium interaction. The data highlight that ROS production, and therefore OS, is enhanced in PCOS, and it is associated with the presence of the MetS, which can increase CVD risk. Moreover, PCOS subjects with the MetS exhibited enhanced levels of proinflammatory cytokines, and these cytokines were correlated with homoeostatic model assessment for IR, reinforcing the importance of the MetS to IR and inflammation in PCOS[68].

The *n*-3 fatty acids are also known to improve the TAC and various associated signalling pathways, probably suppressing lipid peroxidation, which is represented by MDA[69]. MDA levels are significantly higher in PCOS patients and can be considered an important marker for OS[70]. In the present meta-analysis, no relationship was found between *n*-3 fatty acid supplementation and the decline of OS biomarker levels in PCOS. Another systematic review and meta-analysis that aimed to summarise the findings of RCT examining the effects of *n*-3 fatty acids on OS markers in healthy subjects, including thirty-nine trials (*n* 2875 participants), evidenced a significant increase of serum TAC and decrease of MDA in the intervention group when compared with the placebo group[71]. However, the study did not find significant results for NO and GSH, according to our results.

Although the literature search was conducted using multiple databases, without language restriction and following the protocol established in accordance with standardised recommendations, the present meta-analysis has some limitations. The lack of data on the actual consumption of *n*-3 fatty acids must be considered because it may influence inflammatory and OS markers. In addition, none of the studies included in the meta-analysis presented intention-to-treat analysis, a statistical approach that is usually associated with more conservative results. Moreover, only one study[35] showed a low risk of bias due to imprecision, suggesting that our results should have external validity.

In conclusion, the present systematic review with meta-analysis of RCT suggests that *n*-3 fatty acid supplementation in patients with PCOS was associated with a moderate increase in the concentrations of adiponectin and hs-CRP and no effect on the concentrations of visfatin or OS markers when compared with a placebo. Caution must be taken in interpreting these results because important sources of heterogeneity were found in the meta-analyses of *n*-3 fatty acid supplementation. Therefore, future RCT are necessary to confirm these findings.

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The authors' contributions were as follows: J. A. G. T., M. T. A. and K. B. G. designed and performed the study; J. A. G. T., M. T. A., V. E. A. and K. B. G. analysed the data; J. A. G. T., M. T. A., A. L. C., F. M. R., V. E. A. and K. B. G. wrote and reviewed the paper. All the other authors critically reviewed and improved the manuscript.

The authors declare that there are no conflicts of interest.

## Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S0007114520003207

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**Supplementary material 1.** Complete Medline search strategy.

("Polycystic Ovary Syndrome"[Mesh]) OR (Polycystic Ovary Syndrome[Text Word] OR Ovary Syndrome, Polycystic[Text Word] OR Syndrome, Polycystic Ovary[Text Word] OR Stein-Leventhal Syndrome[Text Word] OR Stein Leventhal Syndrome[Text Word] OR Syndrome, Stein-Leventhal[Text Word] OR Sclerocystic Ovarian Degeneration[Text Word] OR Ovarian Degeneration, Sclerocystic[Text Word] OR Sclerocystic Ovary Syndrome[Text Word] OR Polycystic Ovarian Syndrome[Text Word] OR Ovarian Syndrome, Polycystic[Text Word] OR Polycystic Ovary Syndrome 1[Text Word] OR Sclerocystic Ovaries[Text Word] OR Ovary, Sclerocystic[Text Word] OR Sclerocystic Ovary[Text Word]) AND ("Fatty Acids, Omega-3"[Mesh]) OR (Fatty Acids, Omega-3[Text Word] OR n-3 Fatty Acids[Text Word] OR n 3 Fatty Acids[Text Word] OR n-3 Polyunsaturated Fatty Acid[Text Word] OR n 3 Polyunsaturated Fatty Acid[Text Word] OR n-3 PUFA[Text Word] OR PUFA, n-3[Text Word] OR n 3 PUFA[Text Word] OR Omega 3 Fatty Acids[Text Word] OR n3 PUFA[Text Word] OR PUFA, n3[Text Word] OR n3 Polyunsaturated Fatty Acid[Text Word] OR n3 Oils[Text Word] OR n-3 Oils[Text Word] OR n 3 Oils[Text Word] OR Omega-3 Fatty Acids[Text Word] OR n3 Fatty Acid[Text Word] OR Fatty Acid, n3[Text Word]) AND (((((((((randomized controlled trial[Publication Type]) OR controlled clinical trial[Publication Type]) OR randomized[Title/Abstract]) OR placebo[Title/Abstract]) OR drug therapy[ MeSH Subheading]) OR randomly[Title/Abstract]) OR trial[Title/Abstract]) OR groups[Title/Abstract])) NOT ((animals[ MeSH Terms]) NOT humans[ MeSH Terms])

**Supplementary material 2.** Changes on biomarkers of the studies investigating inflammation and oxidative stress markers concentrations from omega-3 fatty acid supplementation.

| Author; Year | Changes in hs-CRP†† (mg/L; % of change) | Changes in adiponectin†† (µg/mL; % of change) | Changes in visfatin†† (µg/mL; % of change) | Changes in NO†† (mmol/L; % of change) | Changes in GSH†† (mmol/L; % of change) | Changes in MDA†† (mmol/L; % of change) | Changes in TAC†† (mmol/L; % of change) |
|---|---|---|---|---|---|---|---|
| Amini *et al.*, 2018 [33] | C: 0.1±1.5; (↑2.2%)<br>I: -2.0±0.8; (↓35.0%) | Not applicable | Not applicable | C: 0.8±5.2; (↑1.7%)<br>I: 0.4±3.1; (↑0.8%) | C: 37.8±55.9; (↑6.6%)<br>I: 73.9±45.9; (↑11.5%) | C: 0.2±0.4; (↑8.3%)<br>I: -0.1±0.1; (↓3.8%) | C: 53.9±67.0; (↑6.2%)<br>I: 80.1±35.9; (↑9.6%) |
| Jamilian *et al.*, 2018 [34] | C: 0.1±0.7; (↑2.5%)<br>I: -1.2±1.9; (↓28.6%) | Not applicable | Not applicable | C: −1.5±6.3; (↓3.4%)<br>I: 0.3±3.9; (↑0.8%) | C: 24.3±116.4; (↑4.6%)<br>I: −15.5±85.8; (↓2.8%) | C: 0.2±0.6; (↑7.1%)<br>I: −0.4±0.4; (↓13.8%) | C: −2.4±168.2; (↓0.2%)<br>I: 114.6±122.2; (↑11.5%) |
| Mejia-Montilla *et al.*, 2017 [35] | Not applicable | C: -0.4±1.2; (↓10.0%)<br>I: 1.4±1.2; (↑35.8%) | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable |
| Mirmasoumi *et al.*, 2017 [36] | C: 0.2±1.5; (↑4.1%)<br>I: -1.6±3.1; (↓32.6%) | Not applicable | Not applicable | C: − 0.5 ± 7.9; (↓1.2%)<br>I: − 2.4 ± 5.3; (↓6.0%) | Not applicable | Not applicable | Not applicable |
| Mohammadi *et al.*, 2012 [37] | C: -0.14±0.8; (↓6.3%)<br>I: -0.15±0.7; (↓6.6%) | C: -0.3±3.4; (↓2.4%)<br>I: 1.7±2.8; (↑14.4%) | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable |
| Nadjarzadeh *et al.*, 2015 [38] | Not applicable | C: -0.51±1.6; (↓ 9.9%)<br>I: 1.17±2.1; (↑26.3%) | C: 0.04±0.5; (↑0.6%)<br>I: 0.07±0.4; (↑ 1.0%) | Not applicable | Not applicable | Not applicable | Not applicable |
| Rafraf *et al.*, 2013 [39] | Not applicable | Not applicable | C: -0.01±0.1; (↓0.9%)<br>I: 0.03±0.1; (↑2.8%) | Not applicable | Not applicable | Not applicable | Not applicable |
| Rahmani *et al.*, 2016 [40] | Not applicable | Not applicable | Not applicable | Not applicable | C: 43.3±66.3; (↑8.5%)<br>I: 19.5±39.3; (↑3.7%) | C: -0.008±0.6; (↓0.3%)<br>I: -0.3±0.4; (↓10.3%) | C: 5.9±116.2; (↑0.6%)<br>I: 89.4±108.9; (↑10.4%) |
| Talari *et al.*, 2018 [41] | C: 0.23±0.7 (↑8.9%)<br>I: -0.39±0.9(↓13.6%) | Not applicable | Not applicable | C: 0.1±2.6 (↑0.2%)<br>I: 1.7±4.7 (↑3.4%) | Not applicable | Not applicable | Not applicable |
| Vargas *et al.*, 2011[42]† | C: -0.2±0.7 (↓5.7%)<br>I: -0.4±0.8 (↓11.1%) | C: -0.3±1.7 (↓4.6%)<br>I: 1.0±1.1 (↑13.3%) | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable |
| Vargas *et al.*, 2011 [42] | C: -0.2±0.7 (↓5.7%)<br>I: 0.5±0.8 (↑16.1%) | C: -0.3±1.7 (↓4.6%)<br>I: -0.4±1.8 (↓5.0%) | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable |

C: control; I: intervention; GSH: glutathione; hs-CRP: high-sensitivity C-reactive protein; MDA: malondialdehyde; NO: nitric oxide; TAC: total antioxidant capacity.

†Fish oil supplementation group.

††Biomarker's concentrations expressed as means and standard deviations.

**Supplementary material 3.** Laboratory characteristics of the participants before and after omega-3 fatty acid supplementation.

| Author; Data; Country. | Baseline and at end-of-trial glucose (mg/dL as mean ± standard deviation) | | Baseline and at end-of-trial insulin (µIU/mL as mean ± standard deviation) | | Baseline HOMA-IR and at end-of-trial (as mean ± standard deviation) | |
|---|---|---|---|---|---|---|
| | Intervention | Control | Intervention | Control | Intervention | Control |
| Amini *et al*., 2018 Iran [33]. | Not reported | Not reported | 12.6 ± 2.8<br>10.2 ± 2.9 | 11.2 ± 3.5<br>12.3 ± 4.2 | 2.8 ± 0.8<br>2.2 ± 0.7 | 2.5 ± 0.8<br>2.7 ± 0.9 |
| Jamilian *et al*., 2018; Iran [34]. | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported |
| Mejia-Montilla *et al*., 2017; Venezuela [35]. | Not reported | Not reported | 18.7 ± 4.2<br>16.5 ± 3.4 | 19.4 ± 4.2<br>19.6 ± 4.3 | 3.6 ± 0.8<br>3.3 ± 0.9 | 3.6 ± 0.9<br>3.7 ± 0.7 |
| Mirmasoumi *et al*., 2017; Iran [36]. | Not reported | Not reported | 13.3 ± 8.7<br>10.7 ± 6.2 | 12.2 ± 4.7<br>13.5 ± 4.9 | 3.1 ± 2.1<br>2.4 ± 1.5 | 2.9 ± 1.2<br>3.2 ± 1.2 |
| Mohammadi *et al*., 2012; Iran [37]. | 95.2 ± 10.3<br>85.4 ± 8.95 | 91.7 ± 12.3<br>92.4 ± 9.92 | 16.5 ± 3.0<br>15.1 ± 2.7 | 16.4 ± 3.5<br>16.4 ± 3.4 | 3.91 ± 1.03<br>3.20 ± 0.80 | 3.79 ± 1.28<br>3.80 ± 1.11 |
| Nadjarzadeh *et al*., 2015; Iran [38]. | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported |
| Rafraf *et al*., 2013; Iran [39]. | 95.2 ± 10.3<br>85.4 ± 8.9 | 91.0 ± 12.3<br>92.4 ± 9.9 | 16.5 ± 2.9<br>15.0 ± 2.7 | 16.4 ± 3.5<br>16.4 ± 3.4 | 3.1 ± 1.0<br>3.2 ± 0.8 | 3.8 ± 1.3<br>3.8 ± 1.1 |
| Rahmani *et al*., 2016; Iran [40]. | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported |
| Talari *et al*., 2018; Iran [41]. | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported |
| Vargas *et al*., 2011; USA [42]. | 93.0 ± 0.9<br>96.0 ± 3.1 | 89.0 ± 2.0<br>91.0 ± 2.0 | 24.9 ± 5.8<br>27.7 ± 4.7 | 17.6 ± 2.5<br>19.0 ± 2.2 | 27.1 ± 3.3<br>28.5 ± 4.0 | 28.1± 3.4<br>30.6± 4.3 |
| Vargas *et al*., 2011; USA [42]. | 98,0 ± 3,1<br>96,9 ± 2,0 | 89.0 ± 2.0<br>91.0 ± 2.0 | 22.0 ± 2.4<br>22.75 ± 0.7 | 17.6 ± 2.5<br>19.0 ± 2.2 | 29.8 ± 3.5<br>29.4 ± 3.2 | 28.1± 3.4<br>30.6± 4.3 |

| Author; Data; Country. | Baseline and at end-of-trial total cholesterol (mg/dL as mean ± standard deviation) | | Baseline and at end-of-trial LDL-c (mg/dL as mean ± standard deviation) | | Baseline and at end-of-trial HDL-c (mg/dL as mean ± standard deviation) | | Baseline and at end-of-trial triglycerides (mg/dL as mean ± standard deviation) | |
|---|---|---|---|---|---|---|---|---|
| | Intervention | Control | Intervention | Control | Intervention | Control | Intervention | Control |
| Amini *et al.*, 2018 Iran [33]. | 164.4 ± 30.9<br>165.9 ± 26.7 | 166.2 ± 30.0<br>167.3 ± 28.4 | 93.5 ± 24.6<br>93.3 ± 23.0 | 96.7 ± 25.7<br>94.2 ± 25.3 | 50.0 ± 9.9<br>49.9 ± 9.3 | 51.3 ± 9.4<br>52.1 ± 10.6 | 104.2 ± 58.0<br>113.4 ± 57.2 | 101.1 ± 40.0<br>105.0 ± 47.9 |
| Jamilian *et al.*, 2018; Iran [34]. | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported |
| Mejia-Montilla *et al.*, 2017; Venezuela [35]. | 180.1 ± 22.1<br>154.6 ± 17.6 | 180.5 ± 21.0<br>176.1 ± 20.8 | 109.3 ± 12.1<br>84.7 ± 11.2 | 111.7 ± 12.9<br>110.4 ± 15.4 | 50.4 ± 5.5<br>52.7 ± 6.7 | 48.5 ± 6.0<br>47.8 ± 5.6 | 106.7 ± 24.7<br>86.3 ± 18.9 | 104.1 ± 22.4<br>102.6 ± 22.9 |
| Mirmasoumi *et al.*, 2017; Iran [36]. | 160.5 ± 37.5<br>166.4 ± 45.9 | 165.4 ± 33.8<br>171.1 ± 33.8 | 89.0 ± 34.7<br>92.5 ± 43.5 | 90.1 ± 28.5<br>92.9 ± 27.8 | 50.4 ± 9.6<br>53.8 ± 8.3 | 52.8 ± 10.1<br>53.8 ± 12.5 | 105.9 ± 61.5<br>100.8 ± 64.1 | 112.3 ± 58.3<br>122.0 ± 75.3 |
| Mohammadi *et al.*, 2012; Iran [37]. | 187 ± 32.5<br>170 ± 32.0 | 188 ± 29.2<br>187 ± 25.9 | 118 ± 29.4<br>102 ± 29.6 | 117 ± 31.5<br>117 ± 27.4 | 43.1 ± 6.55<br>45.9 ± 6.53 | 44.9 ± 6.11<br>45.3 ± 4.49 | 127 ± 29.5<br>119 ± 26.0 | 126 ± 28.5<br>120 ± 28.5 |
| Nadjarzadeh *et al.*, 2015; Iran [38]. | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported |
| Rafraf *et al.*, 2013; Iran [39]. | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported |
| Rahmani *et al.*, 2016; Iran [40]. | 181.8 ± 28.0<br>161.5 ± 31.4 | 166.4 ± 29.2<br>178.6 ± 29.9 | 111.1 ± 26.5<br>94.4 ± 29.8 | 92.9 ± 25.5<br>104.8 ± 26.3 | 46.2 ± 10.0<br>47.0 ± 9.5 | 49.4 ± 8.1<br>48.1 ± 9.3 | 122.7 ± 61.7<br>100.6 ± 54.0 | 120.6 ± 59.4<br>128.3 ± 72.6 |
| Talari *et al.*, 2018; Iran [41]. | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported | Not reported |
| Vargas *et al.*, 2011; USA [42]. | 84.5 ± 4.1<br>84.5 ± 4.1 | 88.6 ± 3.2<br>83.1 ± 3.8 | 49.9 ± 2.9<br>52.8 ± 3.2 | 51.7 ± 3.8<br>46.1 ± 4.7 | 21.1 ± 1.4<br>21.1 ± 0.9 | 22.0 ± 1.6<br>22.0 ± 1.4 | 29.2 ± 4.3<br>23.6 ± 3.1 | 25.0 ± 3.1<br>26.1 ± 3.4 |
| Vargas *et al.*, 2011; USA [42]. | 89.2 ± 3.8<br>91.9 ± 3.8 | 88.6 ± 3.2<br>83.1 ± 3.8 | 59.3 ± 3.2<br>62.0 ± 3.8 | 51.7 ± 3.8<br>46.1 ± 4.7 | 19.1 ± 0.9<br>20.0 ± 0.9 | 22.0 ± 1.6<br>22.0 ± 1.4 | 26.5 ± 2.7<br>21.6 ± 2.5 | 25.0 ± 3.1<br>26.1 ± 3.4 |

| Author; Data; Country. | Baseline and at end-of-trial total testosterone (ng/mL as mean ± standard deviation) | | Baseline and at end-of-trial SHBG (nmol/L as mean ± standard deviation) | |
|---|---|---|---|---|
| | Intervention | Control | Intervention | Control |
| Amini *et al.*, 2018 Iran [33]. | 0.7 ± 0.4<br>0.6 ± 0.3 | 0.8 ± 0.4<br>0.9 ± 0.6 | 49.3 ± 9.6<br>49.9 ± 9.4 | 44.6 ± 13.3<br>43.5 ± 13.4 |
| Jamilian *et al.*, 2018; Iran [34]. | 1.4 ± 0.7<br>1.2 ± 0.6 | 1.2 ± 0.7<br>1.3 ± 0.7 | 46.7 ± 22.9<br>52.7 ± 39.3 | 43.5 ± 16.2<br>47.8 ± 26.8 |
| Mejia-Montilla *et al.*, 2017; Venezuela [35]. | Not reported | Not reported | Not reported | Not reported |
| Mirmasoumi *et al.*, 2017; Iran [36]. | 0.9 ± 0.5<br>0.8 ± 0.6 | 0.9 ± 0.6<br>0.8 ± 0.5 | 71.1 ± 54.7<br>79.2 ± 61.4 | 62.9 ± 47.8<br>63.5 ± 32.3 |
| Mohammadi *et al.*, 2012; Iran [37]. | Not reported | Not reported | Not reported | Not reported |
| Nadjarzadeh *et al.*, 2015; Iran [38]. | Not reported | Not reported | Not reported | Not reported |
| Rafraf *et al.*, 2013; Iran [39]. | Not reported | Not reported | Not reported | Not reported |
| Rahmani *et al.*, 2016; Iran [40]. | Not reported | Not reported | Not reported | Not reported |
| Talari *et al.*, 2018; Iran [41]. | Not reported | Not reported | Not reported | Not reported |
| Vargas *et al.*, 2011; USA [42]. | 0.8 ± 0.1<br>0.8 ± 0.2 | 1.0 ± 0.2<br>0.9 ± 0.2 | 17.7± 3.5<br>16.3 ± 3.7 | 18.4 ± 2.4<br>16.2 ± 2.5 |
| Vargas *et al.*, 2011; USA [42]. | 0.8 ± 0.2<br>0.8 ± 0.3 | 1.0 ± 0.2<br>0.9 ± 0.2 | 17.2 ± 2.5<br>17.2 ± 2.8 | 18.4 ± 2.4<br>16.2 ± 2.5 |

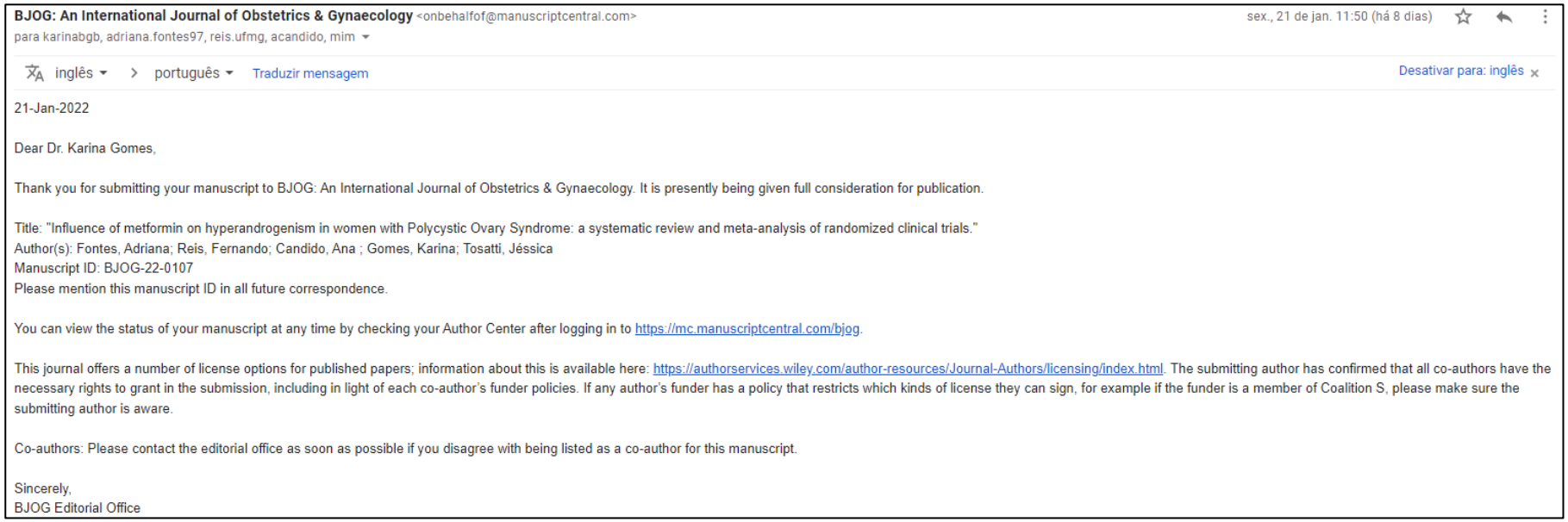
**Supplementary material 4.** Assessment of the quality of the body of evidence for each outcome of the current systematic review.

| Outcomes | Studies | Design/Number of studies | Study limitations | Inconsistency | Directness | Imprecision | Other considerations | Quality | Importance |
|---|---|---|---|---|---|---|---|---|---|
| High-sensitivity C-reactive protein | 7 | Randomized Clinical Trials[7] | Serious[1] | No important inconsistency | Direct | No important imprecision | None | +++, Moderate | Important |
| Adiponectin | 5 | Randomized Clinical Trials[5] | No important limitations | Serious[2] | Direct | No important imprecision | None | +++, Moderate | Important |
| Visfatin | 2 | Randomized Clinical Trials[2] | No important limitations | No important inconsistency | Direct | No important imprecision | None | +++, Moderate | Important |
| Nitric Oxide | 4 | Randomized Clinical Trials[4] | Serious[1] | Serious[2] | Direct | No important imprecision | None | ++, Low | Important |
| Glutathione | 3 | Randomized Clinical Trials[3] | No important limitations | Serious[2] | Direct | No important imprecision | None | +++, Moderate | Important |
| Malondialdehyde | 3 | Randomized Clinical Trials[3] | No important limitations | Serious[2] | Direct | No important imprecision | None | +++, Moderate | Important |
| Total Antioxidant Capacity | 3 | Randomized Clinical Trials[3] | No important limitations | Serious[2] | Direct | No important imprecision | None | +++, Moderate | Important |

(1) One study did not report the randomization process.

(2) Great variability of effect estimates.

6.3 Estudo III. Influence of metformin on hyperandrogenism in women with Polycystic Ovary Syndrome: a systematic review and meta-analysis of randomized clinical trials.



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21-Jan-2022

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Author(s): Fontes, Adriana; Reis, Fernando; Candido, Ana ; Gomes, Karina; Tosatti, Jéssica
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**Influence of metformin on hyperandrogenism in women with Polycystic Ovary Syndrome: a systematic review and meta-analysis of randomized clinical trials.**

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**Running title: Metformin on hyperandrogenism in PCOS**

**Abstract**

**Background:** The treatment of polycystic ovary syndrome (PCOS) includes drugs, such as metformin, to improve insulin sensitivity with the beneficial side effect of reducing hyperandrogenism.

**Objectives:** To summarize the effects of metformin treatment on markers of hyperandrogenism in PCOS.

**Search Strategy:** MEDLINE, CENTRAL, Embase, CINAHL, WOS, and Scopus databases were searched until November 2021.

**Selection Criteria:** Randomized clinical trials that evaluated the effects of metformin treatment on the levels of androgenic markers in adult's women with PCOS.

**Data Collection and Analysis:** Two authors independently conducted selection of studies and data extraction. A meta-analysis to assess changes in the levels of androgenic markers was conducted using a random-effects model. Sensitivity analyzes were performed to explore possible heterogeneity between studies. All studies were evaluated using an appropriate quality assessment tool and the GRADE approach was used to assess the certainty of evidence.

**Main Results:** 19 studies, 954 women with PCOS were eligible for inclusion. A significant reduction in total testosterone levels was seen in the metformin-treated group when compared to the control group SMD: -0.46 (-0.89 to -0.02) points; $I^2$ 85; p=<0.00001. FAI values by the sensitivity analysis were also regulated by metformin treatment SMD: -0.42 (-0.67 to -0.16) points; $I^2$ 0; p=0.58 and total testosterone levels when compared to the control group SMD: -0.24 (-0.40 to -0.07) points; $I^2$ 0%; p=0.56.

**Conclusions:** Metformin proved to be effective in reducing total testosterone levels and FAI values. The certainty of the body of evidence was classified as moderate.

**Keywords:** Polycystic Ovary Syndrome; Metformin; Hyperandrogenism; Systematic review; Meta-analysis.

## Introduction

Polycystic Ovary Syndrome (PCOS) is a heterogeneous endocrine disorder often seen in women of childbearing age, and it is characterized by oligo/anovulation, clinical/ biochemical signs of hyperandrogenism and polycystic ovaries.[1] Based on the Rotterdam criteria of the diagnosis[2], its prevalence is estimated from 8 to 13% worldwide.[3] PCOS can result in numerous clinical complications, such as infertility, obesity, cardiovascular diseases, and insulin resistance (IR), the latter presenting an important role in its pathogenesis, affecting approximately 75% of patients with PCOS.[4] In response to IR, it is observed an increase of serum insulin levels and increased production of androgen hormones by the ovaries, as well as suppression of sex hormone-binding globulin (SHBG), thus increasing free testosterone levels.[5] Furthermore, there is an increase in the activity of the 5α-reductase enzyme, favoring the conversion of testosterone into 5α-dihydrotestosterone (DHT), the most potent form of the hormone, contributing to the typical hyperandrogenism of PCOS.[5] The enzyme 5α-reductase also promotes activation of the hypothalamic-pituitary-adrenal axis, stimulating the production of androgen hormones by the adrenal gland.[5] Up to 80% of women with PCOS manifest hyperandrogenism, clinically demonstrated by hirsutism, acne, and alopecia.[6]

The treatment of PCOS includes medications to improve, directly or indirectly, insulin sensitivity. Metformin acts on IR-related parameters, increasing the sensitivity of peripheral tissues to insulin, inhibiting gluconeogenesis and glycogenolysis in the liver, decreasing intestinal glucose absorption and promoting its use by cells.[7-9] In addition, metformin also favors weight reduction, decreases the concentration of plasma lipids and plasminogen activator inhibitor-1 (PAI-1), a direct risk factor for thrombosis and atherosclerosis, and acts in the prevention of cardiovascular events, commonly seen in patients with PCOS.[8] Moreover, metformin acts indirectly on androgen levels, improving several symptoms, which may favor the reproductive function of these women.[10]

Hyperandrogenism and IR have been widely discussed as risk factors for cardiovascular events in patients with PCOS. Androgen hormones such as testosterone, DHT, dehydroepiandrosterone sulfate (DHEAS), androstenedione and androsterone have direct effects on the blood vessel wall, confirmed by the presence

of androgen receptors in blood vessels[11], related to impairment of vascular functions and a stimulus to the formation of atherosclerotic plaques in these patients.[12] Endothelial dysfunction is also related to IR, resulting in increased production of several biomarkers of atherosclerosis, such as vascular cell adhesion protein 1 (VCAM-1) and von Willebrand factor (vWF).[13,14] Moreover, hyperandrogenemia has an important role in the oxidative stress in PCOS, and metformin could decrease this effect by reducing the levels of androgens and inflammatory markers.[7]

The use of metformin to control IR in PCOS has the beneficial side effect of reducing hyperandrogenism in these patients. However, the magnitude of this effect is not well established, and it is not its main indication. Therefore, the aim of this systematic review with meta-analysis of randomized clinical trials was to assess the effects of metformin treatment on markers of hyperandrogenism in patients with PCOS.

## Methods

The design of this study followed the recommendations of the Cochrane Manual for Systematic Reviews of Interventions[15], being reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)[16] and the protocol published in International Prospective Register of Systematic Reviews (PROSPERO), under the identification CRD42021235761.

### Search strategy

The search strategies applied to electronic databases were built based on the acronym PICO, according to the proposed research question: P (participants) = Women diagnosed with PCOS; I (intervention) = Metformin treatment; C (control) = Placebo; and O (outcome) = Levels of androgenic markers. The literature review was performed based on searches in electronic databases: Medical Literature Analysis and Retrieve System Online (MEDLINE) via PubMed, Cochrane Central Register of Controlled Trials (CENTRAL), Embase, Cumulative Index to Nursing and Allied Health Literature (CINAHL), Web of Science and Scopus to November 2021, aiming to identify placebo-controlled randomized controlled trials (RCTs) reporting the effects of metformin treatment on levels of androgenic markers in adult women with PCOS. The search

included the Medical Subject Headings (MeSH) terms “Polycystic Ovary Syndrome”, “Metformin” and “Hyperandrogenism” or “Androgenism” and their respective entry terms. In order to consider only RCTs, we used the high-sensitivity search strategy developed by the Cochrane Collaboration.[17] The complete search strategy, performed in the MEDLINE database, is presented in **Appendix S1**.

The terms applied to the main search were used for grey literature search in the Open Grey (http://www.opengrey.eu/search/) and Open Access Theses and Dissertations (https://oatd.org/) databases. Searches were also performed for clinical studies and protocols in the databases of the National Institutes of Health (www.clinicaltrials.gov), in the Brazilian Registry of Clinical Trials (ReBEC) (www.ensaiosclinicos.gov.br) and in Turning Research into Practice (www.tripdatabase.com), and a manual search was also performed in the reference lists of relevant reviews.[18-22] All potentially eligible studies were considered for review, regardless of language and publication date.

**Inclusion and Exclusion Criteria**

Only RCTs that evaluated the effects of metformin treatment on the levels of androgenic markers in women over 18 years of age with PCOS were included. The primary outcome was considered as changes in the initial and final values of the androgenic biomarkers evaluated by the primary studies, namely: (1) androstenedione; (2) DHEAS; (3) Free Androgen Index - FAI; (4) SHBG; (5) free testosterone and; (6) total testosterone. Studies were excluded if they used metformin only as adjunctive intervention (associated to primary interventions such as caloric restriction or oral contraceptive), if they did not report at least one of the outcomes of interest, or if they were non-randomized or non-placebo-controlled studies. Review articles, case reports and commentary studies were also excluded.

**Selection of studies and data extraction**

This stage included the recovery of studies obtained in the research in the databases and their insertion in a single electronic library. The duplicates were excluded using the Endnote® software, version 20 for desktop. After removing the duplicates, two authors (AFSF and JAGT) independently analyzed the titles and abstracts of the

remaining articles and, after excluding the studies that did not meet the inclusion criteria, the same reviewers proceeded with the complete reading of the articles and extracted the data using a standardized data extraction tool hosted in the Microsoft Excel® software, version 365. Any disagreement between the reviewers was resolved by a third investigator (KBG).

The data extracted from the included articles were number of participants allocated to each group (intervention and placebo), duration of follow-up of the RCT, demographic characteristics of the studied population, dosage of metformin given to the intervention group and characteristics of the placebo group. Information was also extracted from the levels of androgenic biomarkers, performed at the beginning and at the end of the RCT follow-up, or from the difference between the means, depending on the format of data presentation by the primary studies.

**Assessment of the risk of publication bias**

The risk of bias assessment of the included studies was assessed independently by two reviewers (AFSF and JAGT), in accordance with the recommendations of the Cochrane Manual for Systematic Reviews of Interventions[23], and a third reviewer (KBG) resolved any disparity. The Cochrane Risk of Bias for Randomized Trials tool - Rob 2.0 - beta version 9, hosted in Microsoft Excel software[24], was applied to assess the methodological quality of individual studies, being structured into five domains: (1) bias resulting from the process of randomization; (2) bias due to deviations from intended interventions; (3) bias due to lack of outcome data; (4) bias in outcome measurement and (5) bias in reported outcome selection. The answer options for the questions are: (1) yes; (2) probably yes; (3) probably not; (4) no and; (5) there is no information. In the end, grades – high, with considerations, and low – were assigned to all domains, based on an algorithm of the tool, to each of the included studies.[24]

**Data synthesis and analysis**

To assess changes in the levels of androgenic markers, the values obtained at the beginning and at the end of each study were extracted and included in the meta-analysis. The random effects model was applied because all evaluated outcomes had

more than five studies included. The results were presented as standardized mean difference (SMD) with a 95% confidence interval (CI) as a measure of effect, given the possible differences in the methods of measuring the markers between the included studies.[25]

Heterogeneity in the primary studies was assessed by the Cochran Q test, and p-value ≤0.10 was considered statistically significant. The $I^2$ test was also performed to assess the magnitude of heterogeneity, which was considered high if $I^2 \geq 50.0\%$. The heterogeneity of the outcomes assessed by the meta-analysis was further explored with sensitivity analysis, excluding each study serially. For this purpose, the meta-analysis was performed twice: the first time including all studies, and the second including only those known to be eligible, within the evaluated factors.[26]

Publication bias was assessed through funnel plot asymmetry and the Egger test index for all outcomes except FAI, because, according to the recommendations of the Cochrane Manual for Systematic Reviews of Interventions, the method should be used when there are at least 10 studies included in the meta-analysis.[27] For the Egger test, values of $p < 0.05$ were considered significant.

Statistical analyzes were performed using the Review Manager® software version 5 except for publication bias, which was evaluated using the Comprehensive meta-analysis (CMA)® software, version 3.

**Assessment of certainty of evidence**

The Grading of Recommendations, Assessment, Development and Evaluation (GRADE) approach[28] provided the methods to assess the certainty of evidence for each of the evaluated outcomes: (1) androstenedione; (2) DHEAS; (3) FAI; (4) SHBG; (5) total testosterone and; (6) free testosterone. For this purpose, the data compiled in Review Manager® were imported into the guideline development tool (www.gradepro.org) and the evidence was classified as high, moderate, low, or very low quality, according to certainty assessment criteria (risk of bias, inconsistency, indirect evidence, and imprecision) and other considerations (publication bias and potential confounding factors). In the end, the certainty of the evidence was classified as high, moderate, low, or very low certainty.[29]

## Results

### Qualitative analysis of included studies

Search performed in electronic databases, gray literature and reference lists resulted in 3,694 records. After removing 1637 duplicates, 2,057 unique titles and abstracts were analyzed, and 70 records were selected for the full-text reading stage. After a complete reading of the studies, 19 primary studies[30-48] were included in the qualitative analysis and 18 studies in the quantitative analysis[31-48] (**Figure 1**). The study by Agarwal, et al. (2010)[30] was not included in the quantitative analysis because the measurement of androgenic markers performed at the end of follow-up was not detailed. The study by Onalan et al. (2005)[42] was included in the analysis of six outcomes because it stratified the study results by body mass index (BMI) of participants – less than 25.0 kg/m$^2$; 25.0 to 29.9 kg/m$^2$; ≥ 30.0 kg/m$^2$ - and by insulin levels - normoinsulinemic or hyperinsulinemic.

The characteristics of the studies, as well as the allocated participants, are presented in **Table 1**. The included studies were conducted with volunteers from different countries, most of them residing in the United Kingdom (n = 5)[30,34,37,40,45], followed by Turkey (n = 3)[31,42,48] and Italy (n = 3)[41,43,44], with recruitment performed in centers specialized in endocrinology. Of the 19 placebo controlled RCTs included, five had a crossover design[30,31,37,41,47], two a factorial design[32,35] and twelve a parallel design[33,34,36,38-40,42-46,48], with follow-up duration ranging from 4[36] to 56 weeks[37,43]. As for the intervention with metformin, two studies used the dosage of 1000 mg/day[32,44], five used the dosage of 1500 mg/day[36,37,39,40,44], six used the dosage of 1700 mg /day[31,35,43,45,47,48] and five studies[30,33,34,41,42] performed the intervention with metformin in a staggered manner, increasing the dosage of the drug throughout the design.

The studied population consisted of participants with a mean age ranging from 21.4[41] to 35.0 years[34]. The diagnosis of PCOS was based on the Rotterdam criteria in sixteen studies[30-34,36,37,42-47], National Institute of Child Health and Human Development (NICHHD) criteria in one study[41], World Health Organization (WHO) criteria in one study[48] and one study[35] did not report the criteria applied. The nineteen primary studies included totalized 954 women with PCOS, of whom 465 were allocated to the intervention group (mean age of 28.9 ± 2.9 years and an initial mean BMI of 30.6 ± 4.6

kg/m$^2$) and 324 in the placebo group (mean age of 28.6 ± 3.2 years and an initial mean BMI of 31.6 ± 4.8 kg/m$^2$).

**Androstenedione**

The eleven studies[31,33,36-39,41-44,48] that evaluated androstenedione levels in women with PCOS under metformin compared to placebo had a total of 483 patients randomized, 242 in the metformin intervention group (mean age: 26.9 ± 2.5 years) and 241 in the placebo group (mean age: 27.5 ± 3.0 years), with follow-up duration between 4[36] and 56 weeks[37,43]. Regarding the intervention with metformin, this ranged from 1000[44] to 1700 mg[31,43,48], and three studies[33,41,42] performed the intervention in a staggered manner. The placebo had a similar appearance to metformin and was offered at the same frequency as the drug. The mean concentrations of androstenedione at the end of the follow-up were 297.1 ± 95.0 ng/dL in the intervention group and 284.0 ± 61.5 ng/dL in the placebo group. Based on the eleven studies included, there was no evidence of a significant reduction in androstenedione levels after metformin intervention compared to placebo [SMD: 0.61 (95% CI: -0.30 to 1.52) points; $I^2$ = 94%; p-value for heterogeneity: < 0.00001; **Fig. 2 (a)**]. Considering the risk of bias, a possible asymmetry between the studies was evidenced, based on the analysis of the funnel graph [**Appendix S2, Fig. 3 (a)**], as well as by the Egger test (p = 0.58).

**Dehydroepiandrosterone Sulfate (DHEAS)**

The levels of DHEAS were measured in a total of 546 patients, of whom 275 received metformin (mean age: 26.9 ± 2.3 years) and 271 received placebo (mean age: 27.6 ± 2.9 years)[31-33,36-44,48]. The follow-up duration ranged between 4[36] and 56 weeks[43]. Metformin doses in the intervention group ranged from 1000[38] to 1700 mg/day[31,43,48], and three studies[33,41,42] performed the intervention in a staggered manner. Placebo was described as similar in appearance to the intervention and offered at the same frequency. At the end of the follow-up, DHEAS levels were 264.6 ± 38.0 µg/dL and 263.2 ± 70.6 µg/dL in the metformin and placebo groups, respectively. Combining the results obtained, there was no significant difference in the change of DHEAS levels between the women treated with metformin compared to placebo [SMD: -0.14 (95%

CI: -0.88 to 0.60) points; $I^2$ = 92%; p-value for heterogeneity: < 0.00001; **Fig. 2 (b)**]. A possible asymmetry between the studies was evidenced, based on the evaluation of the funnel plot [**Appendix S2, Fig. 3 (b)**], as well as by the Egger's test (p = 0.86).

**Free Androgen Index – FAI**

Nine studies[30,34,35,37,39,40,43-45] reported FAI as an outcome. The study by Agarwal, et al. (2010)[30] did not present enough data for inclusion in the meta-analysis but reported not having observed a significant difference in FAI values between the groups treated with metformin and placebo. The follow-up duration ranged from 12[30,39] to 56 weeks[37,43] and a total of 378 women were included, being 180 in the metformin groups (mean age: 28.3 ± 3.3 years) and 198 in the placebo groups (mean age: 29.0 ± 3.1 years). The dosage of metformin varied between 1000 mg/day[44], 1700 mg/day[35,43,45] and studies[30,34] performed the intervention in a staggered manner. The placebo was offered at the same frequency and was similar in appearance to the intervention.

In the quantitative analysis, the mean FAI values at the end of follow-up were 10.2 ± 5.1% in the metformin group and 11.7 ± 5.3% in the placebo group. In the combined evaluation of the eight primary studies, no significant difference in the variation of FAI during the intervention period was evidenced between the groups treated with metformin and placebo [SMD: -0.45 (95% CI: -0.94 to 0.04) points; $I^2$ = 72%; p-value for heterogeneity: 0.0007; **Fig. 2 (c)**].

**Sex Hormone Binding Globulin (SHBG)**

Fifteen studies[30,33-37,39-47] evaluated SHBG as an outcome. The studies by Agarwal et al. (2010)[30] and by Trolle et al. (2007)[46] did not present sufficient results for inclusion in the meta-analysis, but both reported not having observed significant differences in SHBG levels between the metformin and placebo intervention groups. In the fifteen studies included in the qualitative analysis, 794 patients were randomized, 385 to metformin (mean age: 27.8 ± 3.1 years) and 409 to placebo (mean age: 28.5 ± 3.5 years), with dosage of metformin ranging from 1000[44,46] to 1700[35,43,45,47] and five studies performed the intervention in a staggered manner[30,33,34,41,42].

To carry out the meta-analysis, thirteen primary studies were included, with follow-up duration ranging from 4[36] to 56 weeks[37,43]. In all, 635 patients diagnosed with PCOS were included, with 308 allocated to receive metformin and 327 to receive placebo. The mean SHBG values at the end of follow-up were 20.5 ± 25.8 nmol/L in the metformin group and 32.9 ± 28.4 nmol/L in the placebo group, but no significant difference was found in the changes of SHBG levels observed during the intervention between the metformin and placebo groups [SMD: 0.23 (95% CI: -0.29 to 0.74) points; $I^2$ = 88%; p-value for heterogeneity: <0.0001; **Fig. 2 (d)**]. A possible asymmetry between the studies was evidenced, based on the evaluation of the funnel graph [**Appendix S2, Fig. 3 (c)**], as well as by the Egger's test (p = 0.68).

**Free Testosterone**

Results from six primary studies[31,36,39,41,42,48] were combined to perform the quantitative analysis in which free testosterone levels were compared between patients receiving metformin and placebo. The metformin groups had a total of 146 patients, with a mean age of 27.5 ± 2.9 years, whereas the placebo groups totalized 147 patients with a mean age of 27.1 ± 3.5 years. The final levels of free testosterone were 15.8 ± 7.1 pg/mL in the metformin group and, 9.9 ± 9.3 pg/mL in the placebo group. When the four studies were combined, no significant difference in the change of free testosterone levels was observed between metformin and placebo [SMD: -0.30 (95% CI: -1.27 to 0.67); $I^2$ = 92%; p-value for heterogeneity: <0.00001; **Fig. 2 (e)**]. Regarding the publication bias analysis, a possible asymmetry between the studies was evidenced, based on the evaluation of the funnel plot [**Appendix S2, Fig. 3 (d)**], as well as by the Egger's test (p = 0.98).

**Total testosterone**

In the qualitative assessment, sixteen studies were included[30-37,39,40,43-48], four with a crossover design[30,31,37,47], two with a factorial design[32,35] and ten parallel RCTs[33,34,36,39,40,43-46,48], with follow-up duration between 4[36] and 56 weeks[37,43] and metformin dosage in the intervention group ranging from 1000[32, 44, 46] at 1700 mg/day[31,35,43,45,47,48], with three studies[30,33,34] performing the intervention in a staggered manner. A total of 712 patients were included in the analyses, with 356 allocated to the

metformin intervention group (mean age: 28.5 ± 2.7 years) and 356 patients allocated to the placebo group (mean age: 28.9 ± 2.7 years). The study by Agarwal, et al. (2010)[30] did not present enough data to be included in the meta-analysis but reported not having observed a significant difference in total testosterone values between the intervention groups with metformin and placebo.

In the quantitative evaluation, fifteen studies were included[30,32-37,40,43-48] totalizing 659 participants. Total testosterone levels at the end of follow-up were 28.3 ± 2.9 nmol/L in the metformin group and 28.8 ± 2.8 nmol/L in the placebo group. After combining the results there was a small but marginally significant difference between metformin and placebo in the mean changes of total testosterone levels [SMD: -0.46 (95% CI: -0.89 to -0.02) points; $I^2$ = 85%; p-value for heterogeneity: <0.00001; **Fig. 2 (f)**]. Regarding the publication bias analysis, no significant asymmetry was evidenced from the analysis of the funnel plot [**Appendix S2, Fig. 3 (e)**], as well as by the Egger's test (p = 0.15).

**Sensitivity analysis**

To explore the substantial heterogeneity between the studies included in the meta-analysis and to examine changes in the size of the combined effect, sensitivity analyzes were performed, proceeding with the serial exclusion of each study from the analyses and for all evaluated outcomes[29,49]. For the androstenedione, DHEAS, SHBG and free testosterone outcomes, no significant differences were found when heterogeneity was explored. For the outcome FAI, a significant reduction of the index was evidenced in the group treated with metformin, when compared to the placebo group, by excluding from the analysis the studies by Kelly et al. (2002)[37] and Lord et al. (2006)[40] [SMD: -0.42 (95% CI: -0.67 to -0.16) points; $I^2$ = 0%; p-value for heterogeneity: 0.58; **Appendix S3, Fig 4 (a)**]. As for the total testosterone outcome, a significant reduction in hormone levels was observed in the intervention group with metformin, when compared to the placebo group, when we removed the studies by Jakubowicz et al. (2000)[36], Kurzthaler et al. (2014)[39] and Lord et al. (2006)[40] [SMD: -0.24 (95% CI: -0.40 to -0.07) points; $I^2$ = 0%; p-value for heterogeneity: 0.56; **Appendix S3, Fig 4 (b)**].

**Assessment of the risk of bias**

The assessment of the risk of bias in each included study is summarized in **Figure 5**. In domain 1, referring to the applied randomization process, one study[32] was considered to have high risk of bias because it did not perform true randomization, just a quasi-randomization based on the day of the week when the participant was enrolled, and another three studies[36,37,43] were evaluated with reservations, as it was not possible to define exactly the randomization method performed. Domain 2, which assessed the risk of bias due to deviations from the intended interventions, was rated low for most studies[31,33,34,36-41,43-48] and with some considerations for four studies[30,32,35,42] for presenting possible negative effects of attribution to the intervention. Domain 3, referring to risk of bias due to lack of outcome data, was classified as low risk for fourteen studies[30, 2-36,38,42-48], with some caveats for three studies[31,39,41] and with a high risk of bias for two studies[37,40], associated with the unavailability of data obtained for at least 95% of the volunteers included throughout the study. In the outcome measurement domain, seventeen studies[31-46,48] were considered to have low risk of bias and two studies[30,47] were classified with caveats due to possible directional biases, i.e., measurement results not consistent with the results that the authors planned to evaluate - directed to one of the interventions proposed by the included studies. Domain 5 - the risk of selecting the reported outcome was rated as low risk for sixteen studies[30-34,36-40,41-47] and with reservations for three studies[35,41,48], related to possible selection biases in reporting the results presented. In the overall assessment, seven studies[31,33,34,38,44-46] were considered at low risk of publication bias, nine[30,35,36,39,41-43,47,48] had some concern and three studies[32,37,40] were evaluated with high risk of bias related to the randomization process and the possible negative effects of imputation to the intervention.

**Assessment of certainty of evidence**

The GRADE[28] results are described in **Appendix S4**. The risk of bias was classified as non-serious for all requirements. The certainty of the body of evidence was classified as moderate for the six outcomes presented.

## Discussion

### Main findings

In the present systematic review with meta-analysis, we evaluated the effects of metformin on androgenic levels in women with PCOS. Treatment with metformin did not cause a significant difference in the levels of androstenedione, DHEAS, SHBG, or free testosterone, when compared to placebo. However, the quantitative analysis of selected studies demonstrated that treatment with metformin resulted in a significant reduction in total testosterone levels and FAI relative to the placebo group.

### Strengths and limitations

Potential limitations should be considered when carrying out the critical analysis of the present study and the primary studies. The different dosages and treatment regimens offered to patients may have interfered with the results obtained from the primary studies included. Furthermore, none of the studies included in the meta-analysis presented intention-to-treat analysis, a statistical approach associated with more conservative results, and three included studies[32,37,40] had a high risk of publication bias. Another important factor to consider was the high heterogeneity presented when the results were combined, which was explored in the sensitivity analysis. However, when the meta-analysis involves many studies, the Cochran Q test may present a statistically significant heterogeneity between studies even when the heterogeneity is not clinically relevant.[49]

Even with the limitations presented, our study has several strengths, such as the publication of the protocol on an electronic basis (PROSPERO); the use of a rigorous methodology based on the PRISMA guidelines; application of well-defined eligibility criteria prioritizing only studies with an emphasis on effects of metformin on hyperandrogenism in women with PCOS and; carrying out a comprehensive literature search, including six electronic databases (CENTRAL, CINAHL, Embase, MEDLINE, Scopus and WOS), as well as performing a search for grey literature; selection and extraction of data from the studies were carried out independently and in duplicate by two researchers, with resolution of disagreement by a third reviewer. Furthermore, this

study presents updated evidence, considering that the latest reviews on the subject were published between 2015 and 2017, in addition to having assessed different outcomes, such as androstenedione, total and free testosterone.

**Interpretation**

Some previous meta-analyses have evaluated outcomes related to hyperandrogenism in women with PCOS treated with metformin. Significant reduction in total testosterone levels after metformin treatment was observed in other meta-analyses[22,50-52], as well as the absence of relevant changes in SHBG[22,50-52], androstenedione[22,50] and FAI.[22] The meta-analysis published by Tang et al. (2012)[51] obtained different results regarding the levels of DHEAS, which showed an increase, and total testosterone, which decreased in women treated with metformin compared to placebo. Tang et al. [[51]] found a reduction in free testosterone and androstenedione levels in association with metformin treatment, as well as a reduction in the levels of total testosterone when a sensitivity analysis removed outlier studies.[51]

Another meta-analysis by Naderpoor et al. (2015)[20] observed that intervention with metformin alone in patients with PCOS resulted in a more pronounced reduction in testosterone levels, compared to the group whose intervention was adopting a healthy lifestyle associated with the administration of placebo. The same result was verified by Kim et al. (2020)[53], who detected a significant reduction in total testosterone levels following metformin treatment compared to intervention in the participants' lifestyle. However, this latter group demonstrated an increase in SHBG compared to the administration of metformin alone.[53]

Hyperinsulinemia, which is characteristic of the syndrome, causes a reduction in SHBG[54]; consequently, an increase in this protein would be expected with the use of metformin in women with PCOS. However, in the present study, there was no difference between placebo and metformin concerning their effects on SHBG levels. Therefore, it is not yet possible to state that metformin has an effect on this outcome in patients with PCOS. As for total testosterone, metformin possibly reduces its levels by improving insulin sensitivity in peripheral tissues[54-57] and promoting increased activity of cytochrome P450c17α, which is involved in androgen synthesis[56].

The action of DHEAS is associated with a reduction in gluconeogenesis and promotes increased hepatic glucose absorption and insulin binding to its receptor.[56, 58] Thus, excess glucose or insulin can cause a decline in DHEAS levels.[58] Brennan et al, in a cohort study, observed a negative relationship between DHEAS and insulin resistance[58], as well as Brahimaj et al. (2017)[59] demonstrated that DHEAS levels are inversely associated with the risk of developing type 2 diabetes by the population. In men, the effect of metformin on DHEAS levels was analyzed by Nestler et al. (1994)[60] who observed that the concentration of this androgen increased substantially in these subjects with administration of the drug. Lerchbaum et al. (2012)[56], in a cohort study, concluded that DHEAS may have a protective role against the metabolic effects caused by excess testosterone, since the group with higher DHEAS and free testosterone had better metabolic status than the group with increased free testosterone alone.[56] In addition, Kolodziejczyk et al. (2000)[61] observed in their prospective study that women whose initial DHEAS values were within the normal range experienced a significant increase after treatment with metformin, while patients who had high DHEAS concentrations at the beginning of the study had this hormone reduced with the use of metformin.[61] Because of this, it is hypothesized that the basal function of the adrenal gland, which is responsible for the synthesis of DHEAS, may influence the effects of metformin, demonstrating the complexity of this system.[61]

Contrary to the results found in the present meta-analysis, Velazquez et al. (1994)[62] observed a significant reduction in DHEAS after metformin treatment.[62] The reduction in DHEAS could be explained by the hypothesis that insulin stimulates the production of this androgen through direct action on the adrenal or on the sulfotransferase enzyme[63] responsible for converting DHEA into DHEAS.[64] However, the study by Velazquez et al. (1994)[62] was not randomized nor it had a control group for a more accurate comparison.

Metformin inhibits the enzyme cytochrome P450c17α, promoting decreased androgen production in ovarian thecal cells and at the level of the adrenal glands.[65,66] In addition, the mechanism of direct inhibition of ovarian steroidogenesis is probably by inhibition of complex I at the mitochondrial level.[67,68] Another theory is the inhibition of the enzyme 3-β-hydroxysteroid dehydrogenase type 2, occasionally increased in some patients with PCOS, and responsible for the conversion of androgen precursors to testosterone and androstenedione.[67,69] An *in vitro* study with human cells[67] showed

that metformin inhibited the activity of the enzyme 17,20-lyase, responsible for the conversion of precursors to DHEA and androstenedione, as well as the enzyme 3 -β-hydroxysteroid dehydrogenase type 2. Furthermore, they also verified the inhibitory action of metformin on the production of androgens in these cells.[67]

As for the results obtained in the sensitivity analysis - significant reduction in FAI values and total testosterone levels, without showing a significant reduction in free testosterone levels, in the group treated with metformin compared to the placebo group, it is important to discuss the role of FAI in this assessment. The free androgen index (FAI) calculated from the values of total testosterone and SHBG becomes an indirect indicator of free testosterone.[69] When free testosterone cannot be accurately measured, FAI can become a sensitive indicator for to evaluate hyperandrogenism in patients with PCOS. Furthermore, FAI can avoid the clinical complexity and analytical uncertainty of direct measurement of free testosterone, indirectly reflecting its biological activity.[70] Consequently, the reduction in total testosterone, combined with the absence of a significant reduction in SHBG levels, results in an effective decrease in the free fraction of plasma testosterone, which is better estimated by the FAI, when compared to assays applied to the direct dosage of free testosterone.[70]

## Conclusion

In conclusion, the present meta-analysis suggests that metformin is effective in reducing total testosterone levels and FAI values in women with PCOS. The results also consistently suggest that SHBG and free testosterone levels are not affected by metformin treatment. The effects of metformin on androstenedione and DHEAS levels, although suggesting no difference from placebo, were so divergent between the studies that further investigation may be required to solve the inconsistency. The GRADE certainty of the body of evidence was classified as moderate for the six outcomes presented.

## Disclosure of interests

None Declared.

**Contribution to Authorship**

AFSF, JAGT, and KGB designed and carried out the study. AFSF, JAGT, and KGB analyzed the data; AFSF, JAGT, ALC, FMR and KGB wrote and reviewed the article. All authors have critically reviewed and improved the manuscript.

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**Table 1.** Descriptive characteristics of studies that investigated the effects of metformin treatment on androgenic biomarkers in women with Polycystic Ovary Syndrome.

| Author, year of publication; Country. | Follow-up duration (weeks) | Study design | No. of participants; age (years; mean ± standard deviation) | | Characteristics of groups | | Body Mass Index (BMI) at the beginning of the follow-up (kg/m$^2$) | | Diagnostic criteria for PCOS | Outcome of interest |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Intervention | Control | Intervention | Control | Intervention | Control | | |
| Agarwal, et al., 2010; United Kingdom[30] | 12 | RCT cross-over | 30; 30.0 ± 2.2 | 30; 30.2 ± 2.5 | Metformin 500 mg orally once a day for the first week, twice a day for the second week, and three times a day for the remaining weeks. | Placebo similar in appearance to metformin. | 34.9 ± 6.8 | | Rotterdam criteria | FAI; SHBG e total testosterone. |
| Açbay et al., 1996; Turkey[31] | 10 | RCT cross-over | 16; 31.0 ± 4.0 | 16; 31.0 ± 4.0 | Metformin 850 mg orally twice per day. | NR | 30.2 ± 1.8 | | Rotterdam criteria | Androstenedione; DHEAS; free testosterone; total testosterone. |
| Bonakdaran et al., 2012; Iran[32] | 12 | RCT factorial | 17; 25.9 ± 4.5 | 16; 25.2 ±7.9 | Metformin 1000 mg per day | Placebo similar in appearance to metformin. | 28.2 ± 5.03 | 25.3 ± 5.1 | Rotterdam criteria | DHEAS; total testosterone. |
| Eisenhardt et al., 2006; Germany[33] | 12 | RCT parallel | 22; 27.0 ± 2.1 | 23; 29.7 ± 3.5 | 1$^{st}$ week: Metformin 500 mg orally twice a day; Other weeks: Metformin 500 mg orally three times a day | Placebo similar in appearance to metformin. | 28.9 ± 3.6 | 32.4 ± 4.1 | Rotterdam criteria | Androstenedione; DHEAS; SHBG; total testosterone. |

| Author, year of publication; Country. | Follow-up duration (weeks) | Study design | No. of participants; age (years; mean ± standard deviation) | | Characteristics of groups | | Body Mass Index (BMI) at the beginning of the follow-up (kg/m$^2$) | | Diagnostic criteria for PCOS | Outcome of interest |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Intervention | Control | Intervention | Control | Intervention | Control | | |
| Fleming et al., 2002; United Kingdom[34] | 16 | RCT parallel | 26; 34.2 ± 3.6 | 39; 35. ± 2.8 | 1st week: Metformin 850 mg orally once daily; Other weeks: Metformin 850 mg orally twice a day | Placebo similar in appearance to metformin | 35.2 | 35.3 | Rotterdam criteria | FAI; SHBG; total testosterone. |
| Hoeger et al., 2004; USA[35] | 48 | RCT factorial | 5; 29.5 ± 6.4 | 7; 27.1 ± 4.5 | Metformin 850 mg orally twice per day | Placebo similar in appearance to metformin | 37.1 ± 4.9 | 37.1 ± 4.6 | Rotterdam criteria | FAI; SHBG; total testosterone |
| Jakubowicz et al., 2000; Venezuela[36] | 4 | RCT parallel | 26; 27 ± 1.0 | 22; 27 ± 1.0 | Metformin 500 mg orally three times a day | Placebo similar in appearance to metformin | 31.8 ± 0.3 | 31.7 ± 0.3 | Rotterdam criteria | Androstenedione; DHEAS; SHBG; free testosterone; total testosterone. |
| Kelly et al., 2002; United Kingdom[37] | 56 | RCT cross-over | 10; NR | 10; NR | Metformin 500 mg orally three times a day | Placebo similar in appearance to metformin | NR | | Rotterdam criteria | Androstenedione; FAI; DHEAS; SHBG; total testosterone. |
| Kjøtrød et al., 2007; Norway[38] | 16 | RCT parallel | 31; 28.9 ± 5.2 | 32; 30.2 ± 4.8 | Metformin 500 mg orally twice a day | Placebo similar in appearance to metformin | 28.6 ± 2.4 | 29.9 ± 2.7 | Rotterdam criteria | Androstenedione; DHEA. |
| Kurzthaler et al., 2010; Austria[39] | 12 | RCT parallel | 10; 26.10 ± 4.25 | 9; 27.9 ± 4.8 | Metformin 500 mg orally three times a day | Placebo similar in appearance to metformin | 29.90 ± 7.41 | 32.15 ± 6.10 | Rotterdam criteria | Androstenedione; FAI; DHEAS; SHBG; free testosterone; total testosterone. |

| Author, year of publication; Country. | Follow-up duration (weeks) | Study design | No. of participants; age (years; mean ± standard deviation) | | Characteristics of groups | | Body Mass Index (BMI) at the beginning of the follow-up (kg/m$^2$) | | Diagnostic criteria for PCOS | Outcome of interest |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Intervention | Control | Intervention | Control | Intervention | Control | | |
| Lord et al., 2006; United Kingdom[40] | 12 | RCT parallel | 16; 27.8 ± 4.9 | 14; 30.6 ± 4.8 | Metformin 500 mg orally three times a day | Placebo similar in appearance to metformin | 33.74 ± 6.74 | 36.37 ± 7.46 | Rotterdam criteria | DHEAS; FAI; SHBG; total testosterone. |
| Moghetti et al., 2000; Italy[41] | 24 | RCT parallel | 11; 23.9 ± 1.2 | 12; 21.4 ± 1.4 | Metformin 500 mg orally once a day for the first week, twice a day for the second week, and three times a day for the next 24 weeks. | Placebo similar in appearance to metformin | 27.1 ± 1.5 | 32.6 ± 1.1 | NICHHD criteria | Androstenedione, DHEAS; SHBG; free testosterone. |
| Onalan et al., 2005; Turkey[42] | 24 | RCT parallel | 55; NR | 61; NR | Metformin 500 mg orally once daily for 5 days and 850 mg twice daily for 6 months | Placebo similar in appearance to metformin | NR | NR | Rotterdam criteria | Androstenedione; DHEAS; SHBG; free testosterone. |
| Palomba et al., 2007; Italy[43] | 56 | RCT parallel | 14; 24.3 ± 3.1 | 13; 24.8 ± 2.7 | Metformin 850 mg orally twice a day | Placebo similar in appearance to metformin | 22.4 ± 2.7 | 22.7 ± 1.9 | Rotterdam criteria | Androstenedione; DHEAS; FAI; SHBG; total testosterone. |
| Romualdi et al., 2010; Italy[44] | 24 | RCT parallel | 13; 24.7 ± 4.4 | 10; 27.2 ± 2.6 | Metformin 500 mg orally twice a day | Placebo similar in appearance to metformin | 22.2 ± 2.2 | 22.3 ±3.9 | Rotterdam criteria | Androstenedione; FAI; DHEAS; SHBG; total testosterone. |
| Tang et al., 2006; United Kingdom[45] | 24 | RCT parallel | 56; 29.7 ± 3.7 | 66; 29.8 ± 3.8 | Metformin 500 mg orally twice a day | Placebo similar in appearance to metformin | 37.6 ± 5.0 | 38.9 ± 9.5 | Rotterdam criteria | FAI; SHBG; total testosterone. |

| Author, year of publication; Country. | Follow-up duration (weeks) | Study design | No. of participants; age (years; mean ± standard deviation) | | Characteristics of groups | | Body Mass Index (BMI) at the beginning of the follow-up (kg/m$^2$) | | Diagnostic criteria for PCOS | Outcome of interest |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Intervention | Control | Intervention | Control | Intervention | Control | | |
| Trolle et al., 2010; Denmark[46] | 24 | RCT parallel | 37; NR | 37; NR | Metformin 500 mg orally twice a day | Placebo similar in appearance to metformin | NR | | Rotterdam criteria | SHBG; total testosterone. |
| Trolle et al., 2007; Denmark[47] | 24 | RCT cross-over | 42; 32 ± 4.7 | 45; 32 ± 4.9 | Metformin 850 mg orally twice a day | Placebo similar in appearance to metformin | 33.8 ± 3.1 | | Rotterdam criteria | SHBG; total testosterone. |
| Yarali et al., 2002; Turkey[48] | 6 | RCT parallel | 16; 29.7 ± 5.6 | 16; 28.4 ± 5.1 | Metformin 850 mg orally twice a day | Placebo similar in appearance to metformin | 28.6 ± 4.0 | 28.6 ± 4.0 | WHO criteria | Androstenedione; DHEAS; free testosterone; total testosterone. |

FAI: Free Androgen Index; DHEAS: dehydroepiandrosterone; NICHHD: National Institute of Child Health and Human Development; NR: not reported; SHBG: Sex Hormone-Binding Globulin; PCOS: Polycystic Ovary Syndrome; WHO: World Health Organization.

**Figure 1.** Flow-diagram of the literature search and the study selection proess according to the PRISMA guidelines.

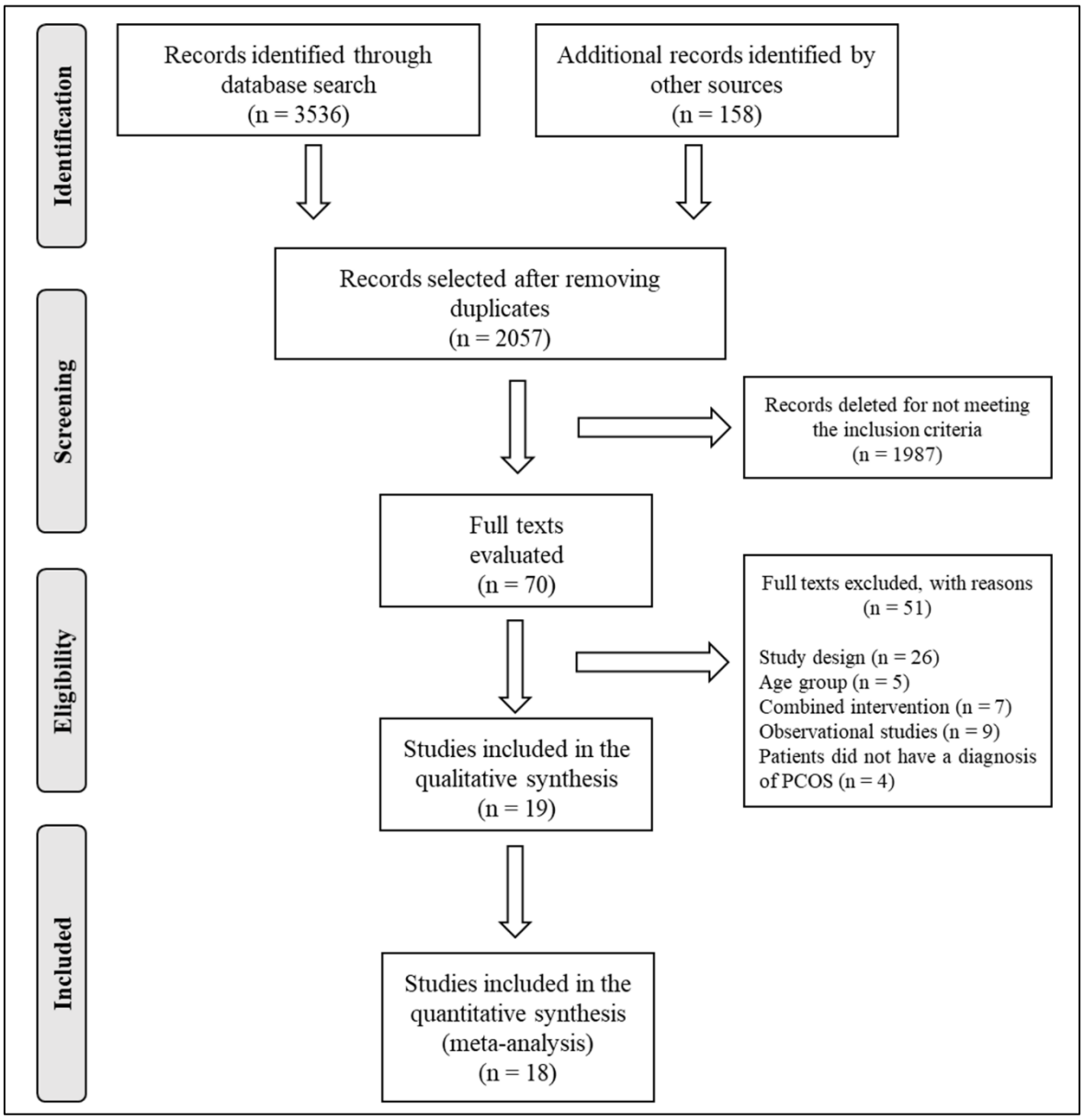


**Fig. 2 (a).** Assessment of mean androstenedione levels between the metformin and placebo treatment groups.

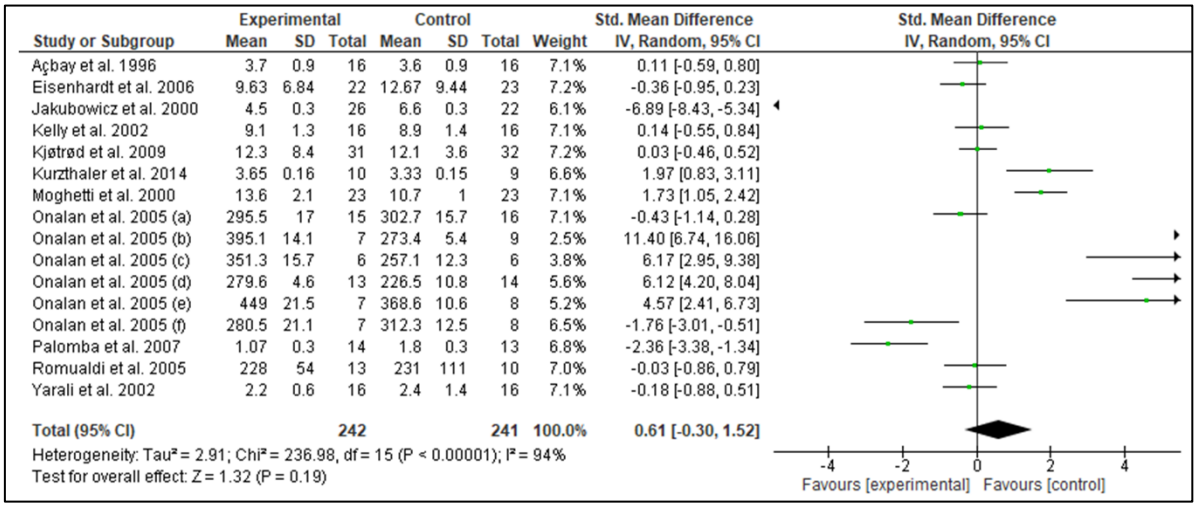



**Fig. 2 (b).** Assessment of mean DHEAS levels between the metformin and placebo treatment groups.

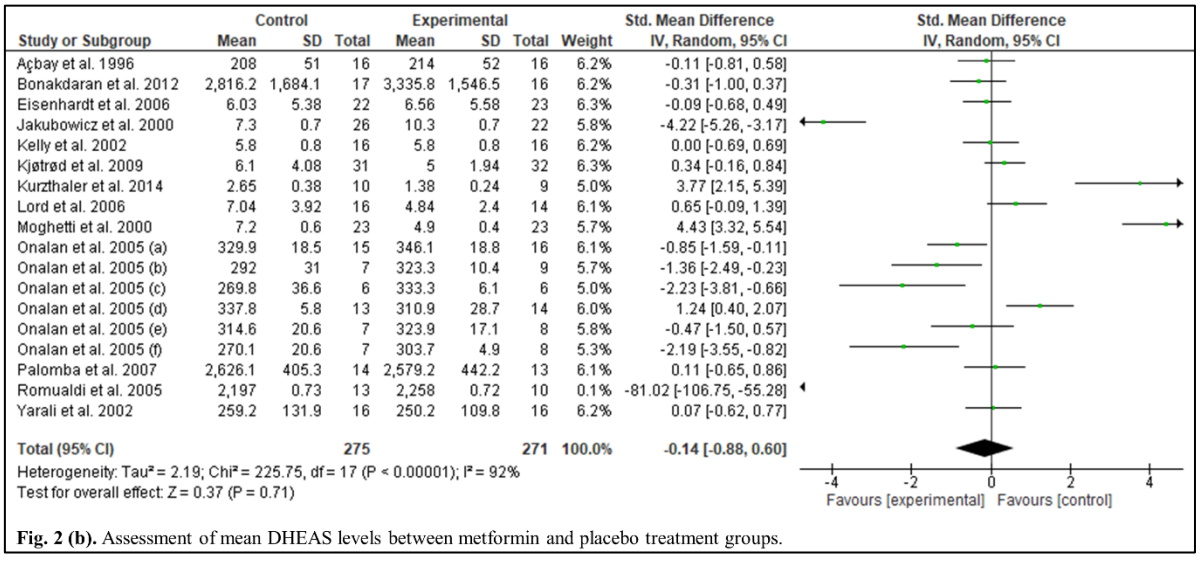



**Fig. 2 (b).** Assessment of mean DHEAS levels between metformin and placebo treatment groups.

**Fig. 2 (c).** Assessment of mean FAI values between the metformin and placebo treatment groups.

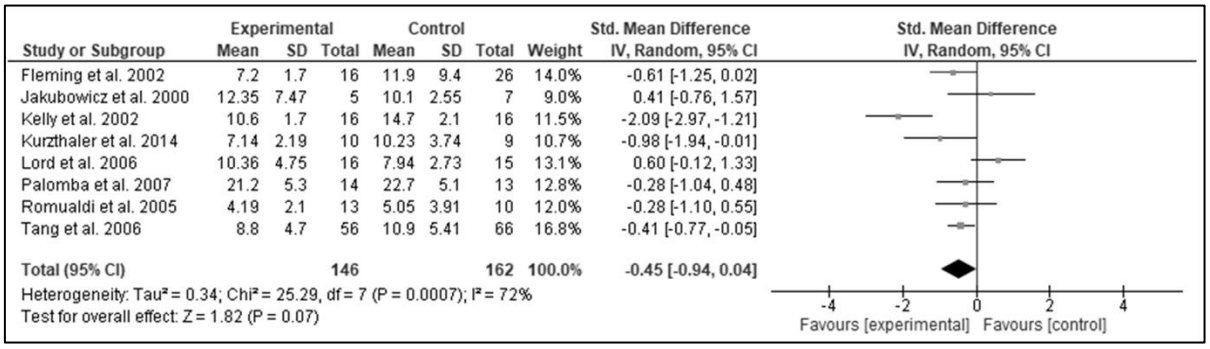



**Fig. 2 (d).** Assessment of mean SHBG levels between the metformin and placebo treatment groups.

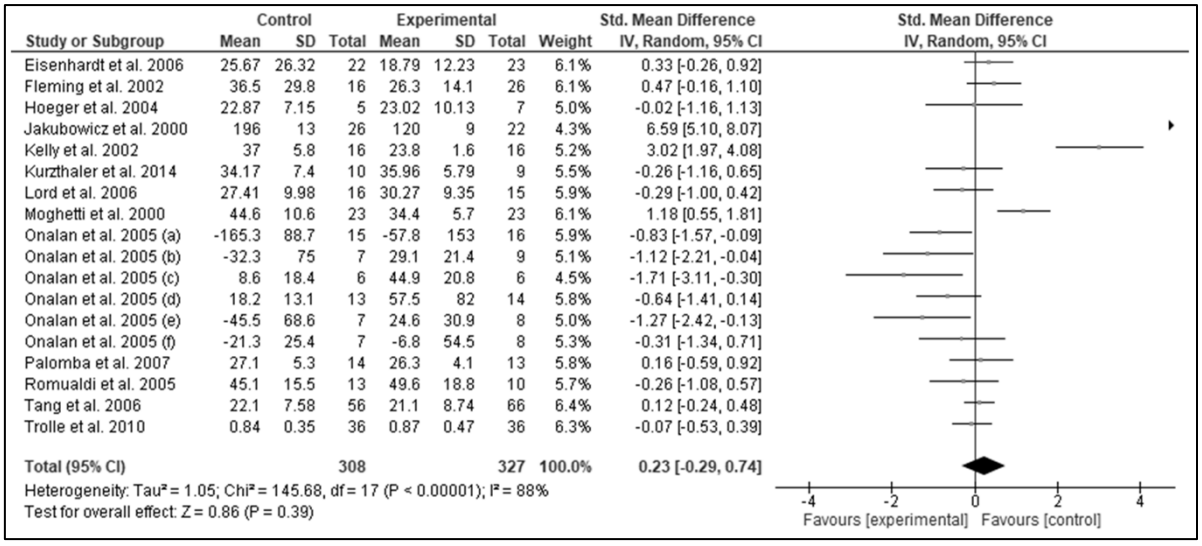


**Fig. 2 (e).** Assessment of mean free testosterone levels between the metformin and placebo treatment groups.

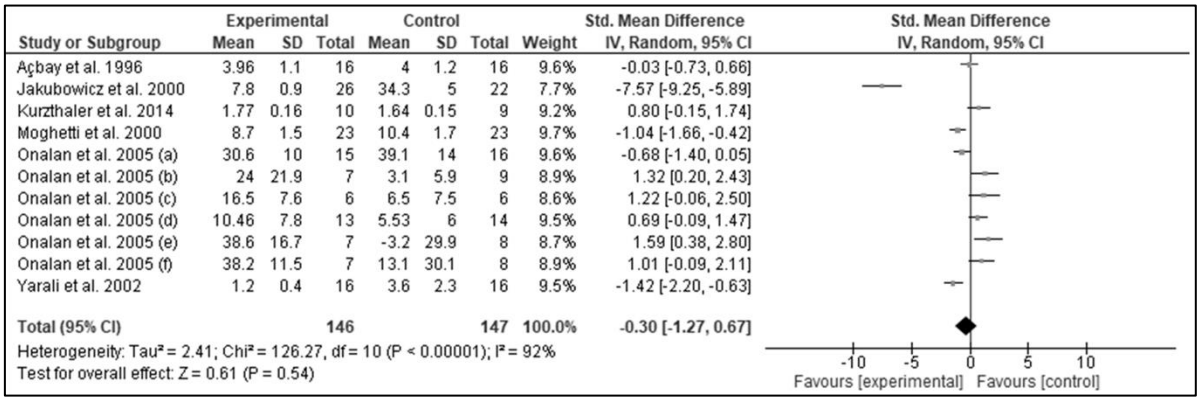



**Fig. 2 (f).** Assessment of mean total testosterone levels between the metformin and placebo treatment groups.

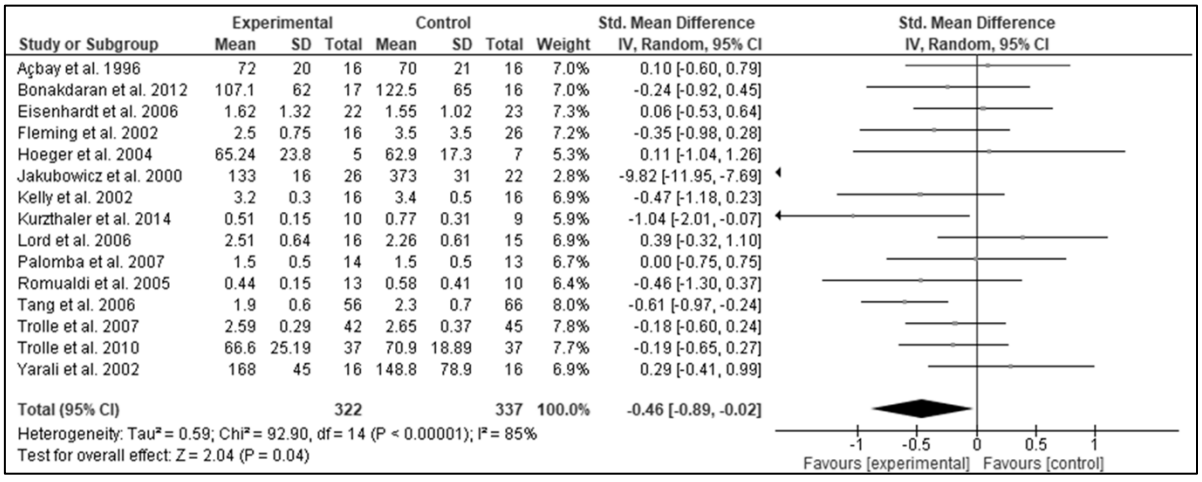
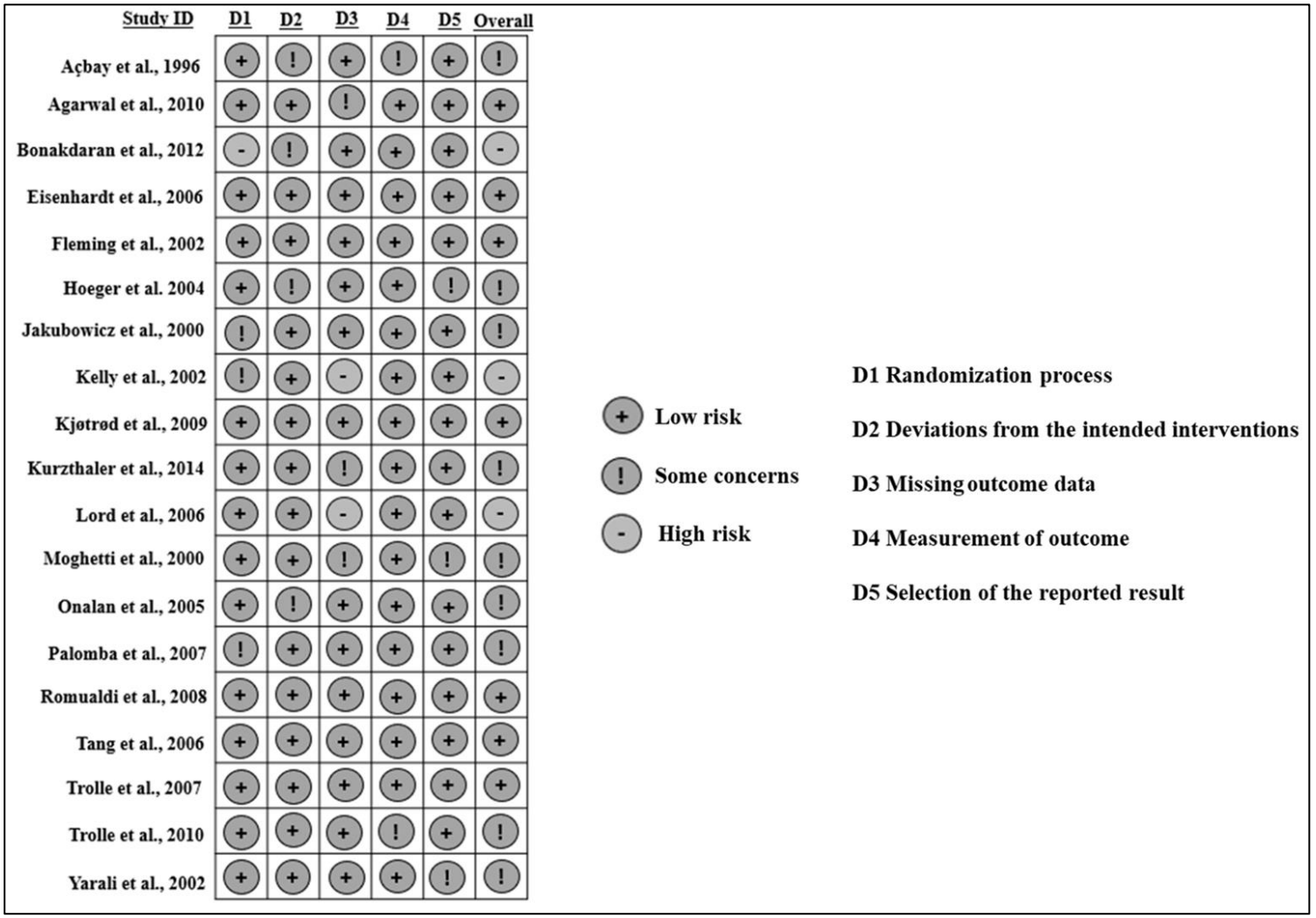


**Fig. 4.** Risk of Bias Assessment Chart – Cochrane Collaboration.



**Appendix S1.** Complete search strategy carried out in the Medical Literature Analysis and Retrieve System Online database (MEDLINE) via Pubmed.

| |
|---|
| ((("Polycystic Ovary Syndrome"[Mesh]) OR (Polycystic Ovary Syndrome[Text Word] OR Ovary Syndrome, Polycystic[Text Word] OR Syndrome, Polycystic Ovary[Text Word] OR Stein-Leventhal Syndrome[Text Word] OR Stein Leventhal Syndrome[Text Word] OR Syndrome, Stein-Leventhal[Text Word] OR Sclerocystic Ovarian Degeneration[Text Word] OR Ovarian Degeneration, Sclerocystic[Text Word] OR Sclerocystic Ovary Syndrome[Text Word] OR Polycystic Ovarian Syndrome[Text Word] OR Ovarian Syndrome, Polycystic[Text Word] OR Polycystic Ovary Syndrome 1[Text Word] OR Sclerocystic Ovaries[Text Word] OR Ovary, Sclerocystic[Text Word] OR Sclerocystic Ovary[Text Word])) AND (("Metformin"[Mesh]) OR (Metformin[Text Word] OR Dimethylbiguanidine[Text Word] OR Dimethylguanylguanidine[Text Word] OR Glucophage[Text Word] OR Metformin Hydrochloride[Text Word] OR Hydrochloride, Metformin[Text Word] OR Metformin HCl[Text Word] OR HCl, Metformin[Text Word]))) AND ((("Hyperandrogenism"[Mesh]) OR (Hyperandrogenism[Text Word])) OR (("Androgens"[Mesh]) OR (Androgens[Text Word] OR Androgen Receptor Agonist[Text Word] OR Agonist, Androgen Receptor[Text Word] OR Receptor Agonist, Androgen[Text Word] OR Androgenic Compounds[Text Word] OR Compounds, Androgenic[Text Word] OR Androgen Receptor Agonists[Text Word] OR Agonists, Androgen Receptor[Text Word] OR Receptor Agonists, Androgen[Text Word] OR Androgenic Agents[Text Word] OR Agents, Androgenic[Text Word] OR Androgen[Text Word] OR Androgen Effect[Text Word] OR Effect, Androgen[Text Word] OR Androgen Effects[Text Word] OR Effects, Androgen[Text Word]))) |

**Appendix S2.** Graphical analysis of publication bias.

**Fig. 3 (a).** Publication bias analysis for the androstenedione outcome – funnel plot.

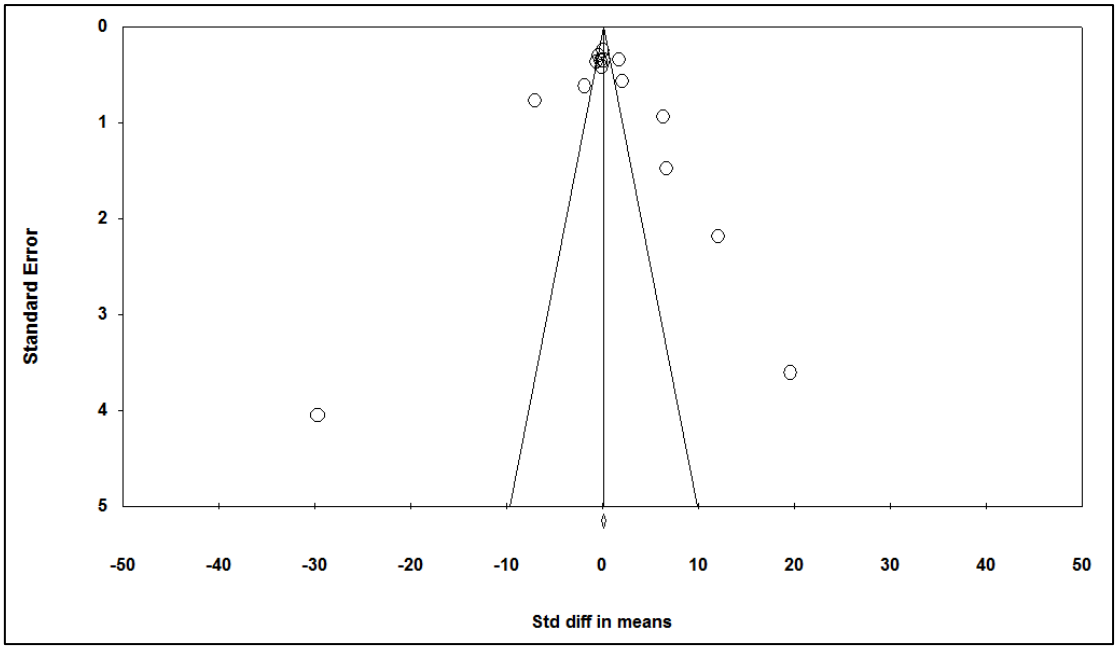



**Fig. 3 (b).** Publication bias analysis for the DHEAS outcome – funnel plot.

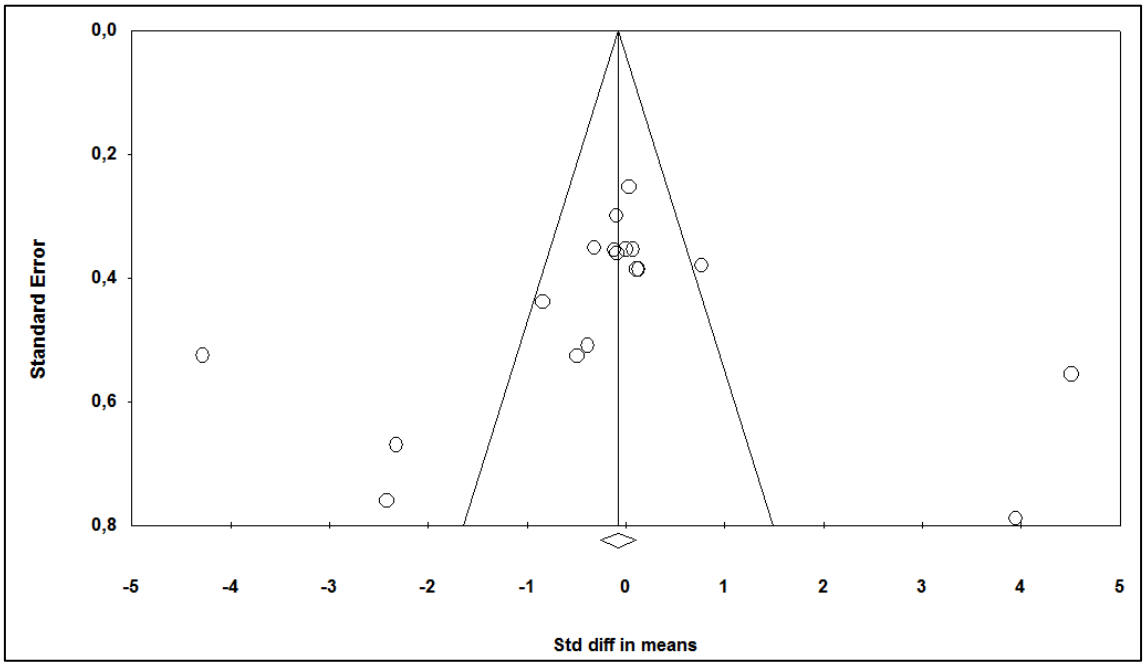


**Fig. 3 (c).** Publication bias analysis for the SHBG outcome – funnel plot.

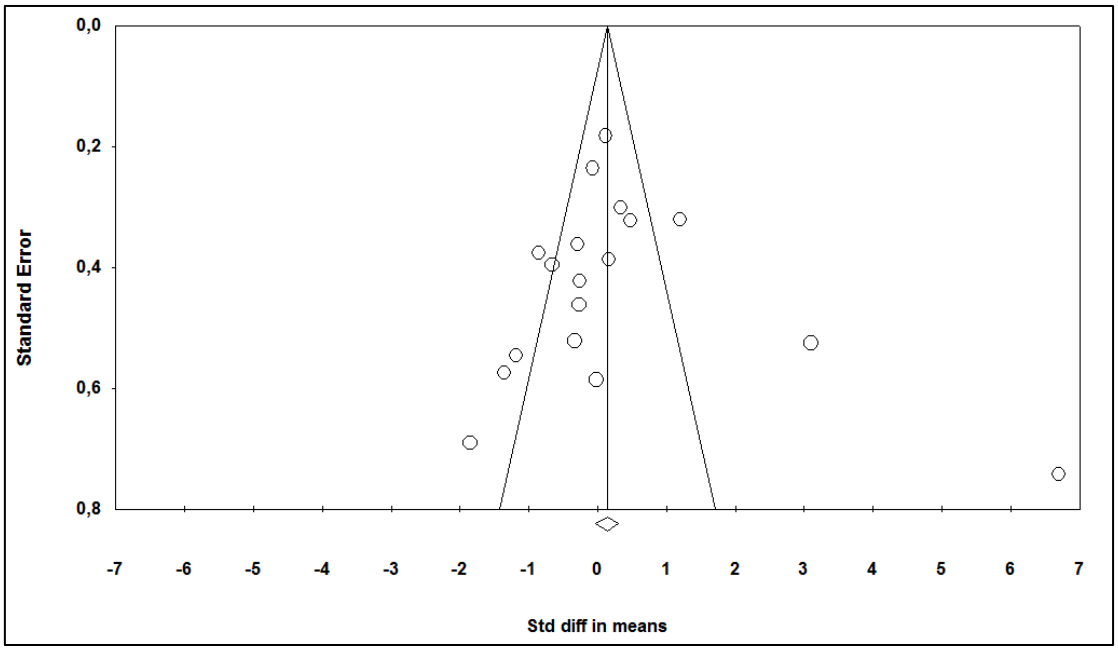



**Fig. 3 (d).** Publication bias analysis for the free testosterone outcome – funnel plot.

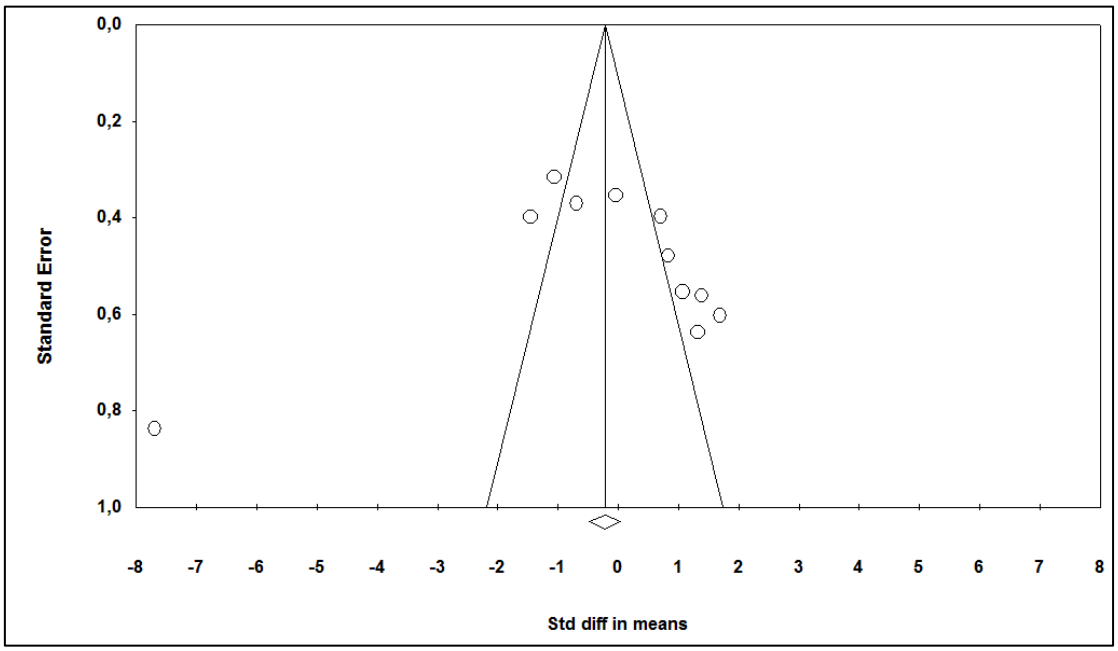
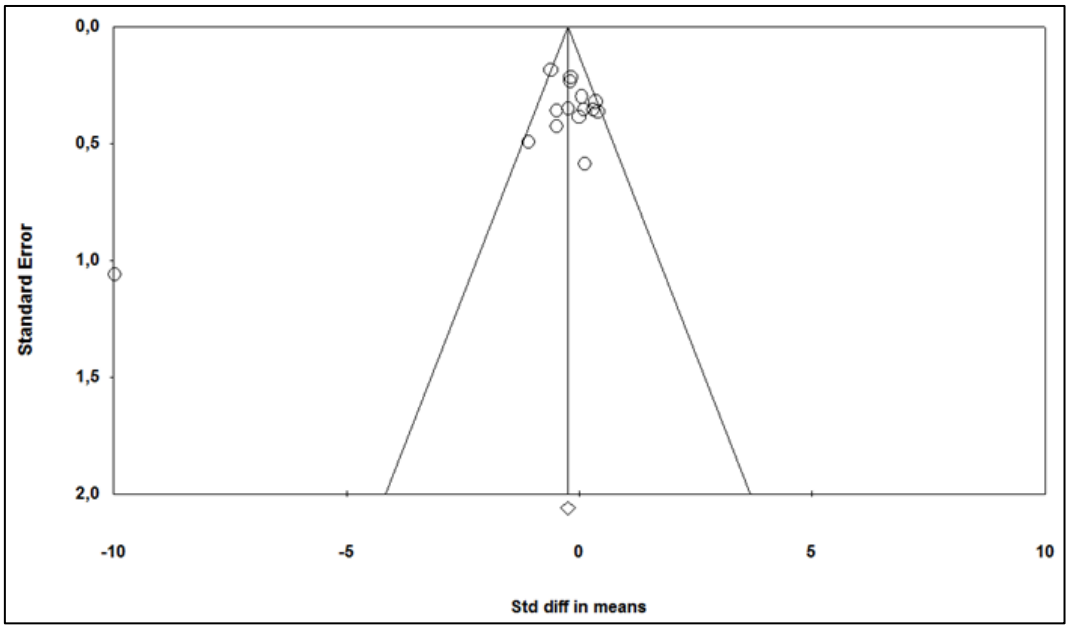


**Fig. 3 (e).** Publication bias analysis for the total testosterone outcome – funnel plot.



**Appendix S3.** Sensitivity analysis

**Fig 4 (a).** Assessment of mean total FAI values between the metformin and placebo treatment groups, after sensitivity analysis.

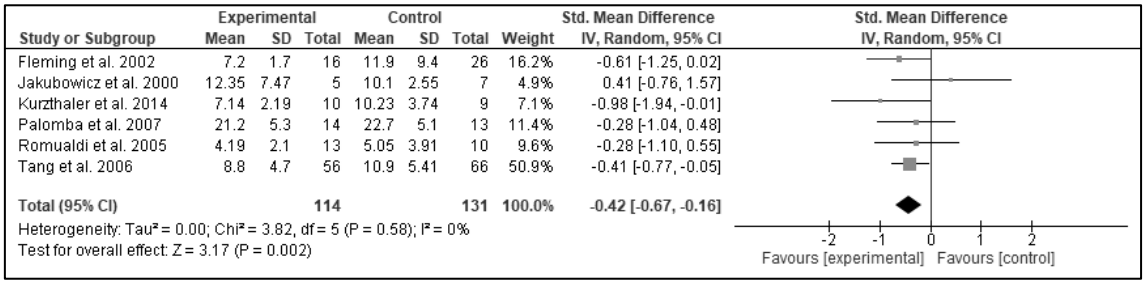
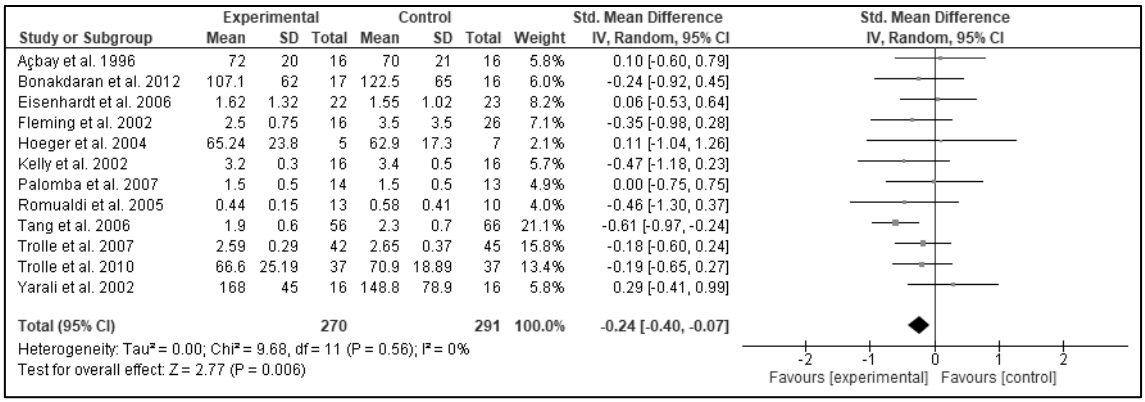



**Fig 4 (b).** Evaluation of mean total testosterone levels between the metformin and placebo treatment groups, after sensitivity analysis.



**Appendix S4.** Assessment of the quality of evidence for each outcome of the systematic review - GRADE System.

| ASSURENCE ASSESSMENT | | | | | | | NUMBER OF PATIENTS | | ABSOLUTE EFFECT | ASSURANCE | IMPORTANCE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Number of studies** | **Study design** | **Risk of bias** | **Inconsistency** | **Indirect evidence** | **Inaccuracy** | **Other considerations** | **Metformin** | **Placebo** | | | |
| **Androstenedione** | | | | | | | | | | | |
| 16 | RCT | Not serious | Not serious | Not serious | Not serious | Strong association | 242 | 241 | SMD: 0.61 [-0.30, 1.52] | ⊕⊕⊕ Moderate | Important |
| **Dehydroepiandrosterone Sulfate (DHEAS)** | | | | | | | | | | | |
| 18 | RCT | Not serious | Not serious | Not serious | Not serious | Strong association | 275 | 271 | SMD: -0.14 [-0.88, 0.60] | ⊕⊕⊕ Moderate | Important |
| **Free Androgen Index - FAI** | | | | | | | | | | | |
| 8 | RCT | Not serious | Not serious | Not serious | Not serious | Strong association | 146 | 162 | SMD: -0.45 [-0.94, 0.04] | ⊕⊕⊕ Moderate | Important |
| **Sex Hormone Binding Globulin (SHBG)** | | | | | | | | | | | |
| 18 | RCT | Not serious | Not serious | Not serious | Not serious | Strong association | 308 | 327 | SMD: 0.23 [-0.29, 0.74] | ⊕⊕⊕ Moderate | Important |
| **Free Testosterone** | | | | | | | | | | | |
| 11 | RCT | Not serious | Not serious | Not serious | Not serious | Strong association | 146 | 147 | SMD: -0.30 [-1.27, 0.67] | ⊕⊕⊕ Moderate | Important |
| **Total Testosterone** | | | | | | | | | | | |
| 15 | RCT | Not serious | Not serious | Not serious | Not serious | Strong association | 322 | 337 | SMD: -0.46 [-0.89, -0.02] | ⊕⊕⊕ Moderate | Important |

6.4 Estudo IV. Effects of resveratrol supplementation on the cognitive function of patients diagnosed with Alzheimer's Disease: a systematic review of randomized controlled trials.

Drugs & Aging
https://doi.org/10.1007/s40266-022-00923-4

SYSTEMATIC REVIEW

# Effects of Resveratrol Supplementation on the Cognitive Function of Patients with Alzheimer's Disease: A Systematic Review of Randomized Controlled Trials

Jéssica Abdo Gonçalves Tosatti[1] · Adriana Fialho da Silva Fontes[1] · Paulo Caramelli[2] · Karina Braga Gomes[1]

Accepted: 2 February 2022


**Abstract**

**Background** Alzheimer's disease (AD) comprises 60–70% of diagnosed dementia cases, and is characterized by the deposition of β-amyloid peptide and the formation of neurofibrillary tangles of tau protein. Resveratrol is a neuroprotective agent acting in the prevention of redox impairment in addition to exerting anti-apoptotic actions on brain cells. An ability to reduce neuronal damage in patients with AD has been suggested by preclinical studies.

**Objectives** The aim of this systematic review was to investigate the evidence in the published literature from studies that evaluated the effects of supplementation with resveratrol, alone or in a solution with glucose and malate (RGM), on the functional and cognitive performance of patients with AD, as assessed by validated instruments.

**Methods** A systematic literature search was performed in MEDLINE, CENTRAL, Embase, CINAHL, Web of Science, and Scopus databases including articles published up to August 2021. Randomized, placebo-controlled, clinical trials that reported cognitive and functional performance, as measured by the Alzheimer's Disease Assessment Scale—Cognitive Subscale (ADAS-cog), Cooperative Study of Alzheimer's Disease—Activities of Daily Living (ADCS-ADL), or the Mini Mental State Examination (MMSE), in AD patients treated with resveratrol, alone or as RGM, were included.

**Results** After 1855 studies were identified, 24 RCTs underwent full-text review, with 20 studies excluded because they did not meet the inclusion criteria. Thus, four RCTs were included in the qualitative analyses. The findings demonstrate that there are still few studies in humans, but they showed that this polyphenol acts in the delay of cognitive impairment in patients with AD, when administered alone or in combination with glucose and malate.

**Conclusions** Supplementation with resveratrol seems to influence the progressive cognitive and functional decline in AD patients, when compared with a placebo group.

**Systematic review registration** PROSPERO CRD42021229234.

## 1 Introduction

Dementia is a neurocognitive disorder characterized by impairment in cognitive or behavioral domains leading to significant functional decline [1, 2]. Among the main causes of dementia, Alzheimer's disease (AD) comprises between 60 and 70% of diagnosed cases [1, 3]. The most common pathophysiological characteristics are the deposition of amyloid β-peptide (Aβ) and the synthesis of neurofibrillary tangles (NFT) of tau protein [4]. In addition, chronic neuronal inflammation and an increase in pro-inflammatory mediators in the cerebral parenchyma can also be identified [5].

Resveratrol, a polyphenolic phytoalexin of the stilbene family, found in berries, wines, peanuts, and grapes, has a chemical structure comprising two phenolic rings linked by a double bond of styrene to produce 3,4′,5-trihydroxystilbene, which occurs in the trans and cis isoforms [6, 7]. The exposition to ultraviolet radiation and heat can transform the resveratrol trans isoform into the cis isoform, whose structure is similar to that of synthetic estrogen diethylstilbestrol [8]. Resveratrol has been considered a neuroprotective agent,

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**Key Points**

Resveratrol is considered to be a neuroprotective agent, being powerful in avoiding redox impairment in brain cells, in addition to exerting anti-apoptotic actions.

There are still few studies in humans, but they are promising regarding the role of this polyphenol in the delay of cognitive impairment in patients with AD, alone or in combination with glucose and malate.

It is noteworthy that supplementation with resveratrol seems to influence the progressive cognitive and functional decline in AD patients, when compared with a placebo group.

being powerful in avoiding redox impairment in brain cells, in addition to exerting anti-apoptotic actions [9]. Because it can cross the blood–brain barrier, resveratrol can modulate memory and learning skills in animals [10, 11], as well as depression in experimental models [12, 13], exerting beneficial effects on neuronal and glial cells [13].

Some reviews [14–16] have assessed the effects of resveratrol on cognitive and memory aspects, under supplementation with resveratrol alone or source foods such as wine and grapes. The findings are controversial, and they did not specifically evaluate patients with AD. Farzaei et al. did not observe impacts on memory and cognitive performance in different clinical groups evaluated by auditory verbal learning tests [14]. Khorshidi et al. discussed a promising effect of resveratrol in animal models, but not in human clinical trials [15]. Marx et al. assessed clinical trial data about the effect of resveratrol supplementation on mood and cognitive performance in healthy populations and showed that resveratrol supplementation can improve some measures of cognitive performance [16]. In addition, a systematic review of preclinical studies suggested the neuroprotective effects of resveratrol in AD animal models [17]. Other studies point out the ability of resveratrol to reduce neuronal damage with a focus on cell cultures and animals [7, 17, 18]. However, these studies did not evaluate the effect of resveratrol on cognitive impairment in patients with AD.

Therefore, we hypothesized that supplementation with resveratrol, isolated or in a compound, could increase cognitive performance in patients with AD. Our aim was to systematically review the literature to evaluate the effects of supplementation with resveratrol, isolated or in a compound, on cognition and functionality in patients with AD based on validated neurocognitive tests and compared with a placebo group.

## 2 Methods

### 2.1 Protocol and Registration

This systematic review of intervention studies was developed in accordance with the precepts determined by the Cochrane Handbook for Systematic Reviews of Interventions (version 6.2) [19] and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guideline (PRISMA) [20]. The protocol was published in the International Prospective Register of Systematic Reviews (PROSPERO), under the identification CRD42021229234.

### 2.2 Search Strategy

The acronym PICOS (participants, intervention, control, outcome, and study design) was applied to structure the search strategies constructed, based on the research question proposed by the study. Therefore, the acronym was composed of the following: P = adults diagnosed with AD; I = resveratrol supplementation; C = placebo; O = assessment of cognitive and functional performance; and S = placebo-controlled randomized clinical trials (RCTs). Searches were performed for primary studies in the electronic databases Cochrane Central Register of Controlled Trials (CENTRAL), Cumulative Index to Nursing and Allied Health Literature (CINAHL), Embase, Medical Literature Analysis and Retrieve System Online (MEDLINE) via PubMed, Scopus and Web of Science (WOS), until August 2021, with the aim of identifying only placebo-controlled RCTs that reported the effect of resveratrol supplementation on cognitive function in adults diagnosed with AD. The search included the Medical Subject Heading (MeSH) terms 'Alzheimer disease' and 'resveratrol' and their respective entry terms using the Boolean operator tools. In order to direct the search to the proposed study design, the high-sensitivity search strategy for RCTs, developed by the Cochrane Collaboration, was added [21]. Online Resource 1 presents the search strategy performed in the PubMed database (see electronic supplementary material [ESM]).

Likewise, the same MeSH terms applied to the main search were used in the search for grey literature in the Open Grey database (http://www.opengrey.eu/search/), for clinical studies and protocols in the National Institutes of Health databases (http://www.clinicaltrials.gov), in the Turning Research into Practice database (http://www.tripdatabase.com), and in the Brazilian Registry of Clinical Trials (ReBEC) (http://www.ensaiosclinicos.gov.br); and for theses and dissertations in the Open Access Theses and Dissertations (https://oatd.org/). Besides, a manual search was also carried out on the reference lists of relevant reviews [7,

22–24]. All potentially eligible studies were considered for review, regardless of date of publication and language.

### 2.3 Inclusion and Exclusion Criteria

We included only placebo-controlled RCTs with no follow-up duration limit. Studies that accessed the effects of resveratrol supplementation, isolated or in combination with other compounds, on cognitive and functional performance, measured using cognitive function assessment instruments, in patients diagnosed with AD were analyzed. The instruments applied to assess the cognitive function of patients with AD in the included studies were restricted to (i) Alzheimer's Disease Assessment Scale—Cognitive Subscale (ADAS-cog); (ii) Alzheimer's Disease Cooperative Study—Activities of Daily Living (ADCS-ADL); or (iii) Mini-Mental State Examination (MMSE).

Considered the gold standard in evaluating the effectiveness of treatments, the ADAS-cog considers changes in mood and behavior and includes 11 items that involve both observer-based assessments and subject-completed tests. Higher scores indicate worse performance [25]. The ADCS-ADL was built specifically for use with AD patients, with a 23-item scale that provides a total score of between 0 and 78, with a lower score being related to greater severity [26]. The MMSE is a 30-question assessment of cognitive function that assesses attention and orientation, recording, memory, calculation, language, and drawing ability. The presence of cognitive decline is decided by the total score, and a lower score is associated with greater severity [27].

The exclusion criteria comprised studies that did not report the outcome of interest, non-placebo-controlled and non-randomized studies, as well as review articles, conference proceedings, case reports, and commentary studies. Studies that included undiagnosed patients with AD, experimental studies, as well as observational design studies were also excluded.

### 2.4 Study Selection, Data-Collection Process, and Data Items

The study selection stage included the union of all exported files, resulting from electronic databases, grey literature, and manual searching, into a single electronic file that was imported to the Mendeley® reference manager, desktop version, to remove duplicates. Two researchers (JAGT and AFSF) first evaluated the titles and abstracts of all primary studies and then read the full texts for relevant studies. A third researcher (KBG or PC) resolved disagreements in both steps. The entire study selection stage was performed using a standardized assessment tool, hosted in Microsoft Excel® software, version 365, to facilitate access for all researchers involved in the development of the systematic review.

The extraction of data from primary studies was performed by two researchers (JAGT and AFSF) in order to resolve any collection errors. They obtained data about the authors of the studies, country and year of publication, duration of follow-up, the total number of included participants, and by the group—intervention and placebo (Online Resource 2, see ESM). Data were also collected on supplementation with resveratrol, alone or in combination, related to the dosage and frequency of supplementation and the scores of the analyzed outcomes (ADAS-cog, ADCS-ADL, and MMSE) at the beginning and the end of the follow-up. For the studies of Moussa et al. [28] and Turner et al. [29], it was necessary to contact the authors for more information about the evaluated scores, but no information was obtained. Therefore, some graphic values were estimated using Web Plot Digitizer® software, version 4.5 (https://apps.automeris.io/wpd/).

### 2.5 Risk of Bias Within and Across Studies

Two investigators (JAGT and AFSF) independently assessed the risk of bias of the studies included in the systematic review based on that indicated by the Cochrane Handbook for Systematic Reviews of Interventions (version 6.2) [30], and a third reviewer (KBG or PC) resolved the disparities. Therefore, the Cochrane Risk of Bias for Randomized Trials—Rob 2.0 [31] bias assessment tool was applied, which is structured into five domains: (1) bias due to the randomization process; (2) bias due to deviations from the intended interventions; (3) bias due to the lack of result data; (4) bias in measuring the result and; (5) bias in selecting the reported result. The answer options for signaling questions are: (1) Yes; (2) Probably yes; (3) Probably not; (4) No, and (5) No information. In the end, three grades (high, with considerations, and low) were attributed to all domains, based on an algorithm of the tool, and to each of the included studies [31].

## 3 Results

Searches in electronic databases and manual searches identified 1855 studies. After removing duplicates, we evaluated titles and abstracts from 709 studies, 685 of which were excluded for not meeting the inclusion criteria. Twenty-four RCTs were selected for full-text review, with 20 studies excluded because they did not meet the inclusion criteria. Thus, four RCTs [28, 29, 32, 33] were included in the qualitative analyses. Fig. 1 presents the PRISMA flowchart of the systematic search used to identify eligible studies for inclusion in this systematic review.

The characteristics of the included studies are summarized in Table 1. All studies had parallel RCT design and

were conducted with volunteers residing in the United States of America (USA), with recruitment carried out in centers specializing in AD. The study populations consisted of older adults (mean age between 69.8 and 80.5 years) with a higher proportion of women (52.0%), when considering the total of patients included. The diagnosis of AD, in three studies [28, 29, 33], was based on criteria determined by the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), with MMSE scores between 12 and 26 points also considered as inclusion criteria for the trials. For the study of Blass and Gibson [32], diagnostic criteria included complete medical and neurological history and physical examination, brain imaging, and clinical laboratory testing [32].

The number of patients included in the systematic review, based on the selected studies, was 241 patients diagnosed with AD, with 117 patients allocated to the placebo group with a mean age of 75.4 ± 5.2 years, and 124 patients in the intervention group (with resveratrol isolated [28, 29] or RGM [32, 33]) with a mean age of 74.8 ± 6.1 years. All included studies had a minimum follow-up duration of 12 weeks [32] and a maximum duration of 52 weeks [28, 29, 33]. The main results observed in the primary studies are summarized in Table 2. The included studies were grouped according to intervention for the narrative review (resveratrol isolated or resveratrol in combination) and to the assessed outcome (ADAS-cog; ADCS-ADL; and MMSE).

### 3.1 Interventions with Resveratrol Alone

Two studies [28, 29] evaluated the effects of supplementation with resveratrol alone on instrument scores to assess cognitive and functional performance in patients with AD when compared with the placebo group. In total, 157 patients were evaluated, with 83 allocated to the intervention group with resveratrol (mean age 69.8 years) and 74 patients allocated to the placebo group (mean age 73.0 years).

One study [28] investigated two of the evaluated outcomes—ADCS-ADL and MMSE—and the other study [29] determined the scores for all three instruments—ADAS-cog,

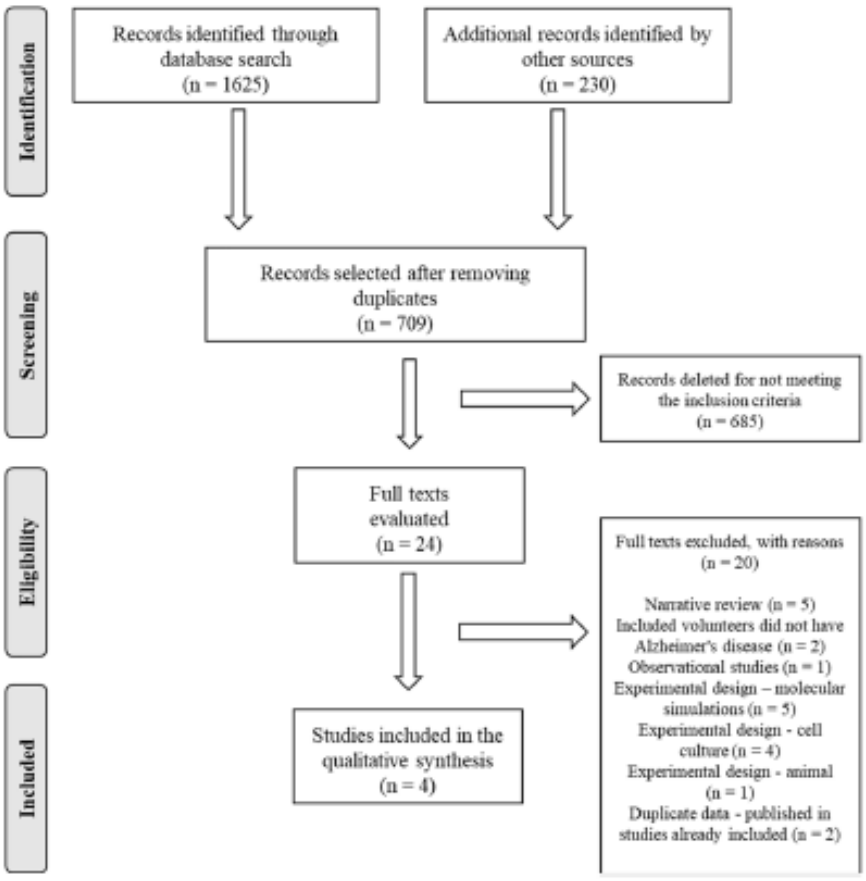


**Fig. 1** Flowchart of the literature search and the study selection process according to the PRISMA guidelines

**Table 1** Descriptive characteristics of the studies that investigated the scores of tests to assess cognition in patients with Alzheimer's disease when supplemented with resveratrol, alone or in combination with glucose and malate (RGM)

| Author, year | Country | Study design | Population baseline characteristics | Diagnosis criteria for AD | No. of patients; age (years; mean ± standard deviation) | | Follow-up (weeks) | Intervention characteristics | Placebo characteristics | Population endpoint characteristics |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Intervention | Placebo | | | | |
| Blass and Gibson, 2006 [32] | USA | RCT parallel | ADAS-cog score<br>IG: 22.0 ± 3.0<br>PG: 23.0 ± 2.0<br>MMSE score:<br>IG: 20.0 ± 1.0<br>PG: 19.0 ± 0.8 | A complete medical and neurological history and physical examination, brain imaging, and clinical laboratory studies | ADAS-cog: 20<br>MMSE: 25; 74.0 ± 2.0 | ADAS-cog: 29<br>MMSE: 30; 74.0 ± 1.0 | 12 | RGM (resveratrol: 5 mg, glucose: 5 g, malate: 5 g) administered twice daily in 15 mL liquid form, dissolved in commercial grape juice | Composed of sucralose and lemon juice and indistinguishable by color or flavor from the active preparation | ADAS-cog score difference:<br>IG: + 0.0 ± 1.0<br>PG: + 3.69 ± 1.1<br>MMSE score difference:<br>IG: + 1.1 ± 0.7<br>PG: − 0.9 ± 0.4 |
| Moussa et al., 2017 [28] | USA | RCT parallel | ADCS-ADL score:<br>IG: 61.2 ± 3.3<br>PG: 65.0 ± 1.7<br>MMSE score:<br>IG: 19.3 ± 1.3<br>PG: 19.4 ± 1.0 | NINCDS/ADRDA | 19; NR | 19; NR | 52 | Resveratrol 500 mg orally once daily (with a dose increase in increments of 500 mg every 13 weeks, ending with 1000 mg twice daily) | The type of placebo used was not reported, but an identical placebo was provided, in accordance with current GMP guidelines | ADCS-ADL score:<br>IG: 53.2 ± 3.7<br>PG: 51.7 ± 2.6<br>MMSE score:<br>IG: 17.4 ± 1.4<br>PG: 16.6 ± 1.3 |
| Turner et al., 2015 [29] | USA | RCT parallel | ADAS-cog score<br>IG: 25.3 ± 10.1<br>PG: 23.7 ± 8.6<br>ADCS-ADL score:<br>IG: 63.7 ± 10.8<br>PG: 60.5 ± 10.7<br>MMSE score:<br>IG: 20.2 ± 4.4<br>PG: 20.7 ± 4.3 | NINCDS/ADRDA | 64; 69.8 ± 7.7 | 55; 73.0 ± 8.2 | 52 | Resveratrol 500 mg orally once daily (with a dose increase in increments of 500 mg every 13 weeks, ending with 1000 mg twice daily) | An identical placebo was provided, in accordance with current GMP guidelines | ADAS-cog score<br>IG vs PG: NSE<br>ADCS-ADL score:<br>IG: 57.4 ± 12.3<br>PG: 51.3 ± 14.5<br>MMSE score:<br>IG vs PG: NSE |
| Zhu et al., 2018 [33] | USA | RCT parallel | ADAS-cog score<br>IG: 26.4 ± 11.9<br>PG: 29.2 ± 8.9<br>ADCS-ADL score:<br>IG: 49.1 ± 10.3<br>PG: 46.6 ± 7.6<br>MMSE score:<br>IG: 18.1 ± 4.9<br>PG: 19.4 ± 3.8 | NINCDS/ADRDA | 16; 80.5 ± 8.6 | 13; 79.3 ± 6.5 | 52 | RGM (resveratrol: 5 mg, glucose: 5 g, malate: 5 g) administered twice daily in 15 mL liquid form, dissolved in commercial grape juice | Composed of sucralose and lemon juice and indistinguishable by color or flavor from the active preparation | ADAS-cog score<br>IG: 29.9 ± 14.1<br>PG: 33.2 ± 18.6<br>ADCS-ADL score:<br>IG: 49.6 ± 10.5<br>PG: 40.5 ± 10.5<br>MMSE score:<br>IG: 16.9 ± 7.7<br>PG: 15.4 ± 6.5 |

*AD* Alzheimer's disease, *ADAS-cog* Alzheimer's Disease Assessment Scale—Cognitive Subscale, *ADCS-ADL* Alzheimer's Disease Cooperative Study—Activities of Daily Living, *GMP* Good Manufacturing Practice, *IG* Intervention group, *MMSE* Mini Mental State Examination, *NINCDS/ADRDA* National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association, *NR* not reported, *NSE* no significant effects, *PG* placebo group, *RCT* randomized clinical trial, *RGM* composed of malate, glucose, and resveratrol, *USA* United States of America

ADCS-ADL, and MMSE. The intervention comprised resveratrol with an initial dose of 500 mg once a day, increasing in increments of 500 mg every 13 weeks, ending at 1000 mg twice a day. The placebo capsule had the same characteristics as the resveratrol capsule, in accordance with Good Manufacturing Practices.

### 3.2 Interventions with Resveratrol in Combination

Two other studies [32, 33] evaluated the effects of supplementation with resveratrol, glucose, and malate on cognitive function assessment scores in AD patients when compared with a placebo control group. Thus, 84 patients were evaluated, 41 of whom were allocated to the intervention group (mean age 77.3 years) and 43 patients allocated to the placebo group (mean age 76.6 years).

One study [32] investigated the effects of RGM on the scores of two instruments—ADAS-cog and MMSE—and the other study [33] carried out the investigation for all three instruments—ADAS-cog, ADCS-ADL, and MMSE. For the intervention, the studies used resveratrol in a compound containing 5 g of glucose, 5 g of malate, and 5 mg of resveratrol, administered twice a day, dissolved in 15 mL of commercial grape juice. The placebo was composed of sucralose and lemon juice, indistinguishable by color or flavor when compared with the solution offered to the intervention group.

### 3.3 Alzheimer's Disease Assessment Scale—Cognition Subscale (ADAS-cog)

Of the four studies included in the systematic review, three [29, 32, 33] investigated the effects of supplementation with resveratrol alone [29] or in combination (RGM) [32, 33] using the ADAS-cog. In total, 197 patients were included, 100 in the intervention group (mean age 74.7 ± 5.4 years) and 97 in the placebo group (mean age 75.4 ± 3.4 years), with follow-up time ranging from 12 to 52 weeks.

Considering the three studies, regardless of the type of intervention, mean ADAS-cog values obtained at baseline for the intervention group were 24.6 ± 2.2 points, and 25.3 ± 3.4 points for the placebo group. Considering the type of intervention (isolated or RGM), the mean values obtained by the two studies [32, 33] at baseline for RGM were 24.2 ± 3.1 points in the intervention group and 26.1 ± 4.4 points in the placebo group. For the study [29] that performed the intervention with resveratrol alone, the mean values obtained at baseline were 25.3 ± 10.1 points for the intervention group and 23.7 ± 8.6 points for the placebo group.

At the end of the studies, it was possible to obtain values only for the group that received RGM (25.9 ± 5.5 points) compared with the placebo group (29.9 ± 4.6 points), since Turner et al. [29] did not present data for the resveratrol intervention group at the end of the design. One study [32]

**Table 2** Main results obtained from primary studies of resveratrol for Alzheimer's disease, by type of intervention

| Author, year | Main result | Systematic review conclusions |
|---|---|---|
| Intervention with resveratrol | | |
| Moussa et al., 2017 [28] | Compared with the placebo-treated group, resveratrol treatment attenuated declines in MMSE scores, change in ADCS-ADL scores, and CSF Aβ42 levels during the 52-week trial, but did not alter tau levels | Supplementation with isolated resveratrol seems to be effective in reducing the production of AD-related biomarkers, which directly influenced the mean values obtained for the scores of the applied evaluations. In this case, the supplementation was offered in high doses, for a long period, in addition to evaluating the patients' tolerance to the dosage, with no significant adverse effects |
| Turner et al., 2015 [29] | Resveratrol and its major metabolites were measurable in plasma and CSF. CSF Aβ40 and plasma Aβ40 levels declined more in the placebo group than the resveratrol treatment group, resulting in a significant difference at week 52. A significant decline in the mean values of the resveratrol group when compared with the placebo group was also reported ($p < 0.03$) | |
| Intervention with RGM | | |
| Blass and Gibson, 2006 [32] | The results showed that changes on both MMSE and ADAS-COG were significantly higher in the placebo group than in those taking the active preparation at the end of the 3-month trial | The difference in the study duration between the studies makes it impossible to compare. However, overall, resveratrol in combination with glucose and malate appears to reduce functional and cognitive decline, but further studies are needed to confirm the findings. Patients showed good tolerance to the treatment, with no related adverse effects |
| Zhu et al., 2018 [33] | At 12 months, changes in ADAS-cog, MMSE, and ADCS-ADL scores showed less decline in the treated than in the control group; however, none of the change scores was significant | |

*Aβ* β-amyloid peptide, *AD* Alzheimer's disease, *ADAS-cog* Alzheimer's Disease Assessment Scale—Cognitive Subscale, *ADCS-ADL* Alzheimer's Disease Cooperative Study—Activities of Daily Living, *CSF* cerebrospinal fluid, *RGM* composed of malate, glucose and resveratrol

reported a significant worsening of the mean ADAS-cog score in the placebo group when compared with the intervention group with RGM ($p < 0.001$). In addition, two studies [29, 32] reported no evidence of significant differences between the intervention and placebo groups in ADAS-cog scores, regardless of the type of intervention—resveratrol alone or RGM.

### 3.4 Alzheimer's Disease Cooperative Study—Activities of Daily Living (ADCS-ADL)

Three studies [28, 29, 33] evaluated the effects of the intervention with resveratrol, alone or in RGM, in a mean ADCS-ADL score, compared with the placebo group. One study [33] performed the intervention with RGM, and two other studies [28, 29] performed the intervention with resveratrol alone. The sample for this outcome was 186 patients diagnosed with AD, consisting of 99 patients allocated to the intervention group (mean age 75.1 years) and 87 patients allocated to the placebo group (mean age 76.1 years), with follow-up in all studies equal to 52 weeks.

For the only study [33] that performed the intervention with RGM, the mean baseline for the ADCS-ADL score was $49.1 \pm 10.3$ points for the intervention group and $46.6 \pm 7.6$ points for the control group. At the end of the study period, the values obtained were $49.6 \pm 10.5$ points for the RGM group and $40.5 \pm 10.5$ points for the placebo group.

The mean ADCS-ADL values obtained by the two studies [28, 29] that performed the intervention with resveratrol alone were $62.4 \pm 1.7$ points for the resveratrol group and $62.7 \pm 3.2$ points for the placebo group. At the end of the study period, the mean ADCS-ADL values were $55.3 \pm 2.9$ points and $51.5 \pm 0.2$ points for the resveratrol alone and placebo groups, respectively.

Two studies [28, 29] observed differences between the intervention group with resveratrol alone and placebo. Turner et al. [29] reported a significant decline in mean values of the resveratrol group compared with the placebo group ($p < 0.03$). Furthermore, it was emphasized that the decline in the placebo group was greater than in the resveratrol group. Moussa et al. [28] reported that ADCS-ADL scores declined at 52 weeks compared with baseline in both groups, placebo ($p < 0.001$) and resveratrol ($p < 0.001$). However, the decline in the placebo group was also greater compared with that for the resveratrol group at week 52. Similarly to the study that performed the intervention with RGM [33], no difference was observed between the intervention and placebo groups when using this combination.

### 3.5 Mini Mental State Examination (MMSE)

All four studies included in the systematic review [28, 29, 32, 33] evaluated the effects of supplementation with resveratrol on MMSE scores in patients with AD, when compared with a placebo group. A total of 235 patients were evaluated, with 119 allocated to the intervention group with resveratrol (mean age 74.8 years) and 116 patients in the placebo group (mean age 75.4 years), with follow-up time ranging from 12 [32] to 52 weeks [28, 29, 33].

Two studies [28, 29] evaluated the effects of resveratrol alone on the mean MMSE values when compared with the placebo group, obtaining mean initial values of $19.3 \pm 0.6$ points in the intervention group and $19.4 \pm 0.9$ points in the placebo group. At the end of the intervention period, the study by Moussa et al. showed $17.4 \pm 1.4$ points in the intervention group and $16.6 \pm 1.3$ points in the placebo group.

The other two studies [32, 33] had mean initial values of $20.0 \pm 1.3$ for the intervention group and $19.0 \pm 0.3$ for the placebo group. At the end of the studies, mean values of $19.3 \pm 0.6$ points and $19.4 \pm 0.9$ points were obtained for the RGM and placebo groups, respectively. No significant difference was evidenced between the analyzed groups in the studies that evaluated the effects of isolated resveratrol. The studies that evaluated the effects of resveratrol as RGM showed significant reduction in the mean MMSE scores in the treated group compared with placebo group ($p < 0.03$).

### 3.6 Assessment of Bias Across Studies

The evaluation of the risk of bias in each included study is summarized in Fig. 2. In domain 1, referring to the applied randomization process, two studies [32, 33] were evaluated with reservations, since it was not possible to define exactly the randomization method performed. Domain 2, in which the risk of bias due to deviations from the intended interventions was assessed, was classified as low for all studies [28, 29, 32, 33] since none had a possible negative effect of the attribution to the intervention. Domain 3, referring to the risk of bias due to the lack of outcome data, was classified as a low risk given the availability of data for 95% of participants in all included studies [28, 29, 32, 33]. In the risk of bias in measuring the result—Domain 4, a possible directional bias (i.e., measurement results that are not suitable for the results that the authors planned to evaluate), was not identified toward the intervention in any of the studies [28, 29, 32, 33]. Domain 5, the risk of selecting the reported result, was assessed as low risk, as none of the studies showed any evidence of selection bias when reporting the results. Overall, two studies [28, 33] were evaluated as having a low risk of bias and the other two studies [29, 32] were assessed 'with some considerations', concerning the allocation and randomization of study participants.

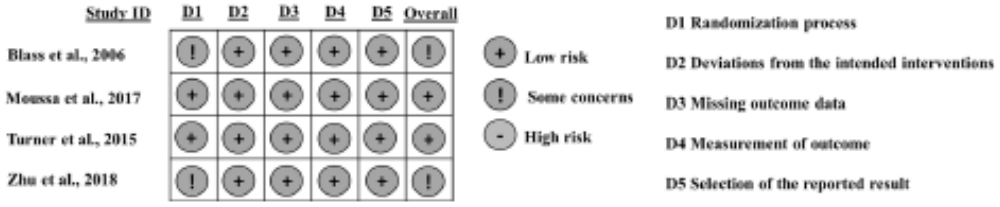


**Fig. 2** Risk of bias graph—Cochrane Collaboration

## 4 Discussion

We conducted this systematic review of randomized clinical trials to examine the possible effect of supplementation with resveratrol, alone or as RGM, on the cognitive and functional performance of patients diagnosed with AD, based on the application of validated tests. Our findings demonstrate that there are still few studies in humans, but they are promising regarding the role of this polyphenol in the delay of cognitive impairment in patients with AD, on its own or when consumed with glucose and malate.

Dementia is a syndrome of increasing prevalence worldwide [34]. The assessment of cognition is of fundamental importance since it is an analysis of functions related to the storage, acquisition, and application of acquired knowledge, including attention, memory, and reasoning, which are important for the patient's interaction with their environment [35]. Therefore, tests to assess cognitive function can help in early diagnosis and monitoring patients as their dementia progresses [36]. According to the European Medicines Agency guideline, cognition, function, and global assessments are considered key domains in the follow-up of patients with AD. Thus, the use of composite scales for the combined assessment of cognition and its impact on daily functioning is considered adequate in this population [37], and these scales were considered as the primary outcomes in the studies included in the present review.

Current attempts to treat AD focus on reducing NFT production, but also inhibiting Aβ production and reducing the negative effects of its accumulation, such as loss of brain function and reduction of brain cortex [38]. A possible mechanism for clearance of these structures would be the overexpression of the enzyme sirtuin-1 (SIRT1) in the brain, since (i) SIRT1 directs the cleavage of the amyloid precursor protein (APP), decreasing its production; and (ii) SIRT1 deacetylates the tau protein, reducing the production of NFT [39]. In this sense, resveratrol has relevance in the pathophysiological factors in AD, due to its anti-aging effects, related to the regulation of SIRT1 [40] production. With the use of resveratrol in animal models, resveratrol-related neuroprotection is based on the activation of SIRT1, which decreases cell death and, consequently, the aggregation and formation of NFT [41].

The mechanism related to the increase of Aβ aggregation in AD patients is related to the formation of advanced glycation end products (AGEs) [34]. The transport of AGEs and Aβ through the blood–brain barrier towards the brain is performed by a transmembrane advanced glycosylation end-product specific receptor (AGER), which is also located on the membrane of neurons, astrocytes, microglia, and vessel walls. AGEs are created during the Maillard reaction, a non-enzymatic reaction between amino acids and reducing sugars, which increases Aβ. After the first formation of Aβ clusters, the Maillard reaction occurs between glucose and the amino groups of proteins existing in the formed Aβ conglomerate [42]. This process generates new AGEs, which accelerate the aggregation of plaques and contribute to the development of reactive oxygen species (ROS), apoptosis, and cell death [43]. It is known that resveratrol acts on reducing the redox effects promoted by ROS. Moreover, brain cells are sensitive to energy stress due to the high demand for adenosine triphosphate (ATP) to maintain neurotransmission, neuronal plasticity, protein synthesis, cell osmolarity, and cell division, among other biological processes that consume ATP [9]. Natural compounds that would be able to modulate this bioenergetic process in brain cells have the potential to be used as neuroprotective agents [44]. In this context, it has been reported that resveratrol is able to modulate mitochondrial function, redox biology, and bioenergy dynamics in several types of cells [9].

The accumulation of Aβ, NFT, AGEs, and increased oxidative stress initiates an inflammatory process, which is observed in the brain of patients with AD [45]. The anti-inflammatory properties of resveratrol are also related to the activation of SIRT1, resulting in the suppression of the expression of the pro-inflammatory nuclear factor-κB (NF-κB) responsible for the increase in the level of inflammatory cytokines, such as interleukin (IL)-1β or tumor necrosis factor (TNF), microglial activation, and production of ROS in the ischemic cortex [46]. In addition, NF-κB can also stimulate cyclooxygenase-2 (COX-2), involved in the conversion of arachidonic acid into prostacyclin and prostaglandins, which, when increased, can trigger a pro-inflammatory

response [47]. Resveratrol would be able to inhibit COX-2, activating the cytokine signaling suppressor-1 (SOCS-1), a protein involved in the negative regulation of the inflammatory response, regardless of SIRT1 signaling. Other studies report that the neuroprotective effects of resveratrol are related to the positive regulation of AMP-activated protein kinase (AMPK) and the decrease in the expression of COX-2 [45, 48]. Because of these properties, resveratrol can have positive effects in maintaining stable cerebral blood flow and, thus, preventing neuronal damage and cognitive function deficits [49].

Resveratrol administered to the cerebral ventricle at a dose of 0.02 mg/kg/day improved memory and reversed the changes induced by Aβ in the inflammatory response and mitochondrial dysfunction in mice [50]. A significant reduction in the levels of NF-κB and IL-1β inflammation markers was achieved in the hippocampus and cortex of the AD mice model. The activities of AMPK and receptor activated by the peroxisome proliferator-activated receptor-gamma coactivator 1 (PGC-1), which is involved in mitochondrial biogenesis, also increased [50]. Finally, all the mechanisms described above promote a reduction in the inflammatory process and, consequently, in microglia activation [23].

The majority of studies relating to dementia and resveratrol involved investigating resveratrol intake via the consumption of wine and grapes [51]. A cohort study evaluated the association between the frequency of alcoholic beverage consumption and dementia in a random sample of 1462 women aged 38–60 years living in Sweden. The results demonstrated that wine was protective for dementia (hazard ratio [HR] 0.6; 95% CI 0.4–0.8), and the association was stronger among women who consumed wine only (HR 0.3; 95% CI 0.1–0.8) [52]. In another study with a semi-experimental design that aimed to verify the effects of the chronic consumption of grape juice on the cognitive function of 35 older female patients, it was observed that the consumption of grape juice contributed to improving the cognitive function of the volunteers over 70 years of age [53].

Preclinical studies indicate that resveratrol crosses the blood–brain barrier, resulting in detectable but low concentrations in the brain, while higher concentrations of resveratrol metabolites are found in the blood. Thus, the bioavailability of oral resveratrol is poor. Since animals are unable to synthesize polyphenols, they must be ingested from a plant-rich diet to achieve pharmacological effects [17]. The bioavailability of resveratrol was then attributed to the several types of formulation used in the included studies—resveratrol isolated in high dosage and resveratrol in low dosage and associated with malate and glucose [28, 33]. The mechanisms involved in interventions with resveratrol in high and low doses may be different. Intervention studies with high doses of resveratrol [29, 32] considered the activation of SIRT1 as a key mechanism, focusing on the high bioactivity of resveratrol to select a maximum safe and well tolerated dose in the studies [29, 32]. The studies that carried out the intervention with a low dosage [29, 33], alongside glucose and malate, focus on increasing the rate of mitochondrial metabolism and on reducing free radicals through the joint action of resveratrol, glucose and malate [54]. Despite these different mechanisms discussed, the safety and efficacy results with resveratrol in high and low doses are not different, although further studies should be conducted to explain resveratrol formulation related to differential ADCS-ADL and MMSE scores.

Two systematic reviews, one with meta-analysis, aimed to assess the effects of resveratrol on AD-related outcomes. In the study of Farzaei et al. [14], four RCTs that assessed the effects of resveratrol on cognitive and memory performance, as well as mood state, were included. The authors reported that there was no evidence that resveratrol acts positively on memory or cognitive performance, or on the mood state of the evaluated patients. However, it should be considered that the volunteers included in the studies were mostly healthy adults, the dosage of resveratrol ranged from 150 to 500 mg/day, and the follow-up ranged from 3 to 26 weeks, all of which could have directly influenced the results obtained in the quantitative analysis. The study of Drygalski et al. [23] aimed to compare recent preclinical studies with previously published studies that evaluated the effects of resveratrol in AD patients. Overall, the authors showed the potential effect of resveratrol not only in reducing the formation of Aβ, but also its role in protecting against neurotoxicity, reducing inflammation and oxidative stress. In addition, resveratrol supplementation prevented apoptosis and loss of neurons that may correspond to improved cognitive functions, effects observed in animal models and in human studies.

Potential limitations should be considered when carrying out the critical analysis of the present study and the primary studies. The small number of studies included ($n=4$) should be considered, as well as the heterogeneity related to the design of the interventions (resveratrol alone and in combination with glucose and malate), study duration (12 weeks vs 52 weeks), resveratrol dosage (isolated resveratrol: 2000 mg/day vs RGM: 5 g/day), and reduced number of evaluated volunteers, which makes it difficult to carry out a quantitative analysis. For the primary studies, considering the assessment of the risk of bias between the studies, the insufficient clarity related to the randomization process in two of the studies [32, 33] should also be considered.

Despite the limitations raised, our study has several strengths, such as the use of a rigorous methodology based on the PRISMA guidelines; publication of the protocol in an online database (PROSPERO); carrying out a comprehensive literature search of six electronic databases (CENTRAL, CINAHL, Embase, MEDLINE, Scopus and WOS) as well as performing a search for grey literature; selection

and extraction of data from the studies were carried out independently and in duplicate by two researchers, with resolution of disagreement by a third reviewer; and application of well-defined eligibility criteria prioritizing only studies with an emphasis on supplementation with resveratrol in patients with AD.

## 5 Conclusion

Supplementation with resveratrol seems to influence the progressive cognitive and functional decline in AD patients when compared with a placebo group. However, further studies with larger samples, longer interventions (study duration of 52 weeks or more), standardization of resveratrol dosage, and better methodological quality of clinical trials should be conducted to elucidate the role of resveratrol in AD patients and its impact on cognitive impairment.

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## Declarations

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## 7 CONSIDERAÇÕES FINAIS

Os resultados do primeiro estudo indicaram que a SOP está relacionada a um desequilíbrio entre citocinas pró e anti-inflamatórias, levando a um status pró-inflamatório em mulheres com SOP. A partir desses dados, surgiu a hipótese de que o tratamento com AG ω-3 poderiam reduzir essa inflamação e, consequentemente, o estresse oxidativo em mulheres com SOP, podendo ser um potencial tratamento nesse grupo.

As evidências do segundo estudo sugerem que a suplementação com AG ω-3 em pacientes com SOP foi associada a um aumento nas concentrações de adiponectina e redução da hs-PCR. Entretanto, nenhum efeito nas concentrações de visfatina ou nos marcadores de estresse oxidativo, quando em comparação com o placebo, foi observado. Pondera-se que os resultados obtidos devem ser interpretados com cautela, visto que, as meta-análises realizadas apresentaram fontes significativas de heterogeneidade.

Foi observado, a partir das evidências obtidas na meta-análise, que a metformina é eficaz na redução dos níveis de testosterona total e valores de FAI em mulheres com SOP. Os resultados também sugerem que os níveis de SHBG e testosterona livre não são afetados pelo tratamento com metformina. Os efeitos da metformina nos níveis de androstenediona e DHEAS, embora não sugerindo nenhuma diferença em relação ao placebo, foram tão divergentes entre os estudos que investigações adicionais são necessárias para resolver a inconsistência.

Considerando que a DA cursa com inflamação, conforme demonstrado por estudos anteriores do nosso grupo, hipotetizou-se que essa condição poderia ser também beneficiada por suplementos que pudessem reduzir o processo inflamatório, medido indiretamente por melhoria no desempenho cognitivo a partir da aplicação de testes neuropsicológicos. Os resultados sugerem que a suplementação com resveratrol parece influenciar no declínio cognitivo e funcional em pacientes com DA, quando comparado ao grupo placebo.

Ademais, para as duas revisões sistemáticas, sugere-se a realização de novos estudos primários com amostras maiores, intervenções mais longas, padronização da

dosagem da intervenção e melhor qualidade metodológica dos ensaios clínicos para validação dos nossos resultados.

## 8 CONCLUSÃO

- A SOP está relacionada a um desequilíbrio entre citocinas pró e anti-inflamatórias, levando a um status pró-inflamatório em mulheres com SOP.
- A suplementação com AG ω-3 em pacientes com SOP foi associada a um efeito anti-inflamatório em mulheres com SOP.
- A metformina mostrou-se eficaz na redução dos níveis de testosterona total e valores de FAI em mulheres com SOP.
- A suplementação com resveratrol parece reduzir o declínio cognitivo e funcional em pacientes com DA.

# 9 PERSPECTIVAS

1) Validação clínica dos tratamentos, com base nas evidências investigadas no presente estudo;
2) Desenvolvimento de novas revisões sistemáticas a partir de possíveis evidências de tratamento da SOP e DA.

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# ANEXOS

## Anexo A – Capítulo de livro publicado durante o desenvolvimento do doutorado

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# The Role of the Mediterranean Dietary Pattern on Metabolic Control of Patients with Diabetes Mellitus: A Narrative Review

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**Abstract**

Diabetes mellitus (DM) is a metabolic disorder characterised by hyperglycemia and abnormalities in carbohydrate, fat and protein metabolism. Several studies demonstrated that foods typical of the Mediterranean diet (MedDiet), including vegetables, fruits, oilseeds, extra virgin olive oil and fish, can promote health benefits for individuals at risk of or with type 2 diabetes (T2DM). In this review, we summarised randomised clinical trials, cohort studies, meta-analyses and systematic reviews that evaluated the effects of the MedDiet on metabolic control of T2DM. The data suggest that the MedDiet influences cardiovascular risk factors, including blood pressure, lipid profile, insulin resistance, inflammation and glucose metabolism, in T2DM patients. In conclusion, the MedDiet appears to protect patients from macro- and microangiopathy and should be considering in the management of diabetic patients.

**Keywords**

Cardiovascular disease · Diabetes mellitus · Diabetic complications · Mediterranean diet

## Abbreviations

| | |
|---|---|
| ADA | American Diabetes Association |
| CRP | C-reactive protein |
| CVD | Cardiovascular disease |
| DKD | Diabetic kidney disease |
| DM | Diabetes mellitus |
| EPIC | European Prospective Investigation of Cancer and Nutrition |
| GLUT-4 | Glucose transporter type 4 |
| HbA1c | Glycated hemoglobin |
| HDL-c | High-density lipoprotein cholesterol |
| IL-6 | Interleukin-6 |
| LDL | Low-density lipoprotein |
| MedDiet | Mediterranean diet |
| T1DM | Type 1 diabetes mellitus |
| T2DM | Type 2 diabetes mellitus |
| TNF-α | Tumor necrosis factor-alpha |

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## 1 Introduction

The term diabetes mellitus (DM) refers to a group of metabolic disorders characterized by the presence of hyperglycemia in the absence of treatment (World Health Organization 2019). The heterogeneous etiopathology of DM includes failure in insulin secretion and/or action and changes in carbohydrate, fat and protein metabolism (Silva et al. 2009). In 2014, it was estimated that 422 million adults lived with DM. Besides, without interventions to halt the increase in diabetes, there will be at least 629 million people living with diabetes by 2045 (World Health Organization 2019). Indeed, it is estimated that a significant percentage of DM cases (30–80%, depending on the country) are undiagnosed (International Diabetes Federation 2017).

DM is commonly associated with, among other factors, changes in dietary patterns, such as increased consumption of ultra-processed foods and sugary drinks, as well as low consumption of fruits, vegetables and fibre, besides an unhealthy lifestyle (World Health Organization 2016). Therefore, the maintenance of controlled plasma glucose levels is essential to prevent or delay the onset of chronic complications associated with DM. The focus in the management of diabetic patients is to obtain an intensified glycemic control, associated with the regulation of serum lipid levels and blood pressure, as well as the maintenance of adequate body weight; these factors are all related to the patient's diet (Silva et al. 2009).

The Mediterranean Diet (MedDiet) is a dietary pattern characterized by high intake of vegetables, fruits, oilseeds, extra virgin olive oil and fish, moderate consumption of wine—depending on the patient's religious beliefs—and rare consumption of red meat, ultra-processed foods, butter and sugary drinks (Itsiopoulos et al. 2011). Recent evidence (Annuzzi et al. 2014; Kastorini et al. 2011; Finicelli et al. 2019) demonstrated the positive relationship between the MedDiet pattern and improvement in T2DM-related parameters, including glycated hemoglobin (HbA1c) (Annuzzi et al. 2014), inflammatory markers, endothelial function (Kastorini et al. 2011) and risk factors related to cardiovascular diseases (CVD) (Finicelli et al. 2019). Nevertheless, the health benefits of the eating pattern in DM patients and key elements that contribute to those benefits are still being investigated.

## 2 MedDiet: General Aspects

The MedDiet was created over the last 5000 years in the Fertile Crescent region, where the first established agricultural communities in the Middle East and the Mediterranean basin are supposed to originate in the early ninth millennium BC. The dietetic pattern was influenced by the achievements of many different civilizations, with consolidated dietary rules coming from the three major monotheistic religions and continuous interactions, additions and exchanges within and outside the region. Consequently, the MedDiet is an expression of the different food cultures present in this region; it represents the historical and environmental diversity that defines the Mediterranean (Dernini and Berry 2015).

The MedDiet was well characterized scientifically by Ancel Keys in the 1960s (Trichopoulou 2001). An important finding of this study was that the low saturated fat content typical of the MedDiet could explain the low incidence of CVD in Mediterranean countries by means of lowering blood cholesterol, one of the major known risk factors for CVD at that time (Trichopoulou 2001). Further work demonstrated that the traditional MedDiet had several additional beneficial health effects (Altomare et al. 2014). Consequently, the MedDiet has been widely studied and disseminated as a healthy eating pattern; it is associated with benefits in the prevention and control of chronic diseases such as cancer, CVD and T2DM (Altomare et al. 2014).

The MedDiet was described on the basis of some dietary characteristics common to Mediterranean countries, with olive oil as the main source of fat, low to moderate intake of fish, dairy

products, poultry and wine with meals, low consumption of red or processed meat and use of herbs and spices as an alternative to refined salt (Trichopoulou et al. 2014). Total lipid intake can be characterized as high (equal to or greater than 40% of total energy consumption) if considering the Greek standard or moderate (about 30% of total energy consumption) considering the Italian dietary standard. In the MedDiet, the ratio of monounsaturated to saturated lipids will be high due to the elevated content of monounsaturated lipids from olive oil; the ratio of polyunsaturated to monounsaturated will also be high due to the elevated consumption of fish and nuts (Trichopoulou et al. 2014).

In 2009 and 2010, based on international scientific consensus, a new pyramid was proposed to the MedDiet standard so that is could be adapted to contemporary lifestyles. This new pyramid proposal was developed in a simplified manner to be adapted to different countries, and specific variations are related to the various geographical, socioeconomic, cultural and contemporary Mediterranean lifestyle contexts. It also considers differences in food portions. Furthermore, the concepts of frugality and regular physical activity were emphasized due to the current major public health challenge: obesity (Bach-Faig et al. 2011). Figure 1 and Table 1 show the adaptation of MedDiet pyramid proposed by Bach-Faig et al. (2011) and a synthesis of the kinds of food, amounts and intake frequency of the MedDiet pattern.

Adherence to the MedDiet pattern is an important factor, given the beneficial results in the control, prevention and mortality from chronic diseases. In a prospective cohort study that involved approximately 22,000 adults, the findings showed that increased MedDiet adherence is associated with a reduction in total mortality. This reduction was evident in both coronary disease and cancer deaths (Trichopoulou et al. 2003).

A randomized clinical trial of approximately 7000 patients at high cardiovascular risk evaluated adoption of the MedDiet pattern and primary prevention of cardiovascular events. It demonstrated a relative risk (RR) reduction of 30% in both intervention groups: MedDiet supplemented with nuts (RR = 0.70; 95% confidence interval [CI]: 0.53–0.94; p = 0.02) and MedDiet supplemented with extra virgin olive oil (RR = 0.70; 95% CI: 0.53–0.91; p = 0.0015). The results support the benefits of the MedDiet for cardiovascular risk reduction (Estruch et al. 2013).

The MedDiet can also improve glycemic control and cardiovascular risk factors, with an evidence rating of B, based on well-conducted cohort, meta-analysis or case-control studies, in a scientific statement published by the American Heart Association and the American Diabetes Association (ADA) (American Diabetes Association (ADA) 2018). Furthermore, the ADA, in its "Standards of Medical Care in Diabetes – 2019", recommends the MedDiet in the prevention and follow-up of patients with T2DM. Regarding the follow-up of patients with type 1 diabetes mellitus (T1DM), the same document argues that there is not enough evidence to support application of the MedDiet standard in this group (American Diabetes Association (ADA) 2018).

## 3 MedDiet and its Effects on DM

### 3.1 Effects of the MedDiet on Postprandial Glycemia and HbA1c

Postprandial blood glucose concentrations refer to plasma glucose levels after ingestion of a meal. The magnitude and peak time of plasma glucose concentration depend directly on the amount and quality of the consumed carbohydrate and factors involved in the response to this carbohydrate (Silva et al. 2009).

HbA1c values close to or below 7% can reduce microvascular and neuropathic complications in patients with DM and possibly macrovascular complications (American Diabetes Association 2009). Postprandial blood glucose is a determining factor in HbA1c values and may account for up to 50% or more of the values of this test

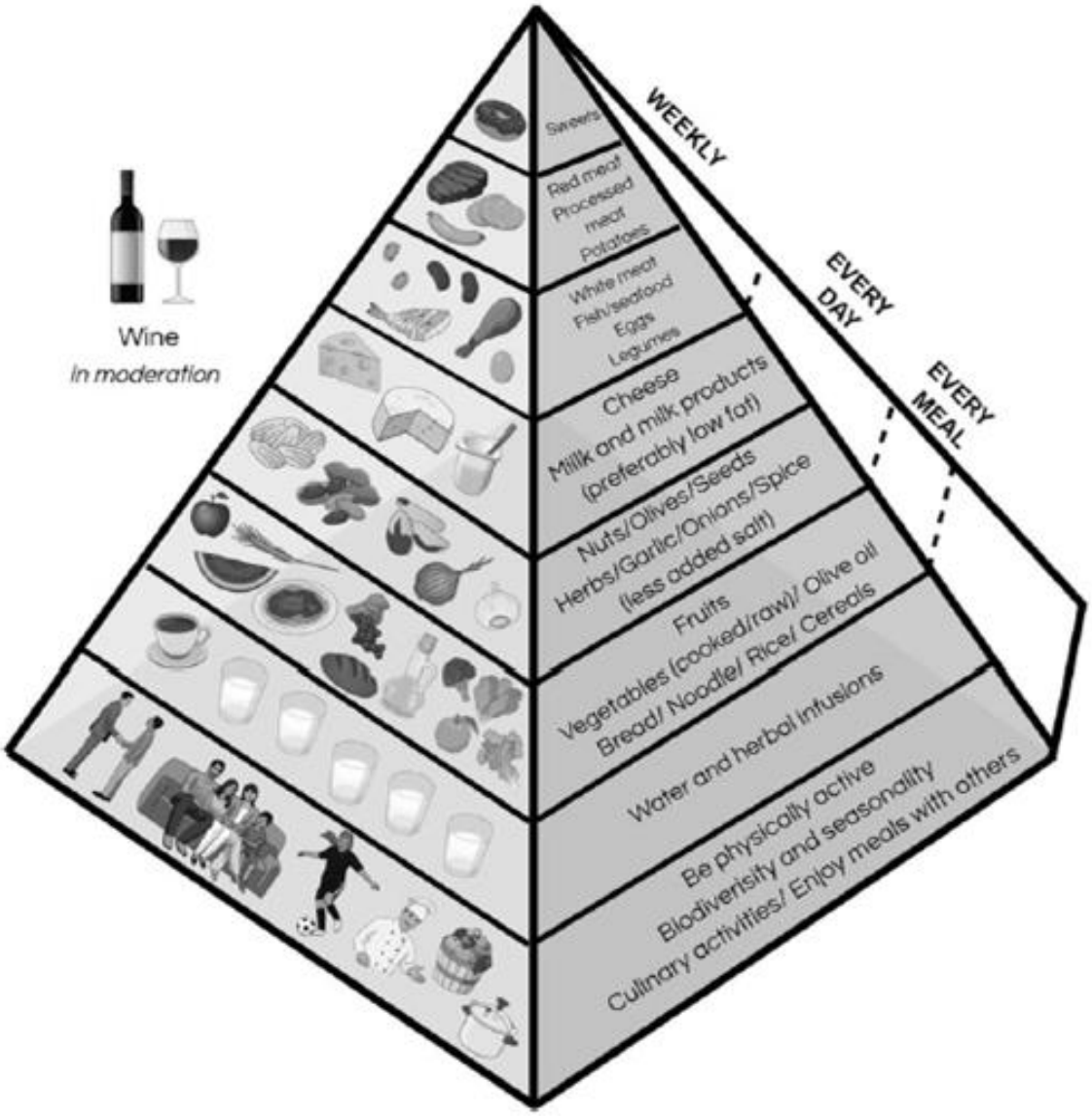


**Fig. 1** Mediterranean diet pyramid: a lifestyle for today; based on Bach-Faig et al. (2011)

(Monnier et al. 2003). Evidence from the European Prospective Investigation of Cancer and Nutrition (EPIC-Norfolk) study revealed that a 1% increase in the HbA1c level increases the risk of death due to all causes by 28% (Khaw et al. 2001).

In randomized controlled trials, reduced glycemic-load diets, including the MedDiet, were associated with better control of weight and risk factors for T2DM (Esposito et al. 2015; Roldan et al. 2019). Besides, a systematic review with meta-analysis assessed three long-term randomized controlled trials in order to evaluate the evidence on the effectiveness of the MedDiet standard in T2DM management. The results showed an estimated global effect of the MedDiet for HbA1c reduction in patients diagnosed with T2DM equal to −0.47% (95% CI: −0.56 to −0.38; $p = 0.0001$) when compared to usual care or a low-fat diet that aimed the glycemic control (Esposito et al. 2015).

In a descriptive, observational study conducted on 107 patients (45.55% men; age: 61.16 ± 23 years) diagnosed with T2DM, with

**Table 1** Types of food, amounts and intake frequency of the Mediterranean dietary pattern (Bach-Faig et al. 2011)

| Food group | Serving size | Frequency |
|---|---|---|
| Sweets | In small amounts | For special occasions |
| Red meat | Less than 2 portions | Weekly |
| Processed meat | Less than 1 portion | |
| Potatoes | Less than 3 portions | |
| White meat | 2 portions | Weekly |
| Fish/Seafood | 2 or more portions | |
| Eggs | 2–4 portions | |
| Legumes | 2 or more portions | |
| Cheese/Milk and milk products | 2 portions | Daily |
| Nuts/Olives/Seeds | 1–2 portions | Daily |
| Herbs/Garlic/Onions/Spice | A reasonable consumption | |
| Fruits | 1–2 portions | Per meal |
| Vegetables | 2 or more portions | |
| Olive oil | One tablespoon per person | |
| Bread/Noodle/Rice/Cereals | 1–2 portions | |
| Water | 1.5–2.0 l | Daily |
| Herbal Infusions | To complete the water requirements | |
| Wine | 1 glass for women and 2 glasses for men | Daily |

poor blood glucose control and a body mass index (BMI) greater than 25 kg/m$^2$, the authors aimed to analyze the relationship between the level of adherence to the MedDiet (low and high dietary adherence) and the control of cardiovascular risk factors. After the 6-month educational intervention, the HbA1c level in the low dietary adherence group was 8.62%, compared to 6.99% in the high dietary adherence group ($p < 0.03$) (Roldan et al. 2019).

In a randomized clinical trial conducted with 27 adults (47–77 years old) with T2DM that aimed to investigate the impact of a diet modeled on the MedDiet pattern on metabolic control and vascular risk in T2DM, the patients were randomly assigned to consume either the intervention MedDiet *ad libitum* or their usual diet for 12 weeks. Compared with the usual diet, the *ad libitum* MedDiet pattern intervention was associated with a reduction in HbA1c levels from 7.1% (95% CI: 6.5–7.7) to 6.8% (95% CI: 6.3–7.3; $p = 0.012$). The authors concluded that a traditional moderate-fat MedDiet improves glycemic control and diet quality in well-controlled T2DM (Itsiopoulos et al. 2011).

In a cross-sectional analysis with 901 outpatients diagnosed with T2DM who attended a diabetes clinic, the authors aimed to evaluate the association of MedDiet pattern with better glycemic control in T2DM patients. The mean HbA1c and 2-h post-meal glucose concentrations were significantly lower in diabetic patients with high adherence to the MedDiet compared to those with low adherence (mean difference: HbA1c 0.9%, 95% CI: 0.5–1.2%; $p < 0.001$; 2-h glucose 2.2 mmol/l, 95% CI: 0.8–2.9 mmol/l; $p < 0.001$) (Esposito et al. 2009a).

These findings suggest that adherence to the MedDiet could benefit glycemic control, as evidenced by the HbA1c results (Estruch et al. 2006; Martins et al. 2014). However, there was no important association when considering each of the MedDiet components, except for a modest association with whole grains and the ratio of monounsaturated/saturated lipids with lower HbA1c levels (Esposito et al. 2009a). Individually, these MedDiet components present small effects that can only promote overall positive changes when they are integrated, for example, into a meal (Trichopoulou et al. 2003).

### 3.2 MedDiet, Inflammation and Oxidative Stress

T2DM is characterized by a chronic and subclinical proinflammatory state and oxidative stress, with increased inflammatory markers, such as C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). These makers are directly related to insulin resistance, a determining factor for T2DM development (Martins et al. 2014). Several studies demonstrated the beneficial effects of diets with a relatively high monounsaturated fatty acid content on DM risk factors. In a prospective study of adiponectin plasma concentrations and dietary data from 987 women with T2DM from the Nurses' Health Study, closer adherence to the MedDiet pattern was associated with higher adiponectin concentrations, which have anti-inflammatory properties (Mantzoros et al. 2006). In fact, the MedDiet significantly increases adiponectin levels and reduces proinflammatory and insulin resistance marker levels (Mattar and Obeid 2009).

Several mechanisms are assumed to explain the metabolic benefits associated with this eating pattern. These benefits include a better lipid profile, rich in monounsaturated fatty acids from regular consumption of extra virgin olive oil and fish; decreased insulin resistance and peripheral inflammation; improved oxidative stress and endothelial function (Estruch et al. 2006). The imbalance between free radical formation and antioxidant factors plays a major role in the onset and development of insulin resistance and pancreatic beta cell dysfunction (Martins et al. 2014). A clinical trial was conducted with 41 healthy individuals submitted to three dietary periods, each lasting 4 weeks, in which the first period comprised a diet rich in saturated fat, the second period a hypolipidaemic diet and the last period a MedDiet. MedDiet consumption reduced the atherogenic index (total cholesterol/high-density lipoprotein cholesterol [HDL-c]) and increased the resistance of low-density lipoproteins (LDL) to oxidation when compared to the low-fat diet period (López-Miranda et al. 2000).

### 3.3 MedDiet Benefits in the Management of DM-Related Complications

Individualized nutritional orientation, which respects patients' preferences and is directed at the prevention and control of overweight and obesity, is one of the most important strategies to achieve the necessary glycemic control for each patient. One of the main goals, and the last step of nutritional therapy recommended for diabetic patients, is to help them reach and maintain metabolic indexes within ideal values in order to prevent chronic complications, including retinopathy, diabetic kidney disease (DKD) and other microvascular complications (Rahati et al. 2014).

The daily intake of varied fruits and vegetables, indicated by the MedDiet pattern, is one of the diet-related DM control aspects that may attenuate the severity and progression of the complications associated with the disease. These flavonoid-rich foods are related to the maintenance of two main factors: better glycemic control and reduced systemic inflammation (Rahati et al. 2014). The mechanism of action of flavonoids *in vitro* reveals that, in addition to their widely known anti-inflammatory characteristic, these compounds exert a beneficial effect on muscle glucose uptake that is mediated by glucose transporter type 4 (GLUT-4) translocation (Mahoney and Loprinzi 2014).

Evidence shows that inflammatory cytokines play a pro-degenerative role in diabetic complications, including proliferative diabetic retinopathy (Donath and Shoelson 2011). Given the anti-inflammatory properties of flavonoids, it is plausible to suggest that adequate intake of quercetin-, kaempferol- and myricetin-containing foods may be an effective strategy for moderating secondary complications associated with diabetes (Mahoney and Loprinzi 2014).

A 7-year, prospective, population-based and observational multicenter study included 192 Spanish patients diagnosed with T1DM and T2DM. The study aimed to evaluate the adherence to the ADA nutritional recommendations—based on 7-day food diaries—and its relation to

targets of metabolic control and onset of diabetic complications. The data did not reveal an association between adherence to each ADA nutritional recommendation and reduction in the onset or progression of diabetic complications. However, a 3.43-to-8.24-fold reduction in the risk of onset of each diabetic complication evaluated (neuropathy, nephropathy, retinopathy, cardiovascular and microvascular complications status) was related with a polyunsaturated fatty acids/saturated fatty acids ratio > 0.4 (Diabetes and Nutrition Study Group of the Spanish Diabetes Association (GSEDNu) 2006).

A randomized clinical trial with approximately 3700 patients diagnosed with T2DM aimed to evaluate the effects of three dietary patterns: MedDiet supplemented with olive oil, MedDiet supplemented with a nut mix and a control diet (following the guidelines based on a low-fat diet, according to ADA) on diabetic complications. After a median 6-year follow-up, there was a significant 43% reduction in the risk of diabetic retinopathy in the group of patients who adhered to the MedDiet standard supplemented with olive oil (hazards ratio [HR] = 0.57; 95% CI: 0.32–0.98). However, there were no significant effects observed in the three dietary patterns in reducing the risk of DKD (Díaz-López et al. 2015).

### 3.4 MedDiet and CVD Risk in T2DM

The risk for coronary heart disease is increased in diabetic patients (Juutilainen et al. 2005). An increase in the prevalence of insulin resistance syndrome may partly explain the recent plateau or increase in CVD rates after several decades of decline (Rosamond et al. 1998). Since the initial studies in the 1970s, the MedDiet has been recognized as a dietary pattern associated with decreased all-cause mortality and reduced cardiovascular risk factor levels and other health outcomes (Martinez-Gonzalez et al. 2015; Ros et al. 2014). Two controlled trials (Shai et al. 2008; Esposito et al. 2009b) evaluated the effects of the MedDiet on cardiovascular risk factors in T2DM patients and found more marked declines in traditional cardiovascular risk factors, including systolic blood pressure, triglyceride levels and the ratio of total cholesterol/HDL-c, in those patients allocated to the MedDiet compared to diabetic subjects who received a control diet.

According to the Standards of Medical Care in Diabetes-2019 from the ADA (American Diabetes Association (ADA) 2018), with regards to the nutrition therapy recommendations for the management of adult diabetics, both the macronutrient composition and the combinations of foods or food groups consumed in a diet should be based on individualized assessment of current eating patterns, personal preferences and metabolic goals. A MedDiet pattern is recommended as an effective alternative to a low-fat and low-carbohydrate diet for T2DM patients due to emerging evidence about the beneficial effects of the MedDiet on glycemic control and CVD risk factors (evidence rating B, indicating supportive evidence from well-conducted cohort studies or case-control studies) (American Diabetes Association (ADA) 2018). Indeed, there is robust epidemiological evidence in the general population that indicates greater adherence to a MedDiet is significantly associated with a reduced risk of both overall and cardiovascular mortality (Sofi et al. 2008).

Other data also indicate a beneficial effect of the MedDiet on the diabetes control. Indeed, this diet reduces blood pressure and modifies the components of fibrinolysis (Bowen et al. 2016). Accumulated evidence from long-term (12–-48 months) randomised controlled trials showed that the MedDiet improves glycemic control and cardiovascular risk in individuals with established diabetes (Esposito and Giugliano 2014).

Apparently, the benefits observed in the CVD risk under MedDiet adherence involve fish intake and heterogeneity in the effects of omega-3 fatty acids (Bowen et al. 2016). Metabolic studies demonstrated that these factors exert beneficial effects on surrogate measures associated with coronary heart disease, including serum levels of triglycerides and thrombotic factors—markers of endothelial dysfunction—and prevention of cardiac arrhythmias (Matsuzawa and Lerman 2014). In the report of a cohort of T2DM patients who

participated in the *PREvención con Dieta MEDiterránea* (PREDIMED) study, a multicenter randomized nutritional intervention trial conducted in a population at high cardiovascular risk, the MedDiet exhibited protective effects on traditional cardiovascular risk factors, including blood pressure, lipid profile and glucose metabolism, and on novel risk factors such as markers of oxidation, inflammation and endothelial dysfunction (Martins et al. 2014).

Current evidence strongly indicates that oxidative modifications of LDL are the key factor in the onset and development of arteriosclerosis. Recent studies in humans and animal models support this hypothesis and show that a diet rich in polyunsaturated fat increases the susceptibility of LDL to oxidation, when compared to a diet rich in monounsaturated fat. The incidence and prevalence of ischaemic heart disease are low in Mediterranean countries, despite a high percentage of their calories (35–40%) coming from fat (Degirolamo and Rudel 2010). This benefit may be due to the high percentage of consumed monounsaturated fat, mainly from olive oil. The Mediterranean experience indicates that the problem of the atherogenic diet depends on the quality rather than the quantity of the ingested fat (Siri-Tarino et al. 2015).

A study was conducted with 41 normolipidemic volunteers submitted to three diets, each lasting 4 weeks: a diet high in saturated fat; a low-fat diet; MedDiet. The results showed that the MedDiet reduced LDL oxidation, in addition to the effect on the already known lipid profile, when compared with the high saturated fat and low-fat diets. In fact, the benefits of the monounsaturated-fat-rich MedDiet highlights its action on plasma lipoproteins, as the risk of CVD is related to the relative tendency of atherogenic lipoproteins, mainly LDL, to suffer oxidative modification (Siri-Tarino et al. 2015). Taken together, these findings suggest that the prevention of atherosclerosis should be based on the type of fat rather than reduction of total fat (López-Mirandaa et al. 2000).

### 3.5 MedDiet and Obesity Control

The prevalence of obesity has increased worldwide, and it is estimated that by 2030 nearly 40% of world's population will be overweight and one in five people will be obese. Obesity is mostly caused by poor health habits related to what is called the "obesogenic environment", including highly processed food, high consumption of red meat and sugary beverages, low consumption of vegetables and fruits and the reduction or replacement of physical activity (Jurado-Fasoli et al. 2019).

As previously described, obesity-induced oxidative stress and insulin resistance activate a signaling cascade that involves a proinflammatory pathway. In general, adherence to the MedDiet is associated with a reduction in different obesity-associated factors, in addition to a high quality of life. There are several physiological explanations that could elucidate why key components of the MedDiet pattern may protect against weight gain. The MedDiet is rich in plant-based foods that provide a large quantity of dietary fiber that increase satiation through various mechanisms, including prolonged mastication, increased gastric detention and enhanced cholecystokinin release (Schroder 2007). The MedDiet pattern also has a low energy density (Schroder 2007) and a low glycemic load (Willett and Leibel 2002) compared with many other dietary patterns. These characteristics, together with its high-water content, lead to increased satiation and lower calorie intake, and thus help to prevent weight gain (Buckland et al. 2008).

The MedDiet pattern is composed of large amounts of dietary fiber and features a high ratio of monounsaturated to saturated fat. It is recognized as one of the healthiest dietary patterns for the treatment of metabolic syndrome. The MedDiet pattern is beneficial for helping to reduce the associated cardiovascular risk and other health outcomes, and evidence from a previous meta-analysis also indicated that the MedDiet can decrease the risk of diabetes in healthy individuals and improve glycemic control, weight loss and

cardiovascular risk factors for T2DM patients (Pan et al. 2019; Huo et al. 2015).

A meta-analysis that aimed to compare the differences among major dietary patterns in improving glycemic control, cardiovascular risk and weight loss for T2DM patients included 10 randomized clinical trials that involved five dietary patterns. Compared to the low-fat diet, the MedDiet pattern showed better effects in reducing body weight (kg) in T2DM patients (RR = −1.18; 95% CI: −1.99–0.37; p = 0.08) and BMI (kg/m$^2$; RR = −0.63; 95% CI: −1.29–0.02; p = 0.0007) (Pan et al. 2019).

In fact, a systematic review and meta-analysis of randomized controlled trials, which aimed to evaluate the effects of the MedDiet pattern on glycemic control, weight loss and cardiovascular risk factors in T2D patients, included nine studies with 1178 total patients. Compared with control diets (usual diet), the MedDiet pattern led to greater reductions in HbA1c, BMI and body weight (mean differences: HbA1c, −0.30, 95% CI: −0.46 to −0.14, p = 0.001; BMI, −0.29 kg/m$^2$, 95% CI: −0.46 to −0.12, p = 0.924; body weight, −0.29 kg, 95% CI: −0.55 to −0.04, p = 0.976). Additionally, the MedDiet pattern was associated with a 1.45 mmHg decline (95% CI: −1.97 to −0.94; p = 0.58) in systolic blood pressure and 1.41 mmHg reductions (95% CI: −1.84 to −0.97; p = 0.95) for diastolic blood pressure (Huo et al. 2015).

## 4 MedDiet and DM: Current Evidence

According to the ADA guideline for T2DM prevention (American Diabetes Association (ADA) 2018), the nutritional instruction to the diabetic patient should be structured in order to include a low-calorie diet that promotes weight loss and stimulate physical activity. These factors are of fundamental importance for those with high risk of developing overweight or obese T2DM. Furthermore, based on intervention testing, dietary patterns should also be useful for patients with pre-diabetes, which include a MedDiet or hypocaloric and hypolipemic diet plan (American Diabetes Association (ADA) 2018).

A European cohort study of prospective population-based research on cancer and nutrition (EPIC) indicated that Mediterranean population groups with good adherence to the MedDiet presented results inversely associated with T2DM risk (odds ratio [OR] = 0.88; 95% CI: 0.77–0.99; p = 0.021). When the MedDiet was combined with a low-glycemic-load dietary profile, the association became stronger (OR = 0.82; 95% CI: 0.71–0.95; p = 0.722). These results suggest that a low-glycemic-load dietary profile combined with a traditional MedDiet can result in 18% increase in T2DM protection (Rossi et al. 2013).

Tripp et al. (Tripp et al. 2019) developed an observational study with 50 healthy overweight and obese subjects with cardiometabolic risk factors and aimed to confirm the safety, tolerability and efficacy of the restricted calorie quantity in the MedDiet. The participants were assigned to a modified MedDiet for 12 weeks; it included protein shakes and targeted supplementation that provided 68–76% of the subject's estimated calorie requirements. The subjects exhibited the following decreases from baseline: 12% in body weight, 18% in body fat and 8.8% in waist circumference. Additionally, inflammation biomarkers, namely oxidized LDL and high-sensitivity C-reactive protein, were reduced by 17% (p < 0.01) and 30% (p < 0.05), respectively, with the modified diet (Tripp et al. 2019).

Another study conducted by Maiorino et al. (2017) assessed the long-term effects of a MedDiet on circulating levels of endothelial progenitor cells (EPCs) and the carotid intima-media thickness (CIMT) in patients with T2DM in a randomized trial with 215 men and women. At the end point, changes in the CIMT were inversely correlated with the changes in EPC and HbA1c levels (mean difference: -0.3; 95% CI: −0.6–0; p = 0.050) and HOMA-IR (mean difference: -0.7; 95% CI: −1.2 to −0.2; p = 0.043) in patients who consumed the MedDiet compared to a low-fat diet (Maiorino et al. 2017).

Another study developed by Tepper et al. (2018) aimed to evaluate whether adherence to

MedDiet was associated with physical function in older T2DM patients. They evaluated 117 patients (age 70.6 ± 6.5 years) at the Center for Successful Aging with Diabetes at Sheba Medical Center. The group with low adherence to MedDiet pattern presented higher HbA1c levels than the group with high adherence to the MedDiet (7.59 ± 1.19 versus 7.35 ± 0.82; $p = 0.7003$) (Tepper et al. 2018).

Grimaldi et al. (2018) in a non-randomized study that assessed the efficacy and durability of a 3-month intensive dietary intervention, aimed to evaluate body weight and cardiometabolic risk factors after implementing the MedDiet in 116 subjects at high risk for cardiac disease. The intensive intervention consisted of 12 weekly group educational meetings and a free-of-charge supply of meals prepared according to the MedDiet pattern. The conventional intervention (control) consisted of an individual education session along with monthly reinforcements of nutritional messages by the general practitioner. All participants were followed for 9 months. In the subgroup of participants with T2DM ($n = 40$), there was a significant reduction in HbA1c levels in the group that followed the intensive intervention group ($n = 24$; from 7.73 to 6.82%; $p < 0.0001$), while this measure remained essentially unchanged in the control group ($n = 16$; from 7.73 to 7.40%) (Grimaldi et al. 2018).

## 5 Other Foods Patterns and Their Effects on T2DM

There are many types of dietary patterns emphasizing the consumption of plant foods besides MedDiet, as Dietary Approaches to Stop Hypertension (DASH), vegetarian and vegan diets (Salas-Salvadó et al. 2019).

The DASH diet was originated in the 1990s and promotes the consumption of vegetables and fruits, lean meat and dairy products, and the inclusion of micronutrients, as vitamins and minerals. It also advocates the reduction of sodium in the diet to about 1500 mg/day and the consumption of minimally processed, stimulating the consumption of fresh food (Kerley 2019). The adherence to DASH dietary pattern is often related to the nutritional management of individuals with hypertension and its role in the T2DM control has also been studied (Campbell 2017). There is a considerable overlap of components of DASH and the MedDiet, such as vegetables, fruits, nuts, and legumes as beneficial components, considering red and processed meat as a rather detrimental component, although evidence for association between DASH diet and diabetes is limited (Mantzioris and Villani 2019). However, unlike the MedDiet, DASH diet includes whole grains, low-fat dairy, a broad group of fats and oils, and sugary drinks, but does not consider alcohol in your composition (Tangney 2014).

A randomized crossover clinical trial that aimed to determine the effects of the DASH eating pattern on cardiometabolic risks in T2DM patients, observed an inverse association between the intake of DASH diet, when compared with control diet, on fasting blood glucose and HbA1c levels (Azadbakht et al. 2011). In fact, a systematic review with meta-analysis that summarized evidences from prospective studies, which examined association of dietary patterns with T2DM, showed an estimated global effect of DASH diet in T2DM incidence equal to 0.82 when compared the highest with the lowest intake of DASH diet (Jannasch et al. 2017).

The term plant-based diet has been extensively used to refer not only to vegan diets, which do not include any food from animal sources, but also to other diets such as vegetarian, which can include eggs and dairy products, or to semi vegetarian diets, which contain small amounts of meat and fish or other animal products (Salas-Salvadó et al. 2019). Several factors may explain the effect of the vegan diet on the glycemic control, such as reduced fat content, especially saturated fat, which can result in decreased accumulation of intracellular fat, improving insulin sensitivity (Salas-Salvadó et al. 2019). Besides, a high intake of fruits and vegetables and adherence to dietary patterns emphasizing the consumption of plant foods has been associated with reduced body weight and lower abdominal adiposity (Hopping et al. 2010). These benefits are also attributed to a

high fiber content and the low glycemic index (Hopping et al. 2010).

The vegetarian dietary pattern may present as an option of easier adhesion in relation to vegan pattern. The Academy of Nutrition and Dietetics of the United States emphasizes that the vegetarian dietary pattern, when properly planned, have positive effects on the treatment and risk of chronic diseases, including T2DM (Melina et al. 2016). In addition, a meta-analysis of six controlled clinical trials observed that the consumption of vegetarian diets was associated with a significant reduction of 0.39% in HbA1c when compared with consumption of other control diets, as omnivorous, conventional diet for diabetics or low fat diets (Yokoyama et al. 2014). Another study that aimed to compare the effects of calorie-restricted vegetarian (experimental) and conventional diabetic diet (controls group) in T2DM patients, observed that the vegetarian diet presented greater capacity to improve insulin sensitivity compared with a conventional diabetic diet over 24 weeks. The metabolic clearance rate of glucose increased more in the experimental group from baseline to 24 weeks (30%) than in the control group (20%) (Kahleova et al. 2011).

Therefore, evidences suggest that healthy patterns, including higher intakes of vegetables and fruits, whole grains, fish, and low-fat dairy, may decrease T2DM risk, and that the unhealthy patterns, including frequent intakes of sugars, processed/red meats, and fried foods, may increase the T2DM risk or harm the treatment. In addition, lifestyle changes are crucial for metabolic control, as well as they may reduce the risk of diabetes complications caused by poor glycemic control (Huang et al. 2019).

## 6 Conclusion

The MedDiet is related to decreased glycemia levels and reduced cardiovascular risk in patients with T2DM. Studies have also shown favourable changes in glycemic control by reducing HbA1c. Regarding CVD, the MedDiet promotes improvements in the lipid profile, blood pressure, insulin resistance and inflammation. Thus, adoption of this dietary pattern is recommended for T2DM patients because of its beneficial metabolic control and its role in reducing the risk of macrovascular complication, such as atherosclerosis.

There are controversial data related to the MedDiet improvement on microvascular complications. Notably, the studies indicate a positive relationship between this dietary pattern and the prevention of diabetic retinopathy and DKD. Crucially, almost all studies were performed in T2DM patients or nondiabetic individuals. Thus, there is a lack of data about its effect on T1DM. Consequently, it is necessary studies with a larger sample size and long-term follow-up for analysis of the MedDiet influence on DM complications.

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## Anexo B – Artigo I publicado em colaboração durante o desenvolvimento do doutorado

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Review

# microRNAs associated to anthracycline-induced cardiotoxicity in women with breast cancer: A systematic review and pathway analysis

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ABSTRACT

*Background:* Cardiotoxicity is a common and serious adverse effect of anthracycline therapy in breast cancer patients. The current criteria for cardiotoxicity are based on imaging and cardiac biomarkers. However, there is a need for new biomarkers to help with early diagnosis. MicroRNAs (miRNAs) are small non-coding RNA molecules that play an important role in the regulation of gene expression. Several miRNAs have been associated with cardiovascular diseases and are biomarkers under investigation for cancer treatment-related cardiotoxicity.
*Methods:* We performed a systematic literature search of Medline/PubMed, Cochrane Central Register of Controlled Trials, Scopus, Lilacs, Web of Science and Embase, until April 2020. Cohort studies that reported miRNA biomarkers in breast cancer patients with anthracycline-induced cardiotoxicity and non-cardiotoxicity patients were included. Moreover, we searched the miRTarBase for experimentally validated miRNA-target interactions.
*Results:* Among the 209 studies retrieved, five fulfilled the inclusion criteria. Let-7f, miR-1, miR-20a, miR-126 and miR-210 were validated in two population-based cohorts. The pro-angiogenic miRNAs let-7f, miR-20a, miR-126 and miR-210 were significantly down-regulated in epirubicin-cardiotoxicity when compared to the non-cardiotoxicity group. miR-1 has been shown to provide diagnostic and prognostic information in the setting of myocardial infarction, but changes in its levels are controversial in doxorubicin-treated breast cancer patients with cardiotoxicity. Reactome pathways relevant to cardiotoxicity were found from the target genes for let-7f, miR-1, miR-20a, miR-126 and miR-210 at miRTarBase.
*Conclusion:* The data suggest that let-7f, miR-1, miR-20a, miR-126 and miR-210 are associated with anthracycline-based cardiotoxicity during chemotherapy in breast cancer patients.

## 1. Introduction

Breast cancer is the most common female cancer and is the leading cause of cancer-related deaths [1]. The World Health Organization has estimated that 627,000 women died of breast cancer in 2018, which represents 15 % of all female cancer deaths [2]. Anthracyclines, such as doxorubicin and epirubicin, are commonly used in the treatment of breast cancer and have significantly improved the disease-specific survival [3]. However, this chemotherapy regimen has been associated with adverse cardiovascular effects and increased cardiovascular mortality, especially in older women. In this sense, appropriate risk factor stratification is essential for early diagnosis and the prevention of cardiovascular disease [4].

The recommendations outlined in recent studies for identify early myocardial injury in cancer patients treated with anthracyclines and/or anti-HER2 (human epithelial growth factor receptor 2) therapy are based on cardiac imaging and cardiac biomarkers [5,6]. A decrease in left ventricular ejection fraction (LVEF) is the most widely recognized echocardiographic profile to cardiotoxicity evaluation. However, LVEF sensitivity is limited for the detection of subtle myocardial dysfunction [7]. Troponins and brain natriuretic peptides, circulating markers of cardiac disease onset, have progressively emerged as useful biomarkers

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to identify patients who are more prone to developing myocardial dysfunction and may be helpful in detecting subclinical cardiotoxicity during patient follow-up [8,9]. However, circulating levels of these biomarkers increase only after tissue damage has occurred.

Both *in vitro* and *in vivo* models have been focused on the association of microRNAs (miRNAs) in anthracyclines-induced toxicity [10]. miRNAs are a class of small noncoding RNAs (21–25 nucleotides), which regulate posttranscriptional gene expression by either inhibiting messenger RNA (mRNA) translation or promoting its degradation [11]. They are involved in many important biological processes such as cellular development and cellular signaling, cell proliferation, cell-to-cell communication, and apoptosis. Importantly, abnormal miRNA expression has been associated with the initiation and progression of pathological conditions, including cardiac diseases [12].

Doxorubicin-treated rats showed significant increase in plasma levels of miR-1, miR-133a, and miR-208 [13,14]. Specifically, miR-133a levels rapidly increased during acute myocardial infarction and were found to be more sensitive than cardiac troponin T [15]. Moreover, higher plasma levels of miR-34a and miR-122 were found in 25 breast cancer patients receiving anthracycline-based chemotherapy after treatment [16]. Although these previous studies have shown that miRNAs represent potential biomarkers for cardiac diseases, further research is needed to investigate the possible involvement of miRNAs in anthracycline-induced cardiotoxicity [17]. Despite the clinical importance, many of the studies have focused on the use of preclinical animal models, which have limitations as predictors of human biology [18].

In the present study, the aim was to perform a systematic review to assess the differential expression levels of circulating miRNAs in breast cancer patients, in order to relate them to anthracycline-induced cardiotoxicity. We also aimed to carry out pathway analysis to investigate molecular pathways related to these miRNAs.

## 2. Materials and methods

This systematic review was designed and conducted in accordance with the Cochrane Handbook recommendations [19]. Results were reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [20]. Supplementary material 1 shows the PRISMA checklist. The protocol of the current study was registered in the International Prospective Register of Systematic Reviews (PROSPERO) (http://www.crd.york.ac.uk/ PROSPERO, number CRD42020177833).

### 2.1. Search strategy

The search strategy was defined based on the PECO question: P (participants) = women with breast cancer treated with anthracyclines; E (exposure) = cardiotoxicity; C (control) = women with breast cancer treated with anthracyclines without cardiotoxicity; and O (outcome) = microRNA expression levels. A literature search was performed in the medical electronic databases Medline via PubMed (Medical Literature Analysis and Retrieve System Online), the Cochrane Central Register of Controlled Trials (CENTRAL), CINAHL EBSCO (Cumulative Index to Nursing and Allied Health Literature), Scopus, LILACS via Virtual Health Library (VHL) (Latin American and Caribbean Health Sciences), Scientific Electronic Library Online (Scielo), ISI Web of Science: Core Collection and Embase until April 2020 to find studies that investigated the differential expression of miRNAs in breast cancer patients. The search included the Mesh terms 'breast neoplasms', 'cardiotoxicity', 'microRNA' and the entry terms. Supplementary material 2 describes the search strategy used for the PubMed database.

The same terms were used to search for clinical studies in Google Scholar, www.scholar.google.com/ and OpenGrey, www.opengrey.eu/. Searches were performed on the following dissertation/thesis databases: ProQUEST Dissertations & Theses Global, Federal University of Minas Gerais (https://repositorio.ufmg.br/), University of São Paulo (https://www.teses.usp.br/), Oswaldo Cruz Foundation - Fiocruz (https://portal.fiocruz.br/repositorio-institucional-arca), University of Brasilia (https://repositorio.unb.br/) and Federal University of Bahia (https://repositorio.ufba.br/ri/). All possibly relevant reports were considered for review, irrespective of language and date of publication. Reference lists of included articles were also checked to identify additional relevant citations.

### 2.2. Inclusion and exclusion criteria

Two authors working independently (J.D.P. and M.T.A.) performed the review of the titles and abstracts of all articles retrieved to evaluate eligibility for inclusion in this study. In cases of disagreement, a third investigator (K.B.G. or J.A.G.T.) contributed to the final decision.

Eligible studies were considered when they evaluated the differential expression of miRNA in breast cancer patients over 18 years old who received cancer therapy with anthracycline with or without cardiotoxicity. We excluded studies that did not present a proper non-cardiotoxicity group, or which were conducted on animal models/cell lines, or did not report the outcomes of interest. Therefore, we excluded reviews and meta-analyses.

### 2.3. Study selection and data extraction

The studies were retrieved from each electronic database and included on a single electronic library, and duplicates were removed using the EndNote® software. Two reviewers (J.D.P. and M.T.A.) independently collected the results using a standardized form. When consensus could not be achieved, a third reviewer (K.B.G. or J.A.G.T.) resolved the differences in data extraction. The extraction of data comprised: 1) characteristics of studies, such as author and year of publication; 2) the sample type (plasma, serum or tissue) that was evaluated; 3) miRNAs measured; 4) method of miRNA detection; and 5) the expression of miRNAs in each study group. Data from the exposure and control group were also collected.

### 2.4. Quality assessment of bias for each study

The risk of bias and methodological quality of the included studies was independently assessed by two reviewers (J.D.P. and M.T.A.) following the Newcastle-Ottawa quality assessment scale (http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp). This scale contains eight items, including representativeness of the sample in the exposed cohort, selection of the unexposed cohort, exposure by the type of measure used (e.g., secure records or structured interviews), how the outcome of interest was assessed, whether the follow-up of the study was long enough for the hypothesis of the results to occur, and if there was adequate follow-up of the cohorts. Stars are assigned to each completed item, with the highest possible score being nine. A score above six means that the study has high methodological quality. Importantly, we considered a p-value $<0.05$ to indicate a statistically significant difference in the expression of miRNAs when the cardiotoxicity and non-cardiotoxicity groups were compared, in at least two population-based cohorts.

### 2.5. Target gene search and pathway analysis

We searched the miRTarBase to find target genes for each of the five miRNAs identified in this systematic review, and considered their different names or aliases, as follows: let-7f (hsa-let-7a-5p), miR-1 (hsa-miR-1-3p), miR-20a (hsa-miR-20a-3p and hsa-miR-20a-5p), miR-126 (hsa-miR-126-3p and hsa-miR-126-5p), and miR-210 (hsa-miR-210-3p). miRTarBase was developed to provide comprehensive information on experimentally validated miRNA-target interactions [21]. For the pathway analysis, we considered only target genes that were experimentally validated by at least one of the validation methods that provide

strong evidence according to miRTarBase, namely reporter assay, western blot, and qPCR [21]. We manually retrieved the target genes for each miRNA (Table 3) and interrogated them for significant well-curated signaling pathways obtained from Reactome 2016 Human Pathway [22] sorted by p-value ranking <0.5 using Enrichr [23].

## 3. Results

### 3.1. Study selection

The flowchart of the strategy used to select studies for inclusion in this systematic review is shown in Fig. 1. The initial search identified 209 studies, of which 53 were excluded as they were duplicates or did not meet the eligibility criteria. Exclusion criteria were: studies that did not evaluate breast cancer therapy with anthracycline, experimental studies, review papers and meta-analyses. The remaining 156 articles were evaluated based on titles and abstracts. In this phase, the Kappa coefficient of agreement between the two investigators (J.D.P and M.T. A) was 0.862.

After reading the titles and abstracts, 133 studies were excluded for not fulfilling the inclusion criteria, and 23 potentially eligible articles were selected. Reasons for excluding studies were: participants aged <18 years old, patients who were not treated with chemotherapy with anthracyclines, studies conducted on animal models or cell lines, studies which did not evaluate microRNA levels, those which did not present a proper non-cardiotoxicity group, review papers and meta-analyses. Eligible studies evaluated miRNA expressions in breast cancer patients aged ≥18 years old and anthracycline-cardiotoxicity (cases) and breast cancer patients without this condition (control groups). However, following full text analysis, 18 studies were excluded due to the following reasons: did not evaluate cancer therapy with anthracycline (n = 4), did not present a non-cardiotoxicity group (n = 4), were conducted on animal models/cell lines (n = 5), or were not a primary study (n = 5). Finally, five articles that fulfilled the eligibility criteria were included in this systematic review [24–28].

### 3.2. Study characteristics and quality assessment

Among the five included studies, one was performed in a population from Italy [24], two from China [25,28], one from Brazil [26] and one from the United States of America [27]. One study [24] was included as two independent reports because the findings were described by the use of different anthracyclines (doxorubicin and epirubicin). The main characteristics of these articles are shown in Table 1.

The studies were designed as cohorts and evaluated the miRNA expression between anthracycline-induced cardiotoxicity and non-cardiotoxicity groups in plasma samples during the follow-up. In particular, Rigaud et al. [26] used data from the CECCY trial (NCT01724450) and Gioffre et al. [24] assessed results from the ICOS-ONE clinical trial (NCT01968200), to investigate miRNA expression as possible circulating markers of cardiotoxicity. One of the studies evaluated only triple negative breast cancer [28] and one excluded HER-2 positive breast cancer patients [26]. In four studies, the cardiac impairment was evaluated in one-year follow-up compared to the baseline, and also included evaluations during the treatment. Only one study [27] was considered as a short follow-up (after first infusion).

The most common parameter used to define cardiotoxicity was LVEF (assessed by echocardiography), which was considered in four studies. Only one study [24] evaluated the occurrence of cardiotoxicity by cardiac troponin (troponin I or T). Sample sizes ranged from 32 to 363 in the included studies. Collectively, these studies investigated a total of 708 subjects (cardiotoxicity, n = 76; non-cardiotoxicity, n = 632) and

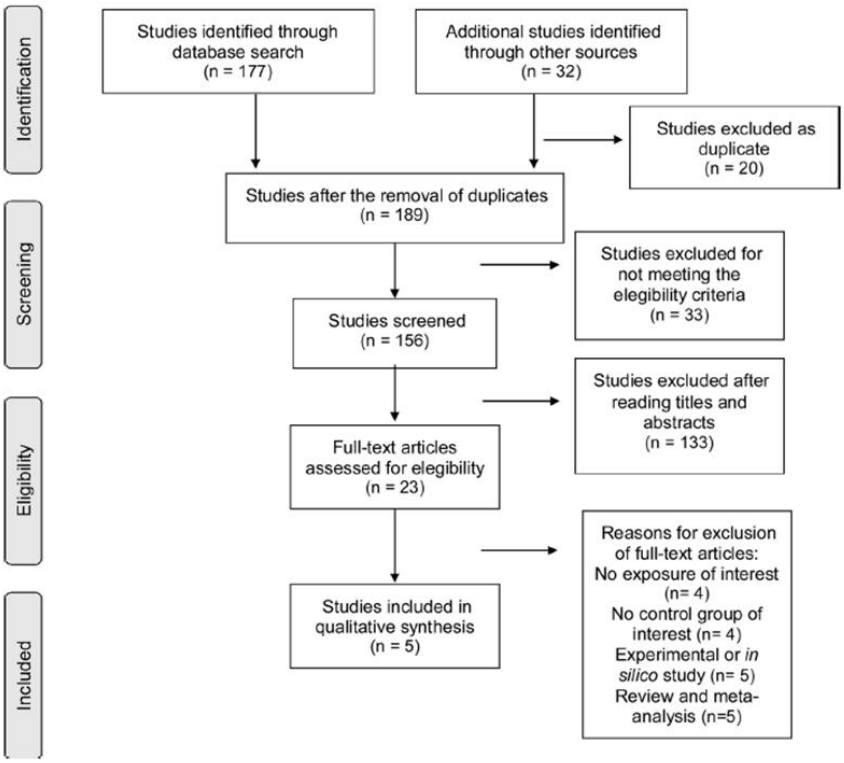


**Fig. 1.** Flowchart of the literature search and the study selection process.

the age ranged from 45.38 to 49.9 years old.

Among the included studies, anthracycline was used in combination with other cytotoxic agents. Todorova et al. [27] used doxorubicin (60 mg/m$^2$) with cyclophosphamide (600 mg/m$^2$), and Rigaud et al. [26] combined doxorubicin (cumulative dose of 240 mg/m$^2$) and cyclophosphamide (600 mg/m$^2$) followed by paclitaxel (80 mg/m$^2$ or docetaxel 75 mg/m$^2$). Only Rigaud et al. [26] excluded patients who used cardio-protective drugs, including ACE inhibitors, angiotensin II receptor blockers, or β-blockers. In another study, neoadjuvant chemotherapy included epirubicin (100 mg/m$^2$) and cyclophosphamide (600 mg/m$^2$), followed by docetaxel (75–100 mg/m$^2$) [28]. The same neoadjuvant chemotherapy regimen was employed in the study developed by Qin et al. [25], which also included HER2 positive patients who received trastuzumab treatment on demand (6 mg/kg, after docetaxel treatment). As mentioned above, one study evaluated the effects of both anthracyclines [24]. According to the clinical trial, epirubicin and doxorubicin have a median cumulative dose of 360 [270–360] and 240 [240–240] mg/m$^2$, respectively. During the trial, 63 % of the patients with breast cancer were treated with taxanes and 22.5 % with trastuzumab; 2 (0.8 %) patients were treated with a tyrosine-kinase inhibitor, imatinib [29].

Two studies used miRNA PCR arrays to assess the plasma miRNA profiles of patients with anthracycline-based therapy [24,27]. Only Gioffre et al. [24] selected miRNA candidates to validate the miRNA array results through single qPCR analysis. Two studies [25,26] selected miRNAs based on a literature search and performed RT-qPCR of these selected miRNAs, while another study did not report the candidate miRNA selection process [28].

Quality assessment of the included studies by using NOS for cohort studies is shown in Supplementary material 3. The median score of NOS was 8. All studies presented a high quality (low risk of bias) score ≥6.

### 3.3. Differentially expressed miRNAs in breast cancer patients with anthracycline-induced cardiotoxicity and non-cardiotoxicity

In two studies, the expression of miRNAs was only assessed at baseline [25,28]. However, one study assessed the expression of miRNAs at baseline and after the first dose of the drug [27], and the other studies performed the analysis at baseline and at least twice during the treatment [24,26].

The number of differentially expressed miRNAs found when comparing cardiotoxicity and non-cardiotoxicity patients varied in the studies from 3 [24] to 32 miRNAs [27]. Considering the five studies, we identified 40 differentially expressed miRNAs ($p < 0.05$) (Table 2). Among them, four miRNAs (let-7f, miR-20a, miR-126 and miR-210) showed concordant results in two studies [25,28], both being down-regulated in the cardiotoxicity group compared to the non-cardiotoxicity group. Only miR-1 showed discordant results, since it was reported as being down-regulated in cardiotoxicity patients in one study [27], and up-regulated in another [26].

Notably, one study found 26 miRNAs to be up-regulated in cardiotoxicity group when compared to non-cardiotoxicity subjects [27]. Also, three miRNAs have shown increased expression levels in cardiotoxicity patients in another study [24]. Moreover, two, three and one miRNAs were found to be down-regulated in anthracycline-treated breast cancer patients with cardiotoxicity in different studies [25,27] and [28], respectively.

The levels of 11 miRNAs (let-7b, miR-17-3p, miR-18a, miR-19b-1, miR-130a, miR-146a, miR-148a-3p, miR-208a, miR-208b, miR-296, miR-423-5p) were not different between the cardiotoxicity and non-cardiotoxicity groups ($p > 0.05$, data not show).

### 3.4. Pathway analysis of target genes for the five differentially expressed miRNAs

We used an approach focused on the target genes identified in the miRTarBase for the five miRNAs (let-7f, miR-1, miR-20a, miR-126 and miR-210) or their aliases. Notably, the number of target genes varied from 46 to 80 for the let-7f and miR-1, respectively (Table 3). We then performed pathway analysis using the target genes for each miRNA in order to search for molecular pathways which may be related to anthracycline-induced cardiotoxicity. Reactome pathways for each of the five miRNAs are shown in Fig. 2. Notably, some pathways are shared among the miRNAs, such as "Signal transduction R-HAS-162582" (let-7f, miR-1, miR-20a, and miR-126) and "Cellular responses to stress R-HAS-2262752" (let-7f, miR-20a, and miR-210) (Fig. 2).

## 4. Discussion

The search for novel biomarkers for the early detection of cardiotoxicity is clinically relevant to the detection of initial cardiac injury before the established dysfunction [17]. In this field, several studies have suggested that miRNAs are key mediators in modulating anthracycline-induced cardiac injury [13–16]. Circulating miRNAs can be potentially non-invasive biomarkers because they are stable in the circulation, resistant to degradation by nucleases, and can be detected before the onset of clinical symptoms [30].

However, studies have shown discordant results regarding miRNA expression profiles, which make the identification of the best miRNA candidates for cardiotoxicity assessment difficult [31]. Furthermore, significant heterogeneity was also observed in different studies related to cardiotoxicity criteria, the number of patients included and the number of miRNAs investigated [32]. Given the importance of miRNAs as diagnostic biomarkers in anthracycline-induced cardiotoxicity in breast cancer patients, we performed this systematic review of all studies that evaluated the differential expression of miRNAs in breast cancer patients. Notably, we found five miRNAs (let-7f, miR-1, miR-20a, miR-126 and miR-210) which were significantly deregulated in two cohorts of breast cancer patients with anthracycline-induced cardiotoxicity.

Let-7f is a pro-angiogenic miRNA, belonging to the let-7 family [27]. This molecule has angiogenic and endothelial function and influences the clinical prognosis for ischemic stroke in young subjects [13]. Let-7f facilitates the vascular network, acting directly on the transformed growth factors (TGF)-β and vascular endothelial growth factor (VEGF) [27]. It was also reported that low levels of let-7f expression were related to LVEF in dilated cardiomyopathy [27]. Thus, let-7f can reduce the risk of cardiac dysfunction and protect patients being treated with anthracyclines against cardiotoxicity [27]. Two Chinese studies included in this systematic review reported that breast cancer patients with anthracycline-induced cardiotoxicity had lower levels of let-7f compared to non-cardiotoxicity patients [25,28]. Both studies selected 14 miRNAs to be evaluated by RT-qPCR, based on their previously proposed pro-angiogenic role. Although miRNA candidates were selected from the literature or based on prior evidences, the validation of miRNAs on an independent cohort of subjects contributed to reinforce the use of miRNAs as minimally invasive screening and triage tools for subsequent diagnostic evaluation. Additionally, the authors enrolled patients undergoing chemotherapy with epirubicin (dose of 100 mg/m$^2$) and considered a follow-up period of 12 months. Cardiotoxicity was defined as a decrease in LVEF by 10 % from baseline to a final value less than 53 % in both studies. The similarity in the study design and miRNA detection method allows the reliable comparison of results. In addition, in these two studies, let-7f was found in plasma miRNA expression profiles from women with breast cancer in China, which limits the extension of the findings to other population groups. Importantly, replication in samples collected from other population groups is important to validate these findings.

Regarding the pathway analysis, LIN28A (Lin-28 Homolog A) was found among the target genes for let-7a-5p. Notably, Lin28a was recently shown to play a pivotal role in pathological cardiac hypertrophy in a mouse model [33]. Through the inhibition of microRNA let-7 maturation or directly binding to mRNAs to regulate their abundance

**Table 2**
Differentially expressed microRNAs in cardiotoxicity compared to non-cardiotoxicity patients in all studies included in the systematic review.

| miRNA | Study [reference] | Change of expression in cardiotoxicity group | P-value | Time of miRNA evaluation | Potential role [Reference] |
|---|---|---|---|---|---|
| let-7f | Qin et al., 2018 [25] | Down | <0.001* | Baseline | Pro-angiogenic [59] |
| | Zhu et al., 2018 [28] | Down | 0.001* | Baseline | |
| | Rigaud et al., 2017 [26] | Up | <0,05* | At cycle 2, 3 and 4 | |
| miR-1 | Todorova et al., 2017 [27] | Down | 0.003* | After first dose | Arrhythmia, myocardial infarction, cardiac hypertrophy and heart failure [60] |
| | Gioffre et al., 2020 [24]a,b | Unreported | >0.05 | Baseline | |
| miR-15a-5p | Todorova et al., 2017 [27] | Up | 0.015* | After first dose | Diffuse myocardial fibrosis [61] |
| miR-15b-5p | Todorova et al., 2017 [27] | Up | 0.029* | After first dose | Arteriogenesis and angiogenesis [62] |
| miR-16-2-3p | Todorova et al., 2017 [27] | Up | 0.008* | After first dose | Sympathetic denervation and PPARγ activation [63] |
| miR-16-5p | Todorova et al., 2017 [27] | Up | 0.016* | After first dose | Cardiac insufficiency [64] |
| miR-17-5p | Qin et al., 2018 [25] | Down | 0.003* | Baseline | Pro-angiogenic, acute myocardial infarction) [65] [66] |
| | Zhu et al., 2018 [28] | Down | 0.332 | Baseline | |
| miR-19a | Qin et al., 2018 [25] | Down | 0.126 | Baseline | Pro-angiogenic, acute myocardial infarction) [65] [66] |
| | Zhu et al., 2018 [28] | Down | 0.023* | Baseline | |
| miR-20a | Qin et al., 2018 [25] | Down | <0.001* | Baseline | Pro-angiogenic [67] |
| | Zhu et al., 2018 [28] | Down | 0.040* | Baseline | |
| miR-23b-3p | Todorova et al., 2017 [27] | Up | 0.041* | After first dose | Cardiac insufficiency [64] |
| miR-25-3p | Todorova et al., 2017 [27] | Up | 0.006* | After first dose | Myocardial Infarction [68] |
| miR-28-3p | Todorova et al., 2017 [27] | Up | 0.008* | After first dose | Type 2 diabetes mellitus [69] |
| miR-30d-5p | Todorova et al., 2017 [27] | Up | 0.034* | After first dose | Myocardial infarction [70] |
| miR-34a-5p | Todorova et al., 2017 [27] | Up | 0.002* | After first dose | Cardiotoxicity and apoptosis with the use of anthracyclines [71] |
| | Gioffre et al., 2020 [24]a,b | Unreported | >0.05 | Baseline | |
| miR-92a | Qin et al., 2018 [25] | Down | 0.882 | Baseline | Pro-angiogenic [72] |
| | Zhu et al., 2018 [28] | Down | 0.160 | Baseline | |
| | Todorova et al., 2017 [27] | Up | 0.019* | After first dose | |
| miR-122-5p | Gioffre et al., 2020 [24]a | Up | 0.007* | Baseline | Acute coronary syndrome, severity of coronary diseases [73,74] |
| | Gioffre et al., 2020 [24]b | Up | 0.50 | Baseline | |
| miR-126 | Qin et al., 2018 [25] | Down | <0.001* | Baseline | Pro-angiogenic [75] |
| | Zhu et al., 2018 [28] | Down | 0.020* | Baseline | |
| miR-133a-3p | Todorova et al., 2017 [27] | Down | 0.010* | After first dose | Proliferation, differentiation, survival, hypertrophic growth [76,77] |
| miR-133b | Rigaud et al., 2017 [26] | Up | >0,05 | At baseline, cycle 2, 3 and 4 | Proliferation, differentiation, survival, hypertrophic growth [76,77] |
| | Todorova et al., 2017 [27] | Down | 0.004* | After first dose | |
| miR-140-3p | Todorova et al., 2017 [27] | Up | 0.014* | After first dose | Myocardial infarction [78] |
| miR-142-5p | Todorova et al., 2017 [27] | Up | 0.024* | After first dose | Inflammation, oxidative stress and apoptosis [79] |
| miR-144-5p | Todorova et al., 2017 [27] | Up | 0.007* | After first dose | Proliferation, migration, invasion and apoptosis of human umbilical vein endothelial cells [80]; identified in heart and colon mouse tissue [81] |
| miR-145-5p | Todorova et al., 2017 [27] | Up | 0.006* | After first dose | Inflammatory response, apoptosis in cardiomyocytes [82] |
| miR-205-5p | Todorova et al., 2017 [27] | Up | 0.034* | After first dose | Differentiation, capture and proliferation of breast cancer [83] |

(*continued on next page*)

**Table 2** (*continued*)

| miRNA | Study [reference] | Change of expression in cardiotoxicity group | P-value | Time of miRNA evaluation | Potential role [Reference] |
|---|---|---|---|---|---|
| miR-210 | Qin et al., 2018 [25] | Down | 0.021* | Baseline | Pro-angiogenic [84] |
| | Zhu et al., 2018 [28] | Down | 0.032* | Baseline | |
| miR-324-5p | Todorova et al., 2017 [27] | Up | 0.025* | After first dose | Mitochondrial fission, apoptosis and myocardial infarction [85] |
| miR-331-3p | Todorova et al., 2017 [27] | Up | 0.023* | After first dose | Tumor suppression [86] |
| miR-363-3p | Todorova et al., 2017 [27] | Up | 0.023* | After first dose | Tumor suppression [87] |
| miR-376a-3p | Todorova et al., 2017 [27] | Down | 0.028* | After first dose | Tumor suppression [87] |
| miR-378 | Qin et al., 2018 [25] | Down | 0.002* | Baseline | Pro-angiogenic [88] |
| | Zhu et al., 2018 [28] | Down | 0.104 | Baseline | |
| miR-421 | Todorova et al., 2017 [27] | Up | 0.015* | After first dose | Cardiomyocyte apoptosis, myocardial infarction [89] |
| miR-486-5p | Todorova et al., 2017 [27] | Up | 0.006* | After first dose | Protection against cardiomyocyte apoptosis [90] |
| miR-499a-5p | Gioffre et al., 2020 [24]a | Up | 0.029* | Baseline | Acute myocardial infarction [91] |
| | Gioffre et al., 2020 [24]b | Up | 0.75 | Baseline | |
| miR-501-3p | Todorova et al., 2017 [27] | Up | 0.013* | After first dose | Alzheimer's disease, metastasis [92] and hepatocellular carcinoma [93] |
| miR-502-3p | Todorova et al., 2017 [27] | Up | 0.010* | After first dose | Triple negative breast cancer [94] |
| miR-532-3p | Todorova et al., 2017 [27] | Up | 0.040* | After first dose | Mitochondrial fission, apoptosis in the presence of doxorubicin [95] |
| miR-532-5p | Todorova et al., 2017 [27] | Up | 0.002* | After first dose | Acute myocardial infarction, apoptotic [96] |
| miR-660-5p | Todorova et al., 2017 [27] | Up | 0.005* | After first dose | Platelets, thrombotic events and acute myocardial infarction [97] |
| miR-885-5p | Gioffre et al., 2020 [24]a | Up | 0.035* | Baseline | Liver toxicity [98] |
| | Gioffre et al., 2020 [24]b | Up | 0.14 | Baseline | |
| miR-1260a | Todorova et al., 2017 [27] | Up | 0.027* | After first dose | Metastatic melanoma [99] |

* $P < 0.05$. Baseline: before treatment.

and translation, the evolutionarily conserved RNA-binding protein Lin28a and its paralog Lin28b play critical roles in pluripotency, organismal growth, tissue repair, and oncogenesis [34,35].

miR-20a is a member of the miR-17 family, which in turn belongs to the miR-17/92 cluster, a gene family with an oncogenic role that is differentially expressed in breast cancer, mainly in tumors negative for estrogen receptors [36]. Notably, the miR-17/92 cluster was deregulated in cardiovascular, immune and neurodegenerative diseases [37]. miR-20a was shown to control angiogenesis in breast cancer and induces abnormalities in the vascular development of the mesh [38]. Moreover, a decreased plasma levels of miR-20a was found when compared cardiotoxicity-affected and non-cardiotoxic patients, which suggest that miR-20a is a potential circulating marker of cancer treatment-related cardiotoxicity [25,28].

miR-126 is involved in angiogenic and inflammatory processes, therefore playing an important role in cancers and autoimmune diseases [39]. miR-126 was shown to be decreased in tumors, because it is able to inhibit the growth, adaptation, migration and invasion of the cancer cell of origin. Notably, miR-126 levels are used as a prognostic pattern for the survival of neoplastic patients [40]. In addition, miR-126 was also shown to play a role in improving myocardial damage after acute myocardial infarction events. Two studies found decreased levels of miR-126 in breast cancer patients when comparing the cardiotoxicity and non-cardiotoxicity groups [25,28], suggesting that miR-126 is a possible marker of cardiotoxicity risk. Conversely, miR-126 was found to be significantly up-regulated after neoadjuvant chemotherapy (cyclophosphamide or fluorouracil and epirubicin followed by docetaxel or paclitaxel) in 25 breast cancer patients before and after chemotherapy [16]. Importantly, this study did not evaluate whether there was a correlation between miR-126 levels and biomarkers of cardiotoxicity [16]. Therefore, these discordant results may be due to the lower level or absence of cardiac toxicity. Moreover, the mechanisms of miR-126 underlying cardiotoxicity is still unclear.

miR-210 modulates endothelial cells response to hypoxia and has a robust anti-hypoxia ability. miR-210 was shown to enhance the formation of capillary networks and the migration and differentiation of endothelial cells [41]. *In vitro* experiments showed that miR-210, when overexpressed, could mitigate hypoxia-induced injury [42]. In addition, the positive regulation of miR-210 was reported in cardiac stem cells under conditions of hypoxia, which prevented apoptosis and promoted cell migration [43]. In cells of breast cancer lineage, when in a hypoxic environment, miR-210 promoted metastasis, proliferation and self-renewal [44]. Importantly, a correlation between miR-210 levels and the decrease in LVEF was observed in a cohort of 97 breast cancer patients under anthracycline treatment; twelve had cardiotoxicity with a reduction in basal LVEF [45]. Decreased levels of miR-126 were also shown in patients affected by cardiotoxicity compared to unaffected subjects [25,28]. Taken together, these data suggest that the differential regulation of miR-126 may modulate cardiotoxicity.

The miR-1 is encoded by *miR-1-1* and *miR1-2,* which are located in two distinct loci on chromosomes 20 and 18, respectively. The two precursors, after being exported to the cytoplasm by the Exportin 5

Table 3
Target genes identified in the miRTarBase for the differentially expressed miRNAs (or their aliases) identified in the systematic review.

| miRNA | Target genes identified in miRTarBase |
|---|---|
| let-7f | EWSR1, PARP1, NF2, UHRF2, E2F2, HMGA2, KRAS, CCR7, RAB40C, ITGB3, IL6, UHRF1, NKIRAS2, HRAS, CCND2, AGO1, RRM2, HMGA1, PKM, PRDM1, PAK1, |
| hsa-let-7a-5p (46 target genes) | EZH2, ARG2, MAP4K4, AURKB, WNT1, RAVER2, TNFAIP3, CDK6, CASP3, NRAS, DICER1, LIN28A, TNFRSF10B, MYC, TGFBR3, CDC34, STAT3, IGF2BP1, TRIM71, EGFR, IGF2, HAS2, AGO4, CDKN1A, LIN28B |
| miR-1 | EDN1, PTBP1, IGF1, API5, TAGLN2, MPL, CDK4, FZD7, PIM1, MET, GJA1, SNAI2, FOXP1, PIK3CA, VEGFA, LASP1, G6PD, XPO6, HCN2, KRAS, CCL2, FRS2, FN1, CCND1, PAX3, RARB, HCN4, PNP, KCNJ2, PTMA, HDAC4, FABP3, NOTCH3, KCNE1, TWF1, ETS1, NAIP, PPP2R5A, |
| hsa-miR-1-3p (80 target genes) | SPRED1, SLC8A1, PGD, TKT, TNKS2, HAND2, MEF2A, ABCB1, TWIST1, BAG4, YWHAZ, CXCL12, FASN, ANXA2, SOX9, ADAR, ND1, SOX6, CNN3, PRKCE, COX1, HSPD1, PGM2, CEBPA, HSPA4, SERP1, CALM3, GATA4, AGO1, BDNF, SRXN1, LARP4, TMSB4X, KIF2A, NETO2, ATP6V1B2, ASPH, TH, POGK, SP1, CAND1, IL11 |
| miR-20a<br>hsa-miR-20a-3p | SMO, PTEN, BID, NR4A3, EGR2, HIF1A, TCEAL1, CCND1, E2F1, BMPR2, CDKN1A, TGFBR2, PTEN, APP, RUNX1, SMAD4, IRF2, KIT, UBE2C, STAT3, LIMK1, GJA1, DUSP2, SMAD7, MAP3K5, MCL1, TP53INP1, EGR2, ABL2, ATG16L1, PRKG1, ETV1, FBXO31, RUNX3, NFKBIB, KIF26B, DAPK3, EGLN3, REST, ITGB8, ZFYVE9, RGS5, |
| hsa-miR-20a-5p (74 target genes) | TGFBR1, ITGB8, MYC, BNIP2, MAP3K12, BCL2, MEF2D, VEGFA, CCND2, E2F3, RB1, RBL1, RBL2, WEE1, PPARG, BAMBI, CRIM1, MAP2K3, PURA, ARHGAP12, TSG101, SIRPA, PHLPP2, ANKH, EPAS1, DNMT1, PKD1, PKNOX1, RB1CC1, TIMP2, PTPRO, PPP2R2A, NRAS, THBS1, MUC17 |
| miR-126<br>hsa-miR-126-3p | PITPNC1, IGFBP2, KRAS, SPRED1, PLK2, EGFL7, RGS3, TOM1, HOXA9, MERTK, CRK, VEGFA, PIK3R2, IRS1, SOX2, TWF1, TWF2, PTPN7, DNMT1, SLC7A5, PIK3CG, TEK, ADAM9, CRKL, FOXO3, BCL2, CXCR4, RHOU, LRP6, |
| hsa-miR-126-5p (48 target genes) | SIRT1, NFKBIA, CADM1, EZH2, ROCK1, SLC45A3, VCAM1, PGR, ADGRE5, AKT1, CCNE2, MMP7, CXCL12, TCF4, ADM, E2F1, SPRED1, PTPN7, HOTAIR, CRK, CYLD, SLC45A3, MYC, ADAM9, MMP7, CXCL12, VEGFA |
| miR-210 | FGFRL1, RAD52, EFNA3, PTPN1, BDNF, ISCU, E2F3, MNT, AIFM3, NDUFA4, SDHD, ALDH5A1, FOXN3, MCM3, IGFBP3, COL4A2, INPP5A, EHD2, SH3BGRL, PTPN2, FOXP3, HIF3A, BNIP3, ATG7, THSD7A, VMP1, BTK, NPTX1, XIST, CPEB2, GPD1L, NCAM1, DDAH1, TFRC, |
| hsa-miR-210-3p (75 target genes) | HSD17B1, STMN1, DIMT1, LDHA, LDHB, P4HB, PTBP3, HIF1A, HOXA9, TP53I11, PIM1, HOXA1, CASP8AP2, KCMF1, PLK1, TWIST1, MRE11A, XPA, SMCHD1, TNPO1, CBX1, ABCB9, CDK10, DENND6A, HOXA3, MYORG, MDGA1, MID1IP1, SEH1L, UBQLN1, SERTAD2, ACVR1B, APC, ATP11C, CHD9, CLASP2, ELK3, PTAR1, NIPBL, MIB1, HECTD1 |

molecule, are processed in mature identical forms of miR-1. It has been seen that miR-1 is related to several types of cancer, including breast cancer [46]. miR-1 has been reported to be elevated in cardiac muscle, but not in other tissues [47]. Patients who have suffered from acute myocardial infarction had higher plasma levels of miR-1 compared to healthy patients. As miR-1 is abundantly expressed in skeletal muscle, it was suggested that miR-1 is released by necrotic cardiac myocytes [48]. While increased levels of miR-1 were found in the plasma of breast cancer patients who had to be treated with doxorubicin and suffered from cardiac dysfunction after cycles 2, 3 and 4 [26], miR-1 was also found to be down-regulated after the first dose of doxorubicin [27]. Although both studies have investigated patients treated with doxorubicin, they have adopted different combined regimes and dosages [26,27]. Considering that cardiotoxicity is related to both the peak plasma concentration and cumulative dose of anticancer drugs [49], their findings may differ because of the chemotherapy cycles (dosage, time and periodicity) and drug combinations. Moreover, their findings probably differed due to different molecular subtypes of breast cancer, which could have distinct clinical outcomes. Importantly, different sample size and detection methods were employed. In particular, Rigaud et al. [26] selected 6 candidate miRNAs, based on the literature, to be evaluated in plasma of 56 breast cancer patients with abnormal cardiac function by qRT-PCR. On the other hand, Todorova et al. [27] performed plasma profiling, using a miRNome PCR panel, in sample of 20 breast cancer patients with cardiotoxicity. Different commercial kits and protocols may lead to different conclusions and make the comparison of results very difficult. Finally, Gioffre et al. [24] did not find any significant differences in miR-1 levels in 88 breast cancer patients treated with doxorubicin or epirubicin. Moreover, this study evaluated the effects of anthracycline by cTnT and cTnI levels, because of the lack of a decrease in LVEF [24]. Conversely, two other studies assessed cardiotoxicity using LVEF [26,27]. Using an OpenArray screening, miR-1 was not found to be differentially expressed at baseline, during treatment and at follow-up, probably because few patients presented cardiac toxicity [24]. Importantly, only this study confirmed the expression of miRNAs by a second RT-qPCR technique using TaqMan assays on the same plasma samples for results validation.

In order to search for pathways that are relevant to anthracycline-induced cardiotoxicity, we searched the miRTarBase for target genes for the five miRNAs (Table 3) and found relevant Reactome pathways (Fig. 2). Notably, the "Cellular responses to stress R-HAS-2262752" pathway was found from the target genes for the miRNAs let-7f, miR-20a, and miR-210. Accordingly, redox cycling and oxidative stress are among the well-known molecular pathways related to doxorubicin-induced cardiotoxicity [50–53]. Notably, recent studies indicate the role of non-coding RNAs, including miRNAs and long non-coding RNAs, in the pathogenic process of oxidative stress and the response of cells to oxidative stress [54,55]. However, recent evidence suggested that doxorubicin cardiotoxicity is not solely due to redox cycling. Novel explanations include anthracycline-dependent regulation of major signaling pathways controlling DNA damage response, cardiomyocyte survival, cardiac inflammation, energetic stress and gene expression modulation [56]. Interestingly, the "Signal transduction R-HAS-162582" pathway was found from the target genes for the miRNAs let-7f, miR-1, miR-20a, and miR-126. Indeed, the review of molecular advances regarding anthracycline-associated cardiomyopathy have uncovered the complex balance between cardiomyocytes and endothelial homeostasis through reactive oxidative stress, interference in apoptosis/growth/metabolism, and angiogenic imbalance [52].

The reliable prediction of who will develop cardiomyopathy and heart failure upon anthracycline exposure have proven elusive [52]. Predictive genomic biomarkers of functional relevance for doxorubicin-induced cardiotoxicity and heart failure were previously identified using human Induced Pluripotent Stem Cells-derived cardiomyocytes [57]. Doxorubicin exposure for more than two days was shown to deregulate genes participating in apoptosis, DNA damage, and the oxidative stress response. Several clusters of genes were found to be down-regulated (sarcomere, myofibril, contractile fiber, and regulation of heart contraction genes) or up-regulated (stress response, p53 signaling pathway, and apoptosis genes) after two and six days of treatment with 156 nM doxorubicin, again becoming up-regulated or down-regulated toward control levels after washing out of the drug [57,58].

The major strength of this systematic review and pathway analysis was the novelty of the study. To the best of our knowledge, this was the first systematic review focusing on the predictive role of miRNAs in anthracycline-induced cardiotoxicity in the prognosis of breast cancer patient. Moreover, we showed two shared pathways among the miRNAs including "Signal transduction R-HAS-162582" and "Cellular responses to stress R-HAS-2262752".

Although the literature search was conducted in accordance with

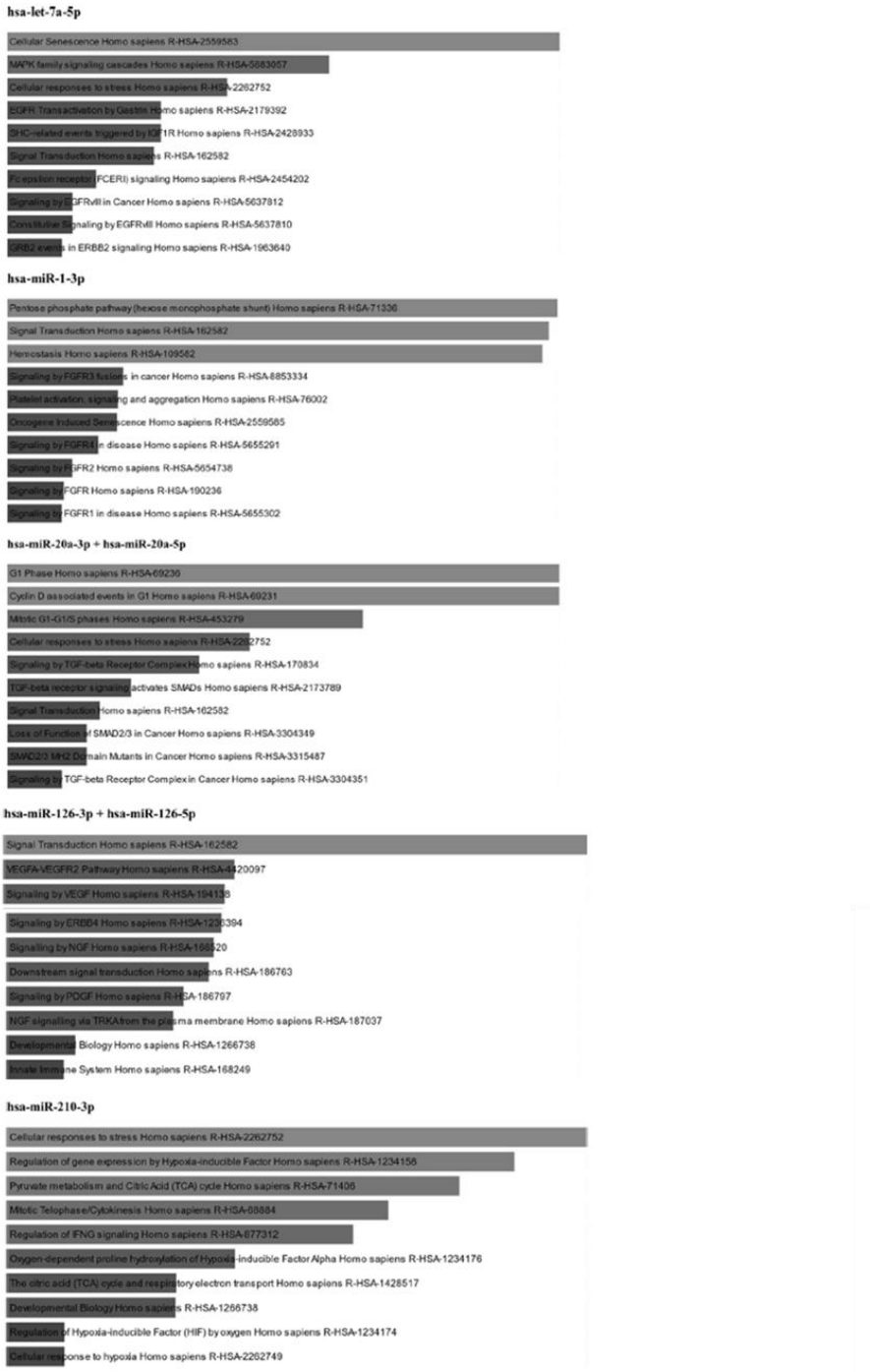


**Fig. 2.** Reactome pathways obtained from the target genes identified in the miRTarBase for the five differentially expressed miRNAs (or their aliases) identified in this systematic review.

**Table 1**
Characteristics of the studies included in the systematic review.

| First author, year, country, reference | Type of breast cancer | Study duration | Cardiotoxicity diagnosis | Number of patients; mean age ± SD or median (interquartile range) in years | | | Chemotherapy regimens; dose (mg/m$^2$) ± SD | | | miRNA detection method | miRNA studied |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Entire cohort | Cardiotoxicity | Non-cardiotoxicity | Entire cohort | Cardiotoxicity | Non-cardiotoxicity | | |
| Gioffre et al., 2020; Italy [24] a | unreported | 12 months | cTn I and cTnT | 32 | 18 | 14 | Doxorubicin | Doxorubicin | Doxorubicin | Open Array screening and RT-qPCR | miR-1-3p miR-34a-5p miR-99b-5p miR-122-5p miR-125b-5p miR-499a-5p miR-532-5p miR-885-5p |
| | | | | 56 | 12 | 44 | Epirubicin | Epirubicin | Epirubicin | | miR-1-3p miR-34a-5p miR-122-5p miR-128-3p miR-181b-5p miR-181c-5p miR-361-3p miR-499a-5p miR-885-5p |
| Gioffre et al., 2020; Italy [24] b | unreported | 12 months | cTn I and cTnT | unreported | 52.2 ± 6.3 | 49.3 ± 11.4 | Median cumulative dose of 360 [270-360] mg/m$^2$ | 239.1 ± 45.3 (Cumulative epirubicin dose converting in terms of doxorubicin equivalents) | 223.3 ± 42.5 (Cumulative epirubicin dose converting in terms of doxorubicin equivalents) | Open Array screening and RT-qPCR | |
| Rigaud et al., 2017; Brazil [26] | Adenocarcinom; HER-2 positive was excluded | 12 months | Reduction in LVEF ≥10% and/or LVEF <50% | 56 49.9±3.3 | 10 48.6 ± 3.2 | 46 49.9 ± 1.2 | Doxorubicin Cumulative dose of 240 mg/m$^2$) and cyclophosphamide (600 mg/m$^2$) followed by paclitaxel (80 mg/m$^2$ or docetaxel 75 mg/m$^2$) | Doxorubicin 408.4 ± 1.4 (Total dose) | Doxorubicin 410.6 ± 11.4 (Total dose) | RT-qPCR | miR-1 miR-133b miR-146a miR-208a miR-208b miR-423-5p |
| Qin et al., 2018; China [25] | unreported | 12 months | LVEF declined by 10% from baseline to below 53% | 363 45.38 ± 6.05 | 19 ≥45 years: 11 (5.5) <45 years: 8 (4.8) | 346 ≥45 years: 189(94.5) <45 years: 157(95.2) | Epirubicin 100 mg/m$^2$, (treatment regimen), cyclophosphamide (600 mg/m$^2$), then followed by docetaxel (75-100 mg/m$^2$), trastuzumab treatment on demand (6 mg/kg, after docetaxel treatment) | Epirubicin unreported | Epirubicin unreported | RT-qPCR | let-7b let-7f miR-17-3p miR-17-5p miR-18a miR-19a miR-19b-1 miR-20a |

(continued on next page)

Table 1 (*continued*)

| First author, year, country, reference | Type of breast cancer | Study duration | Cardiotoxicity diagnosis | Number of patients; mean age ± SD or median (interquartile range) in years | | | Chemotherapy regimens; dose (mg/m$^2$) ± SD | | | miRNA detection method | miRNA studied |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Entire cohort | Cardiotoxicity | Non-cardiotoxicity | Entire cohort | Cardiotoxicity | Non-cardiotoxicity | | |
| | | | | | | | | | | | miR-92a<br>miR-126<br>miR-130a<br>miR-210<br>miR-296<br>miR-378 |
| Todorova et al., 2017; United States [27] | Invasive ductal carcinoma | After one dose of chemotherapy | Decline of LVEF below by >10% or below 50% | 20<br><br>unreported | 8<br><br>unreported | 12<br><br>unreported | Doxorubicin<br>60 mg/m$^2$ (treatment regimen)<br>cyclophosphamide (600 mg/m$^2$) | Doxorubici<br><br>unreported | Doxorubicin<br><br>unreported | RT-qPCR using a miRNome PCR panel | Unreported |
| Zhu et al., 2018; China [28] | Triple negative | 12 months | LVEF declined by 10% from baseline to below 53% | 179<br><br>45.9 ± 6.1 | 9<br><br>unreported | 170<br><br>unreported | Epirubicin<br>100 mg/m$^2$(treatment regimen), cyclophosphamide (600 mg/m$^2$), then followed by docetaxel (75-100 mg/m$^2$) | Epirubicin<br><br>unreported | Epirubicin<br><br>unreported | RT-qPCR | let-7b<br>let-7f<br>miR-17-5p<br>miR-17-3p<br>miR-18a<br>miR-19a<br>miR-19b-1<br>miR-20a<br>miR-92a<br>miR-126<br>miR-130a<br>miR-210<br>miR-296<br>miR-378 |

Abbreviations: cTnI: troponin I; cTnT: troponin T; HER-2: epithelial growth factor receptor 2; LVEF: left ventricular ejection fraction; RT-qPCR: reverse transcriptase quantitative polymerase chain reaction; SD: standard deviation.

standardized guidelines, the current study has some limitations. There were only five eligible studies in this systematic review. While we identified five miRNAs that are associated with the anthracycline-based cardiotoxicity, it was difficult to accurately evaluate their potential use for monitoring of early cardiotoxicity from chemotherapy because they have been reported in a few studies. Importantly, some studies lack a clear and detailed description of the studied population (e.g., histological classification of breast cancer type, number of patients, age), treatment (e.g., total or cumulative anthracycline dose) and different assessment of cardiotoxicity, including image evaluation (e.g., echography) or by the dosage of circulating markers (e.g., troponins). Notably, in almost studies, patients using cardioprotective drugs were not excluded and their beneficial effects on the cardiovascular system were not considered. Importantly, the presence of comorbidities is a factor that can accelerate cardiotoxicity, although the authors did not discuss these confounding factors or adjust the confounders for the analysis. Another limitation was the inclusion of studies that selected only miRNAs reported in the literature (e.g., miR-1). Indeed, the studies showed high variability in breast cancer treatment – including the use of other concomitant agents known to have cardiotoxic effects – and different follow-up periods. Therefore, in this systematic review, the limited number of studies with the same differentially expressed miRNA makes it impossible to perform a quantitative analysis of the data (meta-analysis). In addition, most studies did not report the raw or normalized miRNA expression data, only if the miRNA was significantly up- or down-regulated. Large prospective studies are needed to confirm the involvement of the five miRNAs identified in the current systematic review in breast cancer patients with anthracycline-induced cardiotoxicity. Moreover, it is important to conduct studies using screening methods like microarrays and/or RNAseq techniques in order to investigate additional miRNAs, related to other pathways in cardiotoxic process.

## 5. Conclusion

In conclusion, our systematic review showed five miRNAs (let-7f, miR-1, miR-20a, miR-126 and miR-210) with the potential to predict anthracycline-induced cardiotoxicity in breast cancer patients. These miRNAs and their targets participate in pathways of known relevance for cardiotoxicity pathogenesis, such as pro-angiogenesis and myocardial infarction. Moreover, analysis of the target genes found for the five miRNAs suggests that cellular responses to stress and signal transduction pathways may contribute to anthracycline-induced cardiotoxicity. To the best of our knowledge, this is the first systematic review investigating the differential expression of circulating miRNAs in breast cancer patients affected by anthracycline cardiotoxicity, considering their clinical potential as early prediction tools and prognostic markers.

## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110709.

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## Anexo C – Artigo II publicado em colaboração durante o desenvolvimento do doutorado

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# Global DNA methylation in placental tissues from pregnant with preeclampsia: A systematic review and pathway analysis

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ABSTRACT

Pre-eclampsia (PE) is the major cause of fetal and maternal mortality and can be classified according to gestational age of onset into early-onset (EOPE, <34 weeks of gestation) and late- (LOPE, ≥34 weeks of gestation). DNA methylation (DNAm) may help to understand the abnormal placentation in PE. Therefore, we performed a systematic review to assess the role of global DNAm on pathophysiology of PE, focused on fetal and maternal tissues of placenta from pregnant with PE, including EOPE and LOPE. We searched the databases EMBASE, Medline/PubMed, Cochrane Central Register of Controlled Trials, Scopus, Lilacs, Scielo and Google Scholar, and followed the MOOSE guidelines. Moreover, we performed pathway analysis with the overlapping genes from the included studies. Twelve out of 24 included studies in the qualitative analysis considered the classification into EOPE and LOPE. We did not found heterogeneity in the criteria used for diagnosis of PE, and a few studies evaluated whether confounding factors would influence placental DNAm. Fourteen out of 24 included studies showed hypomethylation in placental tissue from pregnant with PE compared to controls. The differences in DNAm are specific to genes or differentially methylated regions, and more evident in EOPE and preterm PE compared to controls, rather than LOPE and term PE. The overlapping genes from included studies revealed pathways relevant to pathophysiology of PE. Our findings highlighted the heterogeneous results of the included studies, mainly focused on North America and China. Replication studies in different populations should use the same placental tissues, techniques to assess DNAm and pipelines for bioinformatic analysis.

## 1. Introduction

Preeclampsia (PE) is defined as a new-onset hypertension (systolic blood pressure (SBP) ≥ 140 mmHg and diastolic blood pressure (DBP) ≥ 90 mmHg) after 20 weeks of gestation, which may be combined with proteinuria [1]. PE affects up to 9% of all pregnancies and is the major cause of fetal and maternal mortality and morbidity [2]. PE has a heterogeneous etiology and is classified according to gestational age of onset into late- (LOPE, ≥34 weeks of gestation) and early-onset (EOPE, <34 weeks of gestation), which is considered a more severe form of PE [3–5]. However, it is unclear whether EOPE and LOPE have different etiologies and pathogenesis or are the graduation of the same condition [6,7].

DNA methylation (DNAm) is primarily restricted at context of addition of a methyl group to the C5 position of the cytosine-guanine dinucleotide (CpG) [8]. Methylation of CpG sites are naturally associated with transcriptional repression when located in gene promoters, but with increased transcription when located in gene body [9,10]. Most of the human placental methylome is hypermethylated, but 37% of it is covered by partially methylated domains that are hypomethylated and constant through gestation and between individuals [11,12].

Notably, different cell types of placenta exhibit different transcriptional, epigenetic, and morphological features, which can conceal cell-specific signals and lead to spurious associations in different DNAm studies in PE [13]. In this context, epigenomic studies examining tissue or cell-specific signatures may contribute to understand both the normal and abnormal placentation processes, Therefore, it is relevant to assess the available DNAm data in placental tissues from PE pregnant.

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In this study, we aimed to perform a systematic review to assess the role of global DNAm in the pathophysiology of PE focused on the side of placental tissue evaluated and considering the classification into EOPE and LOPE. Moreover, we performed pathway analysis with the overlapping genes found in the included studies.

## 2. Materials and methods

This study was conducted according to The Cochrane Handbook for Systematic Reviews of Interventions guideline [14], and results will be reported in accordance with the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) checklist [15]. The protocol of current study was registered on International Prospective Register of Systematic Reviews (PROSPERO [CRD42020161780]).

### 2.1. Search strategy

The search question was composed by Population, Variable, Outcome (PVO) (Population = pregnant, Variable = preeclampsia, Outcome = methylation). A literature review was conducted by searching the electronic databases EMBASE, Medline/PubMed (Medical Literature Analysis and Retrieve System Online), Cochrane Central Register of Controlled Trials (CENTRAL), Scopus, Lilacs (Latin American and Caribbean Health Sciences), Scielo and Google Scholar to identify studies published until April 2020 that investigated PE, placenta, and global DNAm. The initial search included the Medical Subject Headings (MeSH) entry terms: 'Pregnancy', 'Pre-Eclampsia' and 'DNA Methylation', which were then included for a high-sensitivity search strategy in the Medline/PubMed, as described on Supplementary Material 1.

The same terms were used to search for gray literature and conference proceedings (Google Scholar). All potentially eligible studies were considered for review, regardless the language and publication date.

### 2.2. Inclusion and exclusion criteria

We included case-control studies including EOPE, LOPE or PE as case group, and control groups without chronic hypertension or gestational hypertension, gestational diabetes mellitus (GDM) and other well-known risk factors. The outcome was considered as the comparison of DNAm between PE and control groups.

We excluded studies that did not report the placental tissue or that evaluated only chorionic villous tissue, trophoblast cell lines, blood cells, and whole blood. We further excluded studies without matched control groups, or with control group composed of pregnancies with complications other than PE, such as GDM.

### 2.3. Study selection and data extraction

Initially, the studies retrieved from the databases were input on a single electronic library and duplicates were excluded using the EndNote® software. Two reviewers (J.O.C. and I.M.C.A.C.) independently analyzed the titles and abstracts of articles retrieved, reviewed the full-text articles, and used a standard data extraction protocol. Any disagreements were solved by a third reviewer (M.R.L.). The extracted data included the sample size, study design, maternal age, gestational age, tissue evaluated, applied technique, criteria for diagnosis of PE, and classification into EOPE and LOPE.

### 2.4. Assessment of bias across studies

The risk of bias in individual studies was independently assessed by two reviewers (J.O.C. and I.M.C.A.C.) following the Newcastle-Ottawa Quality Assessment Scale, according to The Cochrane Handbook's recommendations [14]. The tool used is structured into five domains: (1) patient selection (generalization and applicability); (2) comparability of groups in the study; (3) methods for assessing outcomes (cohort studies); (4) evidence of exposure (case-control) and (5) adequate follow-up. Any disparity was solved by a third reviewer (M.R.L.).

### 2.5. Pathway analysis

We manually curated the overlapping genes found in the included studies (Supplementary Table 1), and interrogated them for significant well-curated signaling pathways obtained from KEGG 2019 Human Pathway [16] sorted by p-value ranking <0.5 using Enrichr [17].

## 3. Results

We found 988 publications in the electronic databases (Fig. 1). After exclusion of 353 duplicates, 635 articles were selected for title and abstract analysis. Of these, 515 articles were subsequently excluded for several reasons (Fig. 1), resulting in 120 studies for complete reading. Literature reviews, studies focused on the analysis of specific genes, and studies that did not specify the tissue evaluated were also excluded. Finally, 24 full-text articles remained for the systematic review [18–41] (Table 1).

### 3.1. Included studies

Out of the 24 articles included, 10 (41.6%) had data from North American populations, including Canada and USA [18–22,25,31,35,36,40], eight (33.3%) from China [23,26,29,30,34,37,38,41], three (12.5%) from The Netherlands [24,32,33], and other three (12.5%) from India [28], Republic of Korea [27] and Australia [39]. Among the studies, seven (29.1%) validated their results in another independent cohort [20,25,26,29,36,40,41], and eight (33.3%) presented internal validation with the same samples but using a different technique [19,21–23,30,34,35,39]. Thirteen studies (54.2%) evaluated the fetal side of placenta [18–21,24–26,32–34,36,39,40], four (16.6%) used the maternal side [22,28,29,38], and seven (29.2%) did not specify the placental side used [23,27,30,31,35,37,41]. Twelve studies (50%) considered the classification into EOPE and LOPE [20,23–25,29,32–36,40,41], and 14 (58.3%) used the technique Infinium Human Methylation 450 Bead Chip array [18–20,22,24,25,27,31–36,39] (Table 1).

Regarding the quality assessment according to Newcastle-Ottawa scale, two studies scored nine points [36,40], 11 scored eight [19,20,23–26,28,29,32,33,35] and other 11 scored seven [18,21,22,27,30,31,34,37–39,41] (Supplementary Table 2). The bioinformatic analysis of global DNAm data greatly varied among the included studies. Therefore, a meta-analysis was not possible due to the heterogeneity of placental tissues evaluated, the applied technique used to assess global DNAm, and the different methods used for bioinformatic analysis, which hindered the quantitative comparison between the included studies.

Regarding the criteria used for PE diagnosis, 21 studies (87.5%) were based in evidence of new-onset hypertension (SBP $\geq$ 140 mmHg and DBP $\geq$ 90 mmHg) and proteinuria ($\geq$0.3 g/day or $\geq$2+ dipstick in urine sample of 24 h) after 20 weeks of gestation [18–22,24–36,39–41]. Five of these studies were based in these criteria plus others, such as maternal organ dysfunction, hematological disturbances and uteroplacental dysfunction [21,27,36,39,40]. One study (4.2%) were based in new-onset hypertension (SBP $\geq$160 mmHg and DBP $\geq$110 mmHg) and significant proteinuria (42 g or 3+ in urine sample of 24 h) after 20 weeks of gestation [23]. Two studies (8.3%) did not describe the criteria used for PE diagnosis [37,38] (Table 1).

Fourteen studies (58.3%) found a decreased DNAm level in placentas from PE pregnant compared to controls [20,22,24–27,29–35,38,40]. Although 22 studies (91.6%) used paired maternal age [18–20,22–36,38–41], 18 (75%) used gestational age as covariate for the DNAm analysis [18,20–22,24–27,29,31–36,39–41]. The ratio of male/female of infants varied among studies, and 12 (50%) used gender as covariate [20–22,24,25,29,32,33,35,36,39,40].

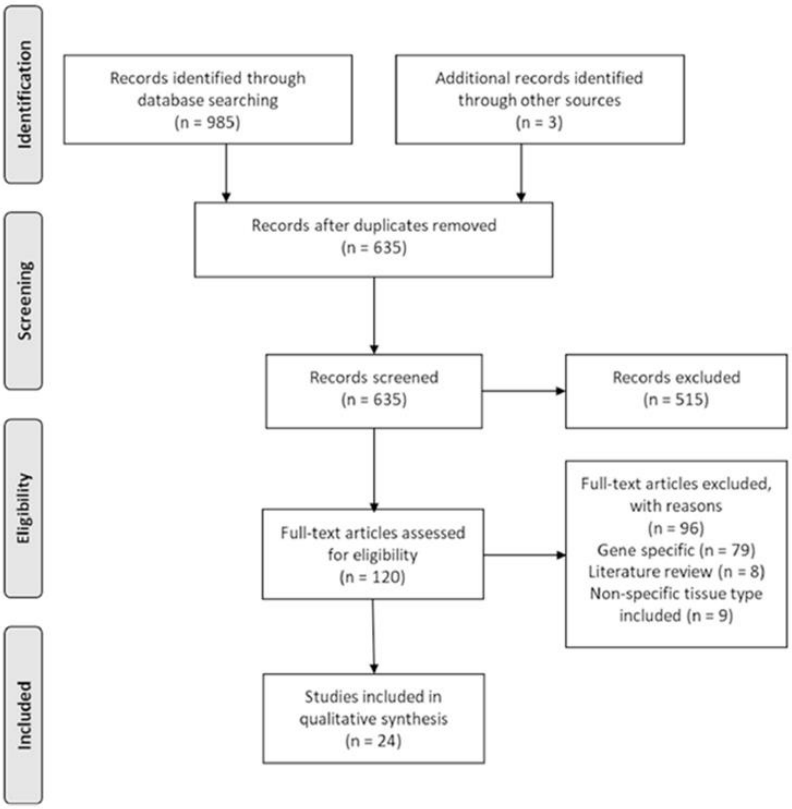


**Fig. 1.** Flow diagram of study selection for systematic review of published articles on the role of DNAm in placental tissue from pregnant with PE.

### 3.2. Global DNAm in placenta of PE pregnant compared to controls

Seven studies did not specify which side of placental tissue was analyzed [23,27,30,31,35,37,41] (Table 1). Four studies were case-control of PE pregnant compared to control, which found heterogeneous patterns of decreased DNAm levels in PE [27,30,31,37]. Remarkably, the number of differentially methylated genes (and the % of hypomethylated) reported were 3.878 (55.2%) [30], 1.664 (60.2%) [37], 617 (80.7%) [31] and 365 (89.9%) [27].

Notably, global DNAm was greatly discordant in maternal peripheral blood and placenta from PE pregnant, with 71 and 365 differentially methylated CpGs loci, respectively [27]. A total of 48 overlapping genes were found in the included studies (Fig. 2; Supplementary Table 1A), which were related to signaling pathways relevant to pathophysiology of PE (Fig. 3A).

Two studies found that global DNAm was significantly higher in EOPE compared to controls, but not statistically higher in LOPE compared to controls [23,41]. The methylation density in the Alu and LINE-1 repeats, and *H19* presented the same results for global methylation analysis [23]. Other study found 403 differentially methylated genes (68.2% hypermethylated) in placentas of LOPE [41].

*PAPPA* gene was exclusively hypomethylated in EOPE in other study, while the promoter and upstream enhancer regions of *INHBA* and *FN1* were hypomethylated in EOPE and LOPE + Intrauterine growth restriction groups. For these candidate genes, a positive correlation between DNAm and gene expression in placenta was found in case, but not in control group. The DNAm of *INHBA* and *FN1* was correlated with protein levels in maternal blood in the second and third trimester of gestation in PE, respectively [35]. Moreover, potential confounding factors in the assessment of DNAm showed an association of birthweight with *INHBA* and *FN1* methylation, and gestational age with *FN1* methylation [35].

### 3.3. Global DNAm in fetal side of placenta from PE pregnant compared to controls

Thirteen studies examined the DNAm in fetal side of placenta [18–21,24–26,32–34,36,39,40] (Table 1). Four case-control studies compared PE to controls [18,21,26,39]. One study did not find differentially methylated CpG sites between cases and controls. However, a significant correlation was found between methylation and gestational age corrected by birthweight, but no correlation with other clinical factors [21].

The methylation profiles of genes between studies were discordant in PE. For example, while one study found that 65.5% of 296 genes were hypomethylated [26], other study found that 70.6% of 303 genes were hypermethylated [39]. Notably, only *PPARG* (hypomethylated) and *ADORA2B* (hypermethylated) were commonly found in these studies [26,39]. In maternal peripheral blood, 207 CpG sites were differentially methylated (64% hypermethylated) in PE, and approximately 75% of them were concordant and hypermethylated in placenta [18].

Eight studies included the classification into EOPE and LOPE [20,24,25,32–34,36,40]. While 192 loci were hypomethylated in EOPE, none was differentially methylated in LOPE [40]. Conversely, 248 and 275 genes were differentially methylated (74.5% and 98.9% hypomethylated) in EOPE [20,34]. Multiple genes related to stress pathways and steroid production were associated with differentially methylated CpG sites in EOPE compared to controls. *NR3C1* and *CRHBP* were

**Table 1**
Characteristics of the studies examining global DNA methylation in placental tissues from pregnant with preeclampsia.

| Author; Data; Country. | Study design; Duration (years); Study validation status | No. of patients; mean age (years mean ± standard deviation) | | Mean gestational age (weeks mean ± standard deviation) | | Sample size in the global analysis | | Diagnosis criteria of PVO | Tissue evaluated | Applied technique |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Case | Control | Case | Control | Case | Control | | | |
| Anderson et al., 2014; USA [18] | Case-control study; NR; NR | PE: 6; 22.8 ± 1.4 | 6; 27.5 ± 3.65 | 38.4 ± 0.58 | 40 ± 0.49 | PE: 6 | 6 | New-onset hypertension (SBP≥ 140 mmHg or DBP≥ 90 mmHg) and proteinuria (≥+ 1 single sample or > 300 mg/24 h) after 20 weeks of gestation | Placental fetal side, white blood cells | Infinium HumanMethylation450 BeadChip array |
| Anton et al., 2014; USA [19] | Case-control study; 4; Internal validation | TPE: 19; 28.0 ± 8.1 PTPE: 12; 27.7 ± 7.6″ | 14; 27.0 ± 7.2 | TPE: 38.9 ± 1.0; PTPE: 31.2 ± 4.0 | 39.2 ± 1.2 | TPE: 19 PTPE: 12 | 14 | New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks | Placental fetal side | Infinium HumanMethylation450 BeadChip array |
| Blair et al., 2013; Canada [20] | Case-control study; NR; Independent validation | EOPE: 20; 33.5 ± NR | 20; 31.5 ± NR | 31.8 ± NR | 31.8 ± NR | EOPE: 20 | 20 | New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks gestation | Placental fetal side | Infinium HumanMethylation450 BeadChip array |
| Bourque et al., 2010; Canada [21] | Case-control study; NR; Internal validation | PE: 17; NR IUGR: 13; NR PE + IUGR: 21; NR | 22; NR | PE: 35.9 ± NR IUGR: 35.4 ± NR PE + IUGR: 32.5 ± NR | 39.0 ± NR | PE: 4IUGR: 5 | 5 | (1) New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks gestation (2) Sibai's criteria (3) British Eclampsia Survey Team criteria to define eclampsia | Placental fetal side | GoldenGate Methylation Cancer Panel 1 arrays |
| Chu et al., 2014; USA [22] | Case-control study; 10; Internal validation | PE; 24; 27.9 ± 7.2 | 24; 29.3 ± 5.4 | 35.9 ± 4.0 | 39.3 ± 1.2 | PE: 24 | 24 | New-onset hypertension (SBP ≥140 mm Hg and/or DBP ≥90 mm Hg) and proteinuria (≥300 mg of protein in 24 h or ≥2+ dipstick) after 20 weeks of gestation | Placental maternal side | Infinium HumanMethylation450 BeadChip array |
| Gao et al., 2011; China [23] | Case-control study; 2; Internal validation | EOPE: 10; 31.2 ± 5.1 LOPE: 10; 30.4 ± 3.7 | 24; 30.6 ± 4.1 | EOPE: 32.3 ± 1.2 LOPE: 36.8 ± 2.1 | 38.3 ± 18.4 | EOPE: 10 LOPE: 14 | 24 | New-onset hypertension (SBP of ≥160 mmHg or DBP of ≥110 mmHg) and significant proteinuria (42 g per 24 h or ≥3+) after 20 weeks of gestation | Placenta | Immunohistochemistry |
| Herzog et al., 2017; The Netherlands [24] | Case-control study; 2; NR | EOPE: 13; 30.0 ± 4.7 LOPE: 16; 33.3 ± 4.5 | Uncomp.: 36; 31.8 ± 5.1 FGR: 27; 29.7 ± 6.0 PTB: 20; 31.0 ± 5.1 | EOPE: 30.7 ± 3.4 LOPE: 37.4 ± 1.9 | Uncomp.: 39.9 ± 1.9 FGR: 38.9 ± 2.6 PTB: 35.4 ± 7.9 | EOPE: 13 LOPE: 16 | Uncomp.: 36 FGR: 27 PTB: 20 | New-onset hypertension (SBP≥140 and DSP ≥90 mmHg) and proteinuria (≥30 mg/mmol) after the 20 weeks of gestation | Placental fetal side, UC-WBC, HUVEC | Infinium HumanMethylation450 BeadChip array |
| Hogg et al., 2013; Canada [25] | Case-control study; NR; Independent validation | EOPE: 19; 34.2 ± 6.0 LOPE: 18; 33.5 ± 5.5 nIUGR: 13; 34.7 ± 5.3 | 111; 33.10 ± 4.74 | EOPE: 31.9 ± 3.3 LOPE: 37.5 ± 2.3 nIUGRn: 36.4 ± 2.3 | 35.1 ± 4.2 | EOPE:19 | 19 | New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/d or ≥2+ dipstick) after 20 weeks of gestation | Placental fetal side | Infinium HumanMethylation450 BeadChip array |
| Jia et al., 2012; China [26] | Case-control study; NR; Independent validation | PE: 9; 29.0 ± 2.9 | 9; 28.0 ± 2.6 | 35.0 ± 2.6 | 39.4 ± 0.2 | PE:3 | 3 | New-onset hypertension (SBP >140 mmHg and DBP >90 mmHg) with proteinuria (300 mg/24 h) after 20 weeks of gestation | Placental fetal side | Methylated DNA immunoprecipitation (MeDIP) |
| Kim et al., 2016; Republic of Korea [27]. | Case-control study; NR; NR | PE: 12; 32.3 ± 5.4 | 12; 31.6 ± 2.4 | 33.1 ± 3.3 | 33.1 ± 3.3 | PE:12 | 12 | New-onset hypertension (SBP ≥ 140 mmHg or DBP ≥ 90 mmHg) and proteinuria (>300 mg/day or >2+ dipstick) or other adverse conditions after 20 weeks of gestation | Placenta and peripheral blood | Infinium HumanMethylation450 BeadChip array |
| | | | 30; 22.9 ± 3.2 | | 39.2 ± 1.2 | | 30 | | | |

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**Table 1** (*continued*)

| Author; Data; Country. | Study design; Duration (years); Study validation status | No. of patients; mean age (years mean ± standard deviation) | | Mean gestational age (weeks mean ± standard deviation) | | Sample size in the global analysis | | Diagnosis criteria of PVO | Tissue evaluated | Applied technique |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Case | Control | Case | Control | Case | Control | | | |
| Kulkarni et al., 2011; India [28] | Case-control study; 2; NR | TPE: 30; 22.3 ± 3.0 PTPE: 27; 24.00 ± 3.7 | | TPE: 38.8 ± 0.9 PTPE: 34.0 ± 1.6 | | TPE: 30 PTPE: 27 | | New-onset hypertension (SBP >140 mmHg and DBP >90 mmHg) with proteinuria (>1þ or 300 mg/24 h) after 20 weeks of gestation | Placental maternal side | Methylamp Global DNA Methylation Quantification Kit |
| Li et al.,2020; China [29] | Case-control study; 1; Independent validation | EOPE: 20; 31.6 ± 4.6 | TB: 20; 32.8 ± 5.1PB: 20; 31.5 ± 4.9 | 32.8 ± 1.4 | TB: 39.1 ± 0.7; PB: 33.7 ± 1.7 | EOPE: 4 | TB:4; PB:4 | New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks of gestation | Placental maternal side | Infinium HumanMethylation850 BeadChip array |
| Liu et al., 2014; China [30]. | Case-control study; NR; Internal validation | PE: 27; 30.1 ± 2.7 GDM: 28; 30.8 ± 1.4 | 30; 29.7 ± 1.8 | PE: 37.7 ± 0.9 GDM: 36.2 ± 0.8 | 36.4 ± 0.5 | PE: 27 GDM: 28 | 30 | New-onset hypertension (SBP ≥ 140 and DBP ≥90 mmHg) and proteinuria (≥2+ or ≥ 300 mg in 24 h) after 20 weeks of gestation | Placenta | 385 K Human CpG Island plus Promoter arrays |
| Martin et al., 2015; USA [31] | Case-control study; NR; NR | PE: 19; 28.4 ± NR | 17; 28.2 ± NR | 38.6 ± NR | 32.8 ± NR | PE:19 | 17 | New onset hypertension (≥140/ 90 mmHg) and proteinuria (>300 mg of protein in a 24 h or protein/creatinine ratio of 0.3 mg/dL) after 20 weeks of gestation | Placenta | Infinium HumanMethylation450 BeadChip array |
| Van Den Berg et al., 2017; The Netherlands [32] | Case-control study; NR; NR | EOPE: 13; 30.0 ± 4.7 LOPE: 16; 33.3 ± 4.5 | Uncomp.: 36; 31.8 ± 5.1 FGR: 27; 29.7 ± 6.0 PTB: 20; 31.0 ± 5.1 | EOPE: 30.7 ± 3.4 LOPE: 37.4 ± 1.9 | Uncomp.: 39.9 ± 1.9 FGR: 38.9 ± 2.6 PTB: 35.4 ± 7.9 | EOPE: 13 LOPE: 16 | Uncomp.: 36 FGR: 27 PTB: 20 | New-onset hypertension (SBP≥140 and DBP ≥90 mmHg) and proteinuria (≥30 mg/mmol) after 20 weeks of gestation | Placental fetal side, UCL, HUVEC | Infinium HumanMethylation450 BeadChip array |
| Van Den Berg et al., 2020; The Netherlands [33]. | Case-control study; NR; NR | EOPE: 13; 30.0 ± 4.7 LOPE: 16; 33.3 ± 4.5 | Uncomp.: 36; 31.8 ± 5.1 FGR: 27; 29.7 ± 6.0 PTB: 20; 31.0 ± 5.1 | EOPE: 30.7 ± 3.4 LOPE: 37.4 ± 1.9 | Uncomp.: 39.9 ± 1.9 FGR: 38.9 ± 2.6 PTB: 35.4 ± 7.9 | EOPE: 13 LOPE: 16 | Uncomp.: 36 FGR: 27 PTB: 20 | New-onset hypertension (SBP≥140 and DBP ≥90 mmHg) and proteinuria (≥30 mg/mmol) after 20 weeks of gestation | Placental fetal side, UC-WBC, HUVEC | Infinium HumanMethylation450 BeadChip array |
| Wang et al., 2019; China [34] | Case-control study; NR; Internal validation | EOPE: 30; 31.23 ± 5.26 | 30; 30.1 ± 4.0 | 33.7 ± 3.5 | 39.1 ± 2.3 | EOPE:20 | 20 | New-onset hypertension (SBP ≥140 mmHg and DBP ≥90 mmHg) and proteinuria (≥300 mg/day from 24 h) after 20 weeks of gestation | Placental fetal side | Infinium HumanMethylation450 BeadChip array |
| Wilson et al., 2015; Canada [35] | Case-control study; NR; Internal validation | EOPE: 20; NR LOPE: 11; NR LOPE + IUGR: 8; NR IUGR: 10; NR | 37; NR | NR | NR | EOPE: 20 LOPE: 11 LOPE + IUGR: 8 IUGR: 10 | 37 | New-onset hypertension (SBP >140 and DBP >90 mm Hg) and proteinuria (>300 g/day) after 20 weeks gestation | Placenta | Infinium HumanMethylation450 BeadChip array |
| Wilson et al., 2018; Canada [36] | Case-control study; NR; Independent validation | EOPE: 22; 33.3 ± NR LOPE: 18; 34.0 ± NR IUGR: 11; 34.3 ± NR | PTC: 24; 32.5 ± NR TC: 19; 34.9 ± NR | EOPE: 32.0 ± NR LOPE: 37.4 ± NR IUGR: 36.6 ± NR | PTC: 32.6 ± NR TC: 38.4 ± NR | EOPE: 22 LOPE: 18 IUGR: 11 | PTC: 24 TC: 19 | New-onset hypertension (BSP >140 mmHg and >90 mmHg) and proteinuria (>300 mg/day) after 20 weeks gestation ii) HELLP syndrome without hypertension or proteinuria; or iii) eclamptic seizure without previous hypertension or proteinuria | Placental fetal side | Infinium HumanMethylation450 BeadChip array |
| Xuan et al., 2016; China [37] | Case-control study; NR; NR | PE: 6; 29.8 ± NR | 6; 30.2 ± NR | 38.0 ± NR | 39.5 ± NR | PE: 6 | 6 | Not reported | Placenta | NimbleGen Human DNA Methylation 3 × 720 K CpG Island Plus RefSeq Promoter Microarray |
| Yan et al., 2013, China [38] | Case-control study; 1; NR | PE: 30; 28.5 ± 3.8 | 30; 27.9 ± 3.0 | 36.1 ± 2.3 35.0 ± 0.8 | 39.2 ± 0.8 39.0 ± 0.2 | PE:5 PE:8 | 5 16 | Not reported | Placental maternal side | Agilent Human CpG Island Microarray |

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**Table 1** (*continued*)

| Author; Data; Country. | Study design; Duration (years); Study validation status | No. of patients; mean age (years mean ± standard deviation) | | Mean gestational age (weeks± mean ± standard deviation) | | Sample size in the global analysis | | Diagnosis criteria of PVO | Tissue evaluated | Applied technique |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Case | Control | Case | Control | Case | Control | | | |
| Yeung et al., 2016; Australia [39] | Case-control study; 13; Internal validation | PE: 8; 28.0 ± 2.0 | 16; 32.0 ± 1.0 | | | | | New-onset hypertension (SBP ≥140 mmHg and/or DSP ≥90 mmHg) and proteinuria (2 on dipstick or 300 mg/24 h) or renal insufficiency, liver disease, neurological problems, and hematological disturbances after 20 weeks of gestation | Placental fetal side | Infinium HumanMethylation450 BeadChip array |
| Yuen et al., 2010; Canada [40] | Case-control study; NR; Independent validation | EOPE: 4; 33.3 ± NR<br>LOPE: 4; 36.4 ± NR<br>IUGR: 4; 37.3 ± NR | EOPE: 4; 36.0 ± NR<br>LOPE: 5; 37.2 ± NR<br>C: 5; 35.9 ± NR | EOPE: 31.0 ± NR<br>LOPE 38.0 ± NR<br>IUGR 33.0 ± NR | EOPE: 29.6 ± NR.<br>LOPE: 38.0 ± NR.<br>C: 37.8 ± NR. | EOPE: 4<br>LOPE: 4<br>IUGR: 4 | EOPE: 4<br>LOPE: 5<br>C: 5 | (1) New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks gestation (2) Sibai's criteria (3) British Eclampsia Survey Team criteria to define eclampsia | Placental fetal side | Illumina GoldenGate Methylation Cancer Panel I array |
| Zhu et al., 2015; China [41] | Case-control study; NR; Independent validation | LOPE: 20; 27.7 ± NR | 20; 26.7 ± NR | 38.2 ± NR | 38.7 ± NR. | LOPE: 20 | 20 | New-onset hypertension (SBP ≥140 mmHg and/or DBP ≥90 mmHg) and proteinuria (≥0.3 g/day or ≥2+ dipstick) after 20 weeks of gestation | Placenta | Methylated DNA immunoprecipitation + deep sequencing |

Abbreviations: C, control; EOPE, early-onset PE; FGR, normotensive fetal growth restricted; GDM, gestational diabetes mellitus; HUVEC, human umbilical vein endothelial cells; IUGR, intrauterine growth restriction; NR, Not reported; LOPE, late-onset PE; nIUGR, normotensive Intrauterine growth restriction; PE, preeclampsia; PTB, normotensive preterm birth; PTC, preterm control; PTPE, preterm preeclampsia; TC, term control; TPE, term preeclampsia; UCL, umbilical cord leukocytes; UC-WBC, umbilical cord white blood cells; Uncomp., Uncomplicated; USA, United States of America.

hypermethylated, while regions associated with *CRH*, *CYP11A1*, *HSD3B1*, *TEAD3* and *CYP19* were hypomethylated in EOPE [25].

The comparison between EOPE and normotensive preterm births (PB) revealed 697 differentially methylated genes (67% hypomethylated) in placenta of EOPE. One differentially methylated CpG was found in EOPE compared to uncomplicated pregnancies controls and normotensive pregnancies with fetal growth restricted in placental tissue [24]. Significant differences in CpG methylation of circadian clock genes were found to be tissue-specific, in umbilical cord leukocytes (31), placenta (7), and HUVEC (1).

In placental tissue, the circadian clock genes *AKT1*, *BHLHE41*, *CSKN1E*, *PRDX1*, and *RORA* were hypomethylated in EOPE and significantly different from spontaneous PB [32]. The *CDH13*, *IGF2BP2* and *LSAMP* genes were also hypomethylated in placental tissue of EOPE and different from spontaneous PB. Notably, *CDH13* was hypermethylated in umbilical cord white blood cells of EOPE, and it was differentially methylated in EOPE compared to all study groups (uncomplicated controls, fetal growth restriction and PB) [33]. Other studies using the same set of samples found no difference in LOPE compared to all groups [24,31–33].

Noteworthy, six studies that examined the fetal side of placenta in EOPE or LOPE compared to controls had similar conclusions [20,24,25,32–34], and the number and methylation status of genes or regions are described above. In summary, five of these studies showed a pattern of hypomethylation in EOPE [20,24,32–34]. Differentially methylated CpGs sites were found in EOPE compared to controls [20,25,34]. EOPE were also different from spontaneous PB, fetal growth restriction and uncomplicated controls, and these differences were more evident when EOPE was compared to spontaneous PB controls [32,33]. Moreover, EOPE differed from spontaneous PB controls but not from fetal growth restriction or uncomplicated controls [24]. Notably, the differentially methylated sites were associated with cardiovascular system, stress pathways, steroid production and circadian clock genes. These findings suggest that EOPE have an increased placental dysregulation of DNAm, and support the hypothesis that EOPE and LOPE have different etiologies.

Most the 1.703 CpG sites were hypomethylated in EOPE compared to PB. Only five sites were differentially methylated between LOPE and term controls, which were not unique to LOPE [36]. Three studies showed that DNAm is affected by gestational age and fetus gender, which is a potential bias for DNAm analysis [20,25,40]. A total of 21




**Fig. 2.** Overlapping genes found in the included studies from PE pregnant compared to control groups (not specified the side of placental tissue evaluated).

overlapping genes were found among these studies (Fig. 4; Supplementary Table 1B), which were related to relevant pathways for PE (Fig. 3B).

Only one study defined preterm and term delivery for PE [19], and found 229 differentially methylated genes between controls and PE (term and preterm, 89.6% hypermethylated in PE) but none differentially methylated in term PE. Nevertheless, 1.448 differentially methylated genes were found between control and preterm PE (91.8% hypermethylated). Moreover, 118 differentially methylated genes were found between term and preterm PE (91.6% hypermethylated in term PE) [19].

### 3.4. Global DNAm in maternal side of placenta from PE pregnant compared to controls

Four studies considered the maternal side of placenta for DNAm analysis, and compared PE pregnant [22,29,38], term and preterm PE [28] to control, and one compared PE pregnant to PB and term birth (TB) [29].

While altered methylation levels were reported for 23 genes (52% hypermethylated) in PE [38], 10 hypomethylated CpG sites were identified in PE, and 49 differentially methylated CpG sites (78% hypomethylated) in EOPE [22]. The mean of global DNAm was higher in preterm and term PE compared to control, but the increase was significant only for term PE. Global DNAm was significant associated with SBP and DBP in term PE [28].

Global DNAm levels in placentas of PE were similar to PB, and the levels for PE and PB were higher than TB and, therefore, placental

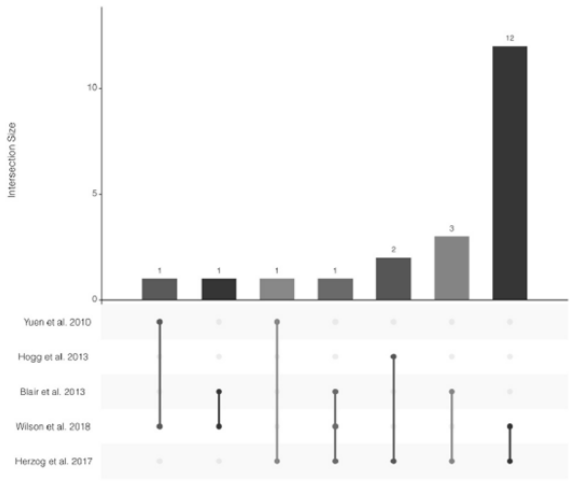


**Fig. 4.** Overlapping genes in the included studies in fetal side of placenta from EOPE and LOPE compared to control groups.

methylation levels were related to gestational age. Moreover, 2.400 differentially methylated genes were found between PE and TB (75.7% hypermethylated in PE), and 808 differentially methylated genes between PE and PB (68.8% hypomethylated in PE). Finally, 3.969

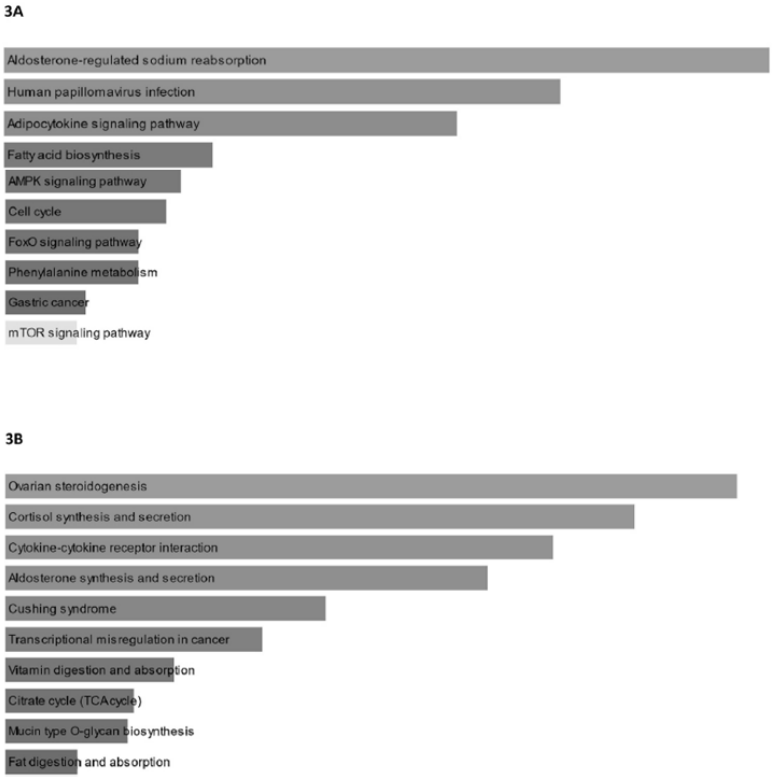


**Fig. 3.** Pathway analysis of the overlapping genes found among the included studies. **(A)** For studies performed with placental tissue from PE pregnant compared to controls (not specified the side of placental tissue). **(B)** For studies performed with fetal side of placenta from EOPE and LOPE compared to controls.

differentially methylated genes were found between PB and TB (80.4% hypermethylated in PB) [29].

## 4. Discussion

PE account for up to 26% of maternal deaths worldwide and is the main complication of pregnancy [3]. Therefore, it is important to understand the mechanisms that may lead to PE. This study is the first systematic review that assessed the role of global DNAm in the pathophysiology of PE, with focus on both maternal and fetal placental tissues. Our novel findings highlighted that the included studies show highly heterogeneous results, and that the differences between PE and controls are specific to genes or differentially methylated regions. These differences are more evident when EOPE and preterm PE were compared to controls, rather than LOPE and term PE. Most of the studies have a pattern of hypomethylation in placental tissue from PE pregnant compared to controls. Conversely, the placental methylome of normal pregnancy is hypermethylated outside the partially methylated domains [12].

It is important to highlight that tissues from the fetal or maternal side of placenta may account for the heterogeneous results of DNAm found among the studies included in this systematic review. We have also included studies that did not specify which side of placental tissue was analyzed, but their results were considered in a separate section. Therefore, the design of upcoming studies should focus on this methodological issue, which could help the understanding of the effects of different levels of DNAm on the different sides of placenta.

### 4.1. Confounding factors of DNA methylation in PE

The heterogeneous findings can be due to the multifactorial nature of PE, biological variation, study design and/or statistical analysis. Studies that examined whether confounding factors (age and fetal gender) would affect DNAm are discordant: some found an effect of maternal age and gestational age [20,22,31,35,39,40,42], but others did not [21]; some showed an effect of fetal gender [21,22,39,40,42], but another did not [21].

Placental DNAm showed a difference according to gestation stages [43], with a hypomethylation in early pregnancy and an increased methylation in later pregnancy [44]. A progressive increase of DNAm levels were found from the first to third trimesters, mainly in genes related to immune regulators, which reflect the placental immune modulation during pregnancy [45]. Therefore, the ideal design is to perform experiments with samples matched for both maternal and gestational age or follow-up studies to avoid and exclude this potential bias, and the exclusion of probes located in the sex chromosomes is required during the analysis, mainly in the X chromosome.

Genetic variation in different populations is another confounding factor that can alter DNAm in specific regions. For instance, probes that overlap with single nucleotide polymorphisms (SNPs) are usually excluded during bioinformatic analysis of global DNAm. However, SNPs that occur within CpG dinucleotide (CpG-SNPs) may lead to alteration of methylation in a region and thereby affect gene expression [46]. The correlation of CpG-SNPs with complex diseases is well-described [47], including type 2 diabetes [48] and cancer [49]. However, the association between CpG-SNPs and PE has not been examined. Further studies focused on potential CpG-SNPs and promoter region methylation may help to interpret findings from candidate gene association studies in PE for *NAMPT* [50,51], *NOS2* [52], *NOS3* [53] and *TIMP1* [54]. Potential CpG-SNPs of these candidate genes were also studied in subgroup of PE who were nonresponsive to antihypertensive therapy, and these follow-up epigenetic studies may further help to reveal targets for PE therapy [55,56].

### 4.2. Use of DNA methylation as a prognostic marker in PE

The placenta is a complex temporary organ that provides the fetal development, and composed by several number of cells and exhibiting regional variations. Placenta is divided into maternal and fetal sides, and the later carry the paternal genome [57]. A cohort analysis found that 35% of the genetic predisposition to PE is attributed to maternal characteristics and 20% to fetal effects that include the paternal genome, which suggest a genetic influence on PE development. The remaining is attributed to couple, environment and unmeasured factors [58].

DNAm is a tissue-specific epigenetic mark [59]. Notably, it is difficult to interpret DNAm data obtained from different pregnant related tissues and cell cultures. For example, only EOPE is related with levels of DNAm of circadian clock and clock-controlled genes in placental and newborn tissues. The same samples of placenta showed a decrease of differentially methylated CpG sites compared to umbilical cord leukocytes [32]. Approximately 75% of differentially methylated CpG sites overlapped between maternal white blood cell in first trimester compared to fetal side of placenta [18]. However, placental tissue showed an increase in differentially methylated CpG loci compared to peripheral blood at delivery [27].

Unfortunately, studies including the maternal side of placenta are scarce, and the DNAm data in the maternal and fetal sides of placenta showed highly discordant results. Therefore, to establish which placental side would be most suitable for studies focused on diagnostic markers for PE is unclear. Moreover, it is difficult to extrapolate the results from placenta to a description of diagnostic markers based on DNAm in the maternal blood. Further studies comparing DNAm between the maternal side of placenta and peripheral blood throughout gestation could help to establish novel diagnostic markers for PE.

### 4.3. Role of DNA methylation in the pathophysiology of PE

Our review highlighted a differential DNAm pattern in EOPE and preterm PE. These findings suggest that methylation have a pathophysiological role on early stages of pregnancy, and that placental epigenetic dysregulation may affect the initial steps of these early severe forms of PE. Indeed, EOPE was shown to exhibit a more severe form of PE [5], and a remarkable placental dysfunction [4].

The causes of PE are heterogeneous. Usually, there is a failure in the remodeling of the spiral uterine arteries by the trophoblastic cells, which can be triggered by an exacerbated immune response at the maternal-fetal interface [60]. These events are associated to poor placental perfusion, leading to physiological changes and gene expression in response to hypoxia and reoxygenation [61].

Transcriptional and epigenetic mechanisms control placental development and cytotrophoblast differentiation, and are activated by oxygen levels during pregnancy [62]. In primary cultures of human cytotrophoblasts and syncytiotrophoblasts, CpGs sites became hypermethylated in cytotrophoblasts exposed for 24 h to <1% oxygen. However, these same sites became hypomethylated upon differentiation of cytotrophoblast into syncytiotrophoblasts [63], and they showed hypomethylation in EOPE [20]. These findings suggest an imbalance of these cells in the expression of hypoxia-related genes in PE.

The number of overlapping genes among studies is low, ranging from 48 in PE versus controls in maternal side of placenta to 21 in EOPE, LOPE versus control in fetal side of placenta, and only *LIMCH1* is repeated in three studies. Some genes are well characterized in hypoxia and trophoblast invasion (*TERT*, *ALDH1A3*, *IRS1*), which are crucial during PE development. Other gene families frequently appear and are differentially methylated between study groups, such as *CXCL*, *SERPIN* and *TMEM*, which are involved in angiogenesis, inflammation, migration, cell proliferation, and invasion in types of cancer [64–66].

Altered methylation of specific genes was shown in PE (Supplementary Table 1). *LEP* [67], *PAPPA2* [68–70] and *YWHAQ* [71] showed increased expression and decreased DNAm in preeclamptic placentas.

Additionally, LEP and PAPPA2 protein was altered in maternal serum before the onset of PE symptoms [72,73], and had an increased expression in placentas from PE pregnant from the third trimester [42]. A decreased expression of *PLXNB1* in preeclamptic placentas may be responsible for the deficiency in Met signaling and in PE development [74].

The increased expression of *HSF1* in endothelial cells from term PE suggests a possible protective role as stress specific natural adaptive response against the generated stress [75,76]. *CRH*, *CYP11A1*, *TEAD3* showed an increased DNAm in preeclamptic placentas, suggesting a hormonal involvement in PE [25]. Moreover, the high expression of *CYP11A1* induces trophoblast autophagy, inhibits trophoblastic invasion and proliferation, as well as increases apoptosis [77,78].

Altered serum levels and placental tissue expression of CXC chemokines, including *CXCL9*, *CXCL10* and *CXCL12*, which participate in several processes triggered by PE, such as neovascularization, embryonic development and inflammatory responses, suggest their role in pathogenesis of PE [79]. Notably, these genes are related to ovarian steroidogenesis, cortisol synthesis and secretion, and cytokine-cytokine receptor interaction (Fig. 3B).

The pathway related to the overlapping genes is already described on process associated with PE, as trophoblast invasion. During pregnancy, AMP-activated protein kinase (AMPK) is necessary for the correct placental differentiation, nutrient transportation, maternal and fetal energy homeostasis, and protection of the fetal membrane. This activation is required for placental differentiation and vasodilation of uterine artery. Therefore, AMPK deficiency induces poor placentation, which results in angiogenic imbalance [80].

Many signaling pathways are involved in PE and are affected by oxidative stress, such as forkhead transcription factors of the O class (FOXO) family. Oxidative stress is responsible for the initiation or progression of pathological process in female reproduction, such as PE. The normal level of reactive oxygen species plays an important regulatory role through various signaling transduction pathways in folliculogenesis, corpus luteum oocyte maturation and feto-placental development, and FOXO is a bond of the different signaling pathway, playing an important role in signaling networks [81]. Insufficient spiral arteries remodeling in PE was associated to higher placental oxidative stress and the generation of oxidized fatty acids [82], as well as an increase of placental dimethyl acetal fatty acid [83], leptin, chemerin and fatty acid binding protein-4 in all pregnancy trimesters and forms of the disease [84].

## 5. Conclusion

In this systematic review, we found that there are significant differences on global DNA methylation levels between PE and controls, and a pronounced effect on DNAm of specific genes in PE, especially in EOPE and preterm PE. However, these studies should be replicated using the same placental tissues, and the same techniques and pipelines for bioinformatic analysis, in order to reduce variations between the studies. Biological variation cannot be avoided, so it is important to carry out studies in different populations, since the available results are mainly focused on samples from North America and China, and studies from literature have already shown the role of CpG-SNPs on epigenomic changes.

## Authors contributions

JOC, IMCA, JAGT, KBG, and MRL have made substantial contributions to the conception or design of the work. All authors have made contributions to the analysis or interpretation of data; All authors have drafted the manuscript or revised it critically for important intellectual content; All authors have read and approved the final version.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2020.09.004.

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**Anexo D – Artigo III publicado em colaboração durante o desenvolvimento do doutorado**

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SYSTEMATIC REVIEW
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# Do Genetic Polymorphisms Affect Fetal Hemoglobin (HbF) Levels in Patients With Sickle Cell Anemia Treated With Hydroxyurea? A Systematic Review and Pathway Analysis

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Hydroxyurea has long been used for the treatment of sickle cell anemia (SCA), and its clinical effectiveness is related to the induction of fetal hemoglobin (HbF), a major modifier of SCA phenotypes. However, there is substantial variability in response to hydroxyurea among patients with SCA. While some patients show an increase in HbF levels and an ameliorated clinical condition under low doses of hydroxyurea, other patients present a poor effect or even develop toxicity. However, the effects of genetic polymorphisms on increasing HbF levels in response to hydroxyurea in patients with SCA (Hb SS) have been less explored. Therefore, we performed a systematic review to assess whether single-nucleotide polymorphisms (SNPs) affect HbF levels in patients with SCA treated with hydroxyurea. Moreover, we performed pathway analysis using the set of genes with SNPs found to be associated with changes in HbF levels in response to hydroxyurea among the included studies. The systematic literature search was conducted on Medline/PubMed, EMBASE, Cochrane Central Register of Controlled Trials, Cumulative Index to Nursing and Allied Health Literature (CINAHL), Scopus, and Web of Science. Seven cohort studies were included following our inclusion and exclusion criteria. From the 728 genetic polymorphisms examined in the included studies, 50 different SNPs of 17 genes were found to be associated with HbF changes in patients with SCA treated with hydroxyurea, which are known to affect baseline HbF but are not restricted to them. Enrichment analysis of this gene set revealed reactome pathways with the lowest adjusted *p*-values and highest combined scores related to VEGF ligand–receptor interactions (R-HSA-194313; R-HSA-195399) and the urea cycle (R-HSA-70635). Pharmacogenetic studies of response to hydroxyurea therapy in patients with SCA are still scarce and markedly heterogeneous regarding candidate genes and SNPs examined for association with HbF changes and outcomes, suggesting that further studies are needed. The reviewed findings highlighted that similar to baseline HbF, changes in HbF levels upon hydroxyurea therapy are likely to

be regulated by multiple loci. There is evidence that SNPs in intron 2 of *BCL11A* affect HbF changes in response to hydroxyurea therapy, a potential application that might improve the clinical management of SCA.

**Systematic Review Registration:** (https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=208790).

**Keywords: *BCL11A* gene, fetal hemoglobin (HbF), genetic polymorphisms, hydroxyurea (HU) therapy, pathway analysis, pharmacogenetics, sickle cell anemia (SCA), systematic review**

## INTRODUCTION

Sickle cell anemia (SCA) is a global health problem, and approximately 300,000 infants are born with SCA every year (Azar and Wong, 2017). SCA is defined as a monogenic hemoglobin disorder caused by homozygosis for A-to-T transversion at codon 7 (c.20A > T, p.E7V) in the hemoglobin subunit beta (*HBB*) gene (den Dunnen and Antonarakis, 2000; Steinberg, 2008). The pathophysiology of SCA is directly related to polymerization of deoxygenated hemoglobin (HbS; α2βS2), leading to a cascade of pathologic events including erythrocyte sickling, vaso-occlusion, and hemolysis (Kato et al., 2018). It is important to note that higher levels of fetal hemoglobin (HbF; α2γ2) ameliorate clinical outcomes and hematological parameters of SCA, since it reduces HbS concentration and inhibit copolymerization between hemoglobin tetramers (Kato et al., 2018). Notably, higher persistent HbF concentration is often observed in patients with SCA than in subjects without SCA (Lettre and Bauer, 2016).

Hydroxyurea (HU) was approved by the U.S. Food and Drug Administration for the treatment of adults with severe SCA in 1998, and it has been associated with improved survival for both adults and children with SCA, as reviewed elsewhere (McGann and Ware, 2015). The clinical effectiveness of HU is related to the induction of the production of HbF, but it is not restricted to it. HU selectively kills cells in the bone marrow and increases the number of erythroblasts producing HbF, which inhibits the intracellular polymerization of HbS and prevents the sickling process in erythrocytes, thereby decreasing the number of sickled cells (McGann and Ware, 2015). Erythrocytes with high HbF have longer survival, thereby attenuating hemolysis (Steinberg, 1999). Furthermore, HU increases the hemoglobin levels; reduces neutrophils, monocytes, and reticulocytes; and alters the expression of adhesion molecules in the endothelium and the generation of nitric oxide. These hematological changes decrease the risk of vaso-occlusion in patients with SCA (Steinberg, 1999; McGann and Ware, 2015; Rigano et al., 2018).

Because HU has dose-related effects, the laboratory and clinical benefits of HU were shown to be optimized when dimensioned for the maximum tolerated dose (MTD). Almost all patients with SCA show a significant increase in HbF concentration at the MTD (McGann and Ware, 2015). The American Society of Hematology guideline panel suggests HU therapy with at least 20 mg/kg/day at a fixed dose or the MTD (DeBaun et al., 2020). However, there is substantial interpatient variability both in the MTD itself and in the percentage of HbF (% HbF) achieved (Ware et al., 2011; McGann and Ware, 2015). For example, the % HbF achieved with the MTD of HU reaches 10–15% in some patients, but it can reach 40% in other patients (Ware et al., 2011). Moreover, while some patients tolerate high HU doses, such as 30–35 mg/kg/day, others develop severe myelosuppression even at lower doses (Lettre et al., 2008). These findings suggest that important individual differences on pharmacokinetics and pharmacodynamics, and genetic factors contribute to the phenotypic variability in both the dosing and response to HU therapy (McGann and Ware, 2015). However, the effect of genetic polymorphisms on increasing HbF levels in response to HU therapy in patients with SCA has been less explored.

Therefore, the aim of the present study was to perform a systematic review to assess whether genetic polymorphisms affect HbF levels in patients with SCA treated with HU. In addition, we performed pathway analysis using the set of genes which had single-nucleotide polymorphisms (SNPs) that were found to be associated with changes in HbF levels in response to HU therapy among the studies included in the systematic review.

## MATERIALS AND METHODS

This study was conducted according to the Cochrane Handbook for Systematic Reviews of Interventions (Higgins et al., 2020), and the results were reported in accordance with the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) checklist (Stroup et al., 2000). The protocol of the current study was registered on the International Prospective Register of Systematic Reviews [PROSPERO (CRD42020208790); https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=208790].

### Search Strategy

The search strategy was defined based on the PECO question: Participants (P) = Sickle cell anemia patients (HbSS); Exposition (E) = Minor alleles; Control (C) = Major alleles of genetic polymorphisms and; Outcomes (O) = Fetal hemoglobin levels. A literature review was conducted by searching the electronic databases EMBASE, Medline/PubMed (Medical Literature Analysis and Retrieve System Online), Cochrane Central Register of Controlled Trials (CENTRAL), Cumulative Index to Nursing and Allied Health Literature (CINAHL), Scopus, and Web of Science (WoS) to identify studies published until July 2021. The initial search included the Medical Subject Headings (MeSH) entry terms: "Anemia, Sickle Cell"; and "Hydroxyurea"; and "Polymorphism, Genetic" or "Amplified Fragment Length Polymorphism Analysis" or "Polymorphism,

Single Nucleotide," or "Polymorphism, Restriction Fragment Length"; and "Fetal Hemoglobin," which were then included for a high-sensitivity search strategy in Medline/PubMed (**Supplementary Table S1**).

The same terms were used to search for gray literature and conference proceedings. The reference lists of included articles were also checked to identify additional relevant citations. All potentially eligible studies were considered for review, regardless of the language and publication date.

## Inclusion and Exclusion Criteria

The inclusion criteria were restricted to studies that described the pharmacogenetics of response to HU therapy in patients with SCA measured by HbF levels (primary outcome). We included only cohort studies that examined patients with the SS genotype, with a minimum age of three y at the time of HU initiation and with a minimum period of six months of HU therapy.

We excluded studies that did not differentiate patients with SCA from patients with another sickle cell disease (SCD), studies that focused on haplotypes and not on individualized SNPs, and studies that did not examine whether SNPs affect HbF levels in patients with SCA treated with HU. Review articles, conference proceedings, case reports, and commentary studies were also excluded.

## Study Selection and Data Extraction

Initially, the studies retrieved from the databases were input into a single electronic library, and duplicates were excluded using EndNote® software. Two reviewers (R.R.S. and B.L.N.) independently analyzed the titles and abstracts of the articles retrieved, reviewed the full text of the published articles, and used a standard data extraction protocol. Any disagreements between the two reviewers were resolved by a third reviewer (J.A.G.T.).

The extracted data from selected studies included study design, country, sample size, follow-up duration, median/mean age of participants, gender of patients, eligibility criteria, median/mean of HU dose, changes in HbF levels after HU therapy, genes, and polymorphisms associated with the primary outcome. The associated genes found in the included studies were used for pathway analysis.

## Assessment of Bias Across Studies

The quality assessment of included studies was carried out independently by two reviewers (R.R.S. and B.L.N.), following the approach of the Joanna Briggs Institute for the synthesis of evidence (Moola et al., 2020), and any disparity between the two reviewers was resolved by a third reviewer (J.A.G.T.). The approach indicates the application of critical assessment tools used in systematic reviews, in which the checklist for cohort studies is applied (Moola et al., 2020). The instrument is structured around eleven questions, in which the selected studies were evaluated: 1) the two groups were similar and recruited from the same population; 2) how they were similarly measured to designate exposed and unexposed groups as people; 3) exposure was measured in a valid and reliable manner; 4) confounding factors have been identified; 5) the instrument was created to deal with confounding factors; 6) the groups were free of the outcome at the beginning of the study; 7) the results were measured in a valid and reliable way; 8) the follow-up time was reported and long enough for the results to occur; 9) the follow-up was complete and, if not, whether the reasons for the loss of follow-up were obtained and explored; (10) the instrument was used to deal with incomplete follow-up; and 11) statistical statistics was applied. The answer options for signaling questions are 1) yes, 2) no, 3) unclear, and 4) not applicable (Moola et al., 2020).

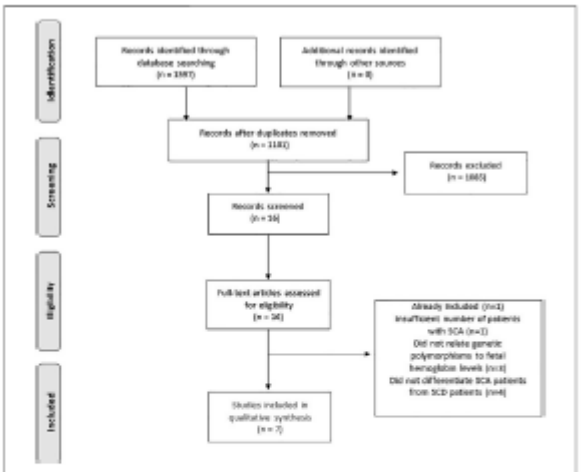


**FIGURE 1 |** Flow diagram of the literature search and the study selection for this systematic review of published articles related to the effect of genetic polymorphisms on HbF levels in patients with SCA (Hb SS) treated with hydroxyurea.

## Gene Set Enrichment Analysis and Pathway Analysis

After the data extraction, we manually curated the genes which had SNPs that were found to be associated with changes in HbF levels in patients with SCA treated with HU among the seven studies included in the systematic review (listed in **Table 2**). Next, we interrogated this gene set for significant well-curated signaling pathways obtained from the Reactome Pathway Knowledgebase (Jassal et al., 2020). The pathways found were sorted both by the adjusted *p*-values ranking <0.05, which were calculated using a Benjamini–Hochberg method (Benjamini and Hochberg, 1995), and the combined scores according to the gene set enrichment analysis web server Enrichr (Kuleshov et al., 2016; Xie et al., 2021).

# RESULTS

## Study Selection

We identified 1,597 records in the initial search (**Figure 1**). After the exclusion of duplicates, 1,101 articles were selected for title and abstract analyses. Of these, 1,085 articles were subsequently excluded due to the following reasons (as stated before in the "exclusion criteria"): 1) studies that focused on haplotypes rather than individualized SNPs; 2) studies that did not differentiate patients with SCA from patients with another SCD; or 3) studies

**TABLE 1 |** Characteristics of the seven cohort studies included in the systematic review, which examined the effects of genetic polymorphisms on fetal hemoglobin (HbF) levels in patients with SCA treated with hydroxyurea (HU).

| Author, data; country | Sample (n) | Average age (years)* | Gender (M/F) | Dose of HU (mean ± SD; mg/kg/day) | Time of follow-up on HU therapy (months) | HbF changes | HbF measurement | Number of genes (SNPs) studied | Multiple test correction |
|---|---|---|---|---|---|---|---|---|---|
| Friedrisch et al. (2016); Brazil | 111 | 21 ± 14 (from 4 to 54) | 38/62 | 23 ± 7.6 | Minimum of 6 | Δ MTD HbF (%)[a] | Capillary electrophoresis | 3 (6) | Not applied |
| Ware et al. (2011); United States | 88 | 9.6 ± 4.8 | 57/31 | 23.9 ± 5.1 | Minimum of 6 | Δ MTD HbF (%)[b] | HPLC | Not informed (331) | Applied |
| Aleluia et al. (2017); Brazil | 42 | 15.2 ± 11.1 | 70/71 | 15 (47.6%) 20 (23.8) 25 (26.2%)** | Mean of 13.4 ± 9.7 | Not informed | HPLC | 3 (6) | Not applied |
| Green et al. (2013); United States | 38 | 12.5 ± 4.9 | 57/60 | 25.3 ± 3.0 | Minimum of 6 | Δ HbF (%)[c] | HPLC | 9 (20) | Applied |
| Kumkhaek et al. (2008); United States | 32 | Not informed | Not informed | Not informed | Minimum of 8 | Δ HbF (% and g/dl)[b] | HPLC | 1 (20) | Not applied |
| Sheehan et al. (2014); United States | Discovery cohort (171) | 10.4 ± 4.5 | Not informed | 25.1 ± 4.5 | Minimum of 6 | Δ MTD HbF (%)[b]; Final HbF | HPLC | Whole exome | Unclear |
| | Validation cohort (130) | 8.1 ± 4.0 | Not informed | 27.1 ± 4.3 | Minimum of 6 | Δ MTD HbF (%)[b]; Final HbF | HPLC | 24 (25) | Unclear |
| Ma et al. (2007); United States | 137 | Not informed | Not informed | Not informed | Minimum of 21 | Δ HbF (% and g/dl)[a] | Alkali denaturation | 29 (320) | Not applied |

*Abbreviations: HbF, fetal hemoglobin; M, male; F, female. All selected studies were part of cohort studies.*
**age at the time of hydroxyurea initiation.*
***dose (case percentage).*
*[a](ΔHbF = MTD HbF—baseline HbF).*
*[b](ΔHbF = final HbF—baseline HbF).*
*[c](ΔHbF = maximum HbF—baseline HbF).*

that did not focus on SNPs related to HbF levels in patients with SCA treated with HU. Subsequently, 16 full-text articles were thoroughly assessed for inclusion. Following review, nine articles were removed due to the following reasons (**Figure 1**): One cohort study had an insufficient number of patients with SCA (Sclafani et al., 2016). Three studies did not assess whether the SNPs affected HbF levels (Italia et al., 2010; Zhu et al., 2017; Yahouedehou et al., 2020). Four studies did not differentiate patients with SCA from patients with SCD (Borg et al., 2012; Gravia et al., 2016; Chondrou et al., 2017; Elalfy et al., 2017). One study was part of an oral session and their results were later published in an original article already included in this systematic review (Wyszynski et al., 2004). Finally, seven cohort studies were included in this systematic review (Ma et al., 2007; Kumkhaek et al., 2008; Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017) (**Figure 1**).

## Characteristics of the Included Studies

Out of the seven included studies, five studies had data from the United States and two studies had data from Brazil. Sample size in the included studies ranged from 42 to 174 patients with SCA. The publication date ranged from 2007 to 2018, and the sample mean age ranged from 8.1 to 21 y. The mean dose of HU ranged from 19 to 27.1 ± 4.3 mg/kg/day. The mean duration of treatment with HU ranged from 13.4 to 102 months (**Table 1**). Two studies calculated the change in HbF levels for each patient from baseline to the MTD (delta HbF) (Ware et al., 2011; Friedrisch et al., 2016), while other four studies used the increment in HbF after treatment with HU (final HbF) (Ma et al., 2007; Kumkhaek et al., 2008; Green et al., 2013; Sheehan et al., 2014), and one study calculated from the baseline to maximum HbF during treatment with HU (Aleluia et al., 2017).

Overall, 728 genetic polymorphisms were assessed for their association with changes in HbF levels in patients with SCA under treatment with HU, and 11 candidate genes were the most examined in the seven included studies. Four studies examined *BCL11A* and the *HBSIL-MYB* intergenic region (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017). Three studies focused on arginase 1 and 2 (*ARG1* and *ARG2*) genes (Ma et al., 2007; Ware et al., 2011; Green et al., 2013). Two studies evaluated the secretion-associated Ras-related GTPase 1A (*SAR1A*) gene (Ma et al., 2007; Green et al., 2013). Two studies examined the *XmnI* gene (Ware et al., 2011; Friedrisch et al., 2016). The Fms-related receptor tyrosine kinase 1 (*FLT1*), hydroxyacid oxidase 2 (*HA O 2*), nitric oxide synthase 1 (*NOS1*), and olfactory receptor family 51 subfamily B member 5 and 6 (*OR51B5/6*), genes were mentioned only once by two studies (Ma et al., 2007; Aleluia et al., 2017).

Regarding the quality assessment according to the Joanna Briggs Institute checklist (**Supplementary Table S2**), one of the seven articles answered affirmative in all the 11 questions. Five studies responded affirmative to ten out of the 11 questions. One

study answered affirmative to six out of the 11 questions, while three of the questions were negative and two were not applicable.

## Pharmacogenetics of Response to HU Therapy in Patients With SCA

Among the included studies, a cohort study involving 137 adult African-Americans with SCA from the Multicenter Study of Hydroxyurea in Patients With Sickle Cell Anemia (MSH) examined the association of 320 tagging SNPs from 29 candidate genes with changes in HbF concentrations (measured by %, g/dL and F-cell count) after two years of HU treatment (Ma et al., 2007). Candidate genes were involved in the regulation of DNA transcription, cell proliferation/differentiation, and drug metabolism functions. The daily dose of HU started at 15 mg/kg and increased by 5 mg/kg each week up to the MTD, which means 35 mg/kg, unless toxicity was established. The authors found 17 SNPs to be associated with % HbF change and 20 SNPs to be associated with change of absolute HbF (g/dl), most of them being located in introns or untranslated genomic regions. The SNPs found to be associated with the higher mean of squared were rs2182008 in the *FLT1* gene and rs10483801 in the *ARG2* gene, which is involved in the metabolism of HU. This MSH cohort study performed analysis with age, sex, and *β-globin* haplotypes as covariates and showed several SNPs in other genes as predictors for HbF response. In the random forest algorithm, the SNP rs21822998 in *FLT1* and the SNP rs9376173 in the phosphodiesterase 7B (*PDE7B*) gene had a higher mean of squared residuals as predictors for % HbF and absolute HbF, respectively (Ma et al., 2007).

The hypothesis for a later pharmacogenetic study (Kumkhaek et al., 2008) was supported by an experimental study on the molecular mechanisms underlying the increase in HbF levels induced by HU (Tang et al., 2005). The authors searched for differential gene expression in human adult erythroid cells and identified a small guanosine triphosphate (GTP)–binding protein, whose secretion is associated with Ras-related (SAR) protein, as a specific gene induced by HU. SAR was shown to play a key role in γ-globin (*HBG*) gene induction by promoting cell apoptosis and G1/S-phase arrest by the reduction of PI3K and extracellular signal–regulated kinase phosphorylation and increasing p21 and GATA-binding protein 2 expression (Tang et al., 2005). From these experimental findings, variations of the *SAR1A* gene were hypothesized to explain differences in individual responses to HU treatment (Kumkhaek et al., 2008). The authors tested whether 20 variants in the upstream promoter region, exon 1, and intron 1 of *SAR1A* were associated with HbF changes in response to HU compared to baseline in 32 adults with SCA from the Sickle Cell Pulmonary Hypertension Screening Study, prospectively followed up during two y of HU therapy. The SNP rs231099 was found to be associated with the change in % HbF, and the SNPs rs2310991, rs76901216, rs76901216, and rs4282891 were found to be associated with the change in absolute HbF (g/dl). The intronic SNP rs4282891 showed stronger association, which is phylogenetically conserved in vertebrates (Kumkhaek et al., 2008).

The Hydroxyurea Study of Long-Term Effects (HUSTLE) was a prospective clinical trial for pediatric patients with SCA receiving HU designed to understand the interpatient variability in the responses and toxicities to HU (Ware et al., 2011). A candidate gene study was conducted to carry out pharmacogenetic analyses for the HU end points of % HbF and the MTD. The dose administrated in patients who were included before beginning HU therapy (new cohort; $n = 88$) started with 15 mg/kg/day, and it was escalated every eight weeks to a maximum dose of 30 mg/kg/day or the MTD, with careful monitoring of blood counts every two weeks. If hematologic toxicity occurred twice at the same dose, the MTD was set at 2.5 mg/kg below the toxic dose. Pharmacogenetic analyses included 331 SNPs in candidate genes that were selected based on their presumed pharmacogenetic and pharmacodynamic effects of HU. The *ARG1* rs175999586 and *ARG2* rs2295644 SNPs were associated with the change in % HbF between baseline and MTD. The SNP rs1427407 of the *BCL11A* gene was associated with the MTD, but none was associated with the MTD after adjustment for baseline % HbF (Ware et al., 2011).

The association of several SNPs with HbF levels induced by HU was also examined in a multi-site observational study of 117 pediatric patients (5–21 y), which was mainly composed of SCA patients (93% of HbSS and 7% of Sβ0-thalassemia) (Green et al., 2013). SNPs of *BCL11A*, *HBSIL-MYB*, *HBB*, hemoglobin subunit beta (*HBE*), *OR51B6*, glucagon-like peptide 2 receptor (*GLP2R*), *SAR1A*, *ARG1*, and *ARG2* genes, which were reported as associated with baseline HbF levels, were also examined for their association with HbF under HU therapy ("maximum HbF" and "delta HbF," from baseline to maximum). Clinical indications for HU therapy were comparable across sites (nearly all for repetitive painful crises and/or acute chest episodes) at least for six months. Stable dosing was reached at three months or near maximal dose by absolute neutrophil count criteria, excluding data from subjects on less than 20 mg/kg/day, even for dose-limiting toxicity. The SNPs of *BCL11A* (rs766432, rs11886868, rs4671393, and rs7557939), *HBE* (rs7130110), and *GLP2R* (rs12103880) were associated with maximum HbF under HU. Only the SNP rs7130110 of *HBE* was associated with delta (Δ) HbF (Green et al., 2013).

A cohort composed of 171 patients from the HUSTLE study and 51 patients from the Stroke with Transfusions Changing to Hydroxyurea (SWiTCH) (called "discovery") was examined to identify genetic predictors of HbF response to HU, with focus on protein coding regions (Sheehan et al., 2014). Whole-exome sequencing was performed in two prospective pediatric cohorts with robust HbF phenotype data and a standardized dose escalation regimen to the MTD, which were genotyped for SNPs of *BCL11A* (rs1427407, rs4671393, rs11886868, and rs7599488) and *HBSIL-MYB* (rs9399137 and rs9402686). HbF responses to HU were measured by maximum % HbF at the MTD ("final HbF") or the change in % HbF from baseline to final ("delta HbF"). The patients had baseline HbF measured after three y of age. The HU therapy initiated with 20 mg/kg, and then dose was escalated to mild myelosuppression. The average age of the patients at the time of HU initiation was 10.4 ± 4.5 y. However, they found no associations of *BCL11A* or *HBSIL-MYB* variants with HbF response. Whole-exome sequencing identified 13 and 12 variants associated with final HbF and delta HbF ($p$-value $< 5 \times 10^{-4}$), respectively. Although these

**TABLE 2** | Genes and chromosomes for the 50 different SNPs found to be associated with changes on HbF [described as delta (Δ) % HbF, Δ HbF (g/dl), maximum tolerated dose (MTD) % HbF, or maximum HbF] in response to hydroxyurea therapy in the seven cohort studies included in the systematic review. *The SNP rs17599586 of *ARG1* and three SNPs of *BCL11A* (rs1427407, rs4671393, and rs11886868) were found to be associated by two different cohort studies.

| Gene | Chromosome | SNP | HbF response | Reference |
|---|---|---|---|---|
| *ARG1* | 6 | *rs17599586 | Δ % HbF; Δ HbF (g/dL) | Ma et al. (2007) |
| *ARG1* | 6 | rs2781667 | Δ % HbF; Δ HbF (g/dL) | Ma et al. (2007) |
| *ARG1* | 6 | *rs17599586 | Δ % HbF | Ware et al. (2011) |
| *ARG2* | 14 | rs2246012 | Δ HbF (g/dl) | Ma et al. (2007) |
| *ARG2* | 14 | rs2295644 | Δ % HbF | Ware et al. (2011) |
| *ARG2* | 14 | rs10483801 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *ARG2* | 14 | rs10483802 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *ASS* | 9 | rs7860909 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *ASS* | 9 | rs10793902 | Δ % HbF | Ma et al. (2007) |
| *ASS* | 9 | rs10901080 | Δ % HbF | Ma et al. (2007) |
| *ASS* | 9 | rs543048 | Δ HbF (g/dl) | Ma et al. (2007) |
| *BCL11A* | 2 | rs766432 | Δ % HbF | Aleluia et al. (2017) |
| *BCL11A* | 2 | *rs1427407 | MTD % HbF; Δ % HbF | Friedrisch et al. (2016) |
| *BCL11A* | 2 | *rs4671393 | MTD % HbF | Friedrisch et al. (2016) |
| *BCL11A* | 2 | *rs11886868 | MTD % HbF; Δ % HbF | Friedrisch et al. (2016) |
| *BCL11A* | 2 | rs766432 | Maximum HbF | Green et al. (2013) |
| *BCL11A* | 2 | *rs11886868 | Maximum HbF | Green et al. (2013) |
| *BCL11A* | 2 | *rs4671393 | Maximum HbF | Green et al. (2013) |
| *BCL11A* | 2 | rs7557939 | Maximum HbF | Green et al. (2013) |
| *BCL11A* | 2 | *rs1427407 | MTD, mg/kg | Ware et al. (2011) |
| *FIGF* | X | rs6632521 | Δ % HbF | Ma et al. (2007) |
| *FLT1* | 13 | rs9319428 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *FLT1* | 13 | rs2182008 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *FLT1* | 13 | rs3751395 | Δ HbF (g/dl) | Ma et al. (2007) |
| *FLT1* | 13 | rs8002446 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *FLT1* | 13 | rs2387634 | Δ HbF (g/dl) | Ma et al. (2007) |
| *GLP2R* | 17 | rs12103880 | Maximum HbF | Green et al. (2013) |
| *HAO2* | 1 | rs10494225 | Δ % HbF | Ma et al. (2007) |
| *HBE* | 11 | rs7130110 | Maximum HbF; Δ % HbF | Green et al. (2013) |
| *MAP3K5* | 6 | rs9376230 | Δ % HbF | Ma et al. (2007) |
| *MAP3K5* | 6 | rs9483947 | Δ % HbF | Ma et al. (2007) |
| *NOS1* | 12 | rs7309163 | Δ HbF (g/dl) | Ma et al. (2007) |
| *NOS1* | 12 | rs816361 | Δ % HbF | Ma et al. (2007) |
| *NOS1* | 12 | rs7977109 | Δ % HbF; Δ HbF (g/dL) | Ma et al. (2007) |
| *NOS2A* | 17 | rs1137933 | Δ % HbF | Ma et al. (2007) |
| *NOS2A* | 17 | rs944725 | Δ % HbF | Ma et al. (2007) |
| *PDE7B* | 6 | rs2327669 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PDE7B* | 6 | rs11154849 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PDE7B* | 6 | rs9376173 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PDE7B* | 6 | rs1480642 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PDE7B* | 6 | rs487278 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PIR* | X | rs2071182 | Δ HbF (g/dl) | Ma et al. (2007) |
| *SALL2* | 14 | rs61743453 | Δ % HbF | Sheehan et al. (2014) |
| *SAR1* | 10 | rs2310991 | Δ % HbF; Δ HbF (g/l) | Kumkhaek et al. (2008) |
| *SAR1* | 10 | rs76901216 | Δ HbF (g/dl) | Kumkhaek et al. (2008) |
| *SAR1* | 10 | rs76901220 | Δ HbF (g/dl) | Kumkhaek et al. (2008) |
| *SAR1* | 10 | rs4282891 | Δ HbF (g/dl) | Kumkhaek et al. (2008) |
| *TOX* | 8 | rs2693430 | Δ HbF (g/dl) | Ma et al. (2007) |
| *TOX* | 8 | rs826729 | Δ % HbF | Ma et al. (2007) |
| *TOX* | 8 | rs765587 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *TOX* | 8 | rs9693712 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *TOX* | 8 | rs172652 | Δ % HbF | Ma et al. (2007) |
| *TOX* | 8 | rs380620 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *TOX* | 8 | rs12155519 | Δ HbF (g/dl) | Ma et al. (2007) |

to be associated with HbF changes in response to HU therapy in the included studies. There is evidence that SNPs of intron 2 of *BCL11A* affect HbF changes in patients with SCA treated with HU. Five out of the seven included studies examined the role of the three main QTLs associated with baseline HbF levels: *BCL11A*, *Xmn1*, and *HBS1L-MYB* intergenic region (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017). Noteworthy, BCL11A is a negative regulator of HbF expression. Subjects with variation in any of the established SNPs of *BCL11A* are known to have

associations did not achieve the genome-wide significance level ($p$-value < $1.3 \times 10^{-6}$), they did provide suggestive signals (Sheehan et al., 2014). By using functional prediction methods, the authors identified that 13 variants associated with HbF response to HU were predicted to introduce an amino acid change, inducing damage in the protein structure or function (Sheehan et al., 2014). These 13 variants were then genotyped in a validated cohort composed of 130 unrelated children with SCA receiving HU at Texas Children's Hospital Hematology Center for at least six months prior to the MTD. One variant (P840R; rs61743453) in the spalt-like transcription factor 2 (*SALL2*) gene was associated with higher delta HbF and with final HbF in the discovery and the validated cohorts, respectively. A meta-analysis combining the discovery and validation cohorts ($n = 301$) found that the P840R variant was associated with both delta HbF ($p = 8.30 \times 10^{-4}$) and final HbF ($p = 1.48 \times 10^{-4}$) (Sheehan et al., 2014).

A cohort of 121 patients with SCA under regular HU therapy for at least six months at the Sickle Cell Center of the Clinical Hospital from Porto Alegre (Southern Brazil) was examined for the effect of genetic variants at the major loci modifier of baseline HbF on HU-induced HbF levels (Friedrisch et al., 2016). Patients who received any other drugs stimulating HbF (e.g., erythropoietin) or blood transfusion within three months prior to the study were excluded. HbF measurements were obtained before HU (baseline HbF) and at the MTD (MTD HbF), and the change from baseline to the MTD (delta HbF) was calculated for each patient. The association tests were performed by linear regression analyses adjusted for age at start HU, gender, and absolute neutrophil count at MTD. SNPs of hemoglobin subunit gamma 2 (*HBG2*) (rs7482144), *BCL11A* (rs1427407, rs4671393, and rs11886868), and *HBS1L-MYB* (rs9399137 and rs9402686) were assessed, and patients with variations in SNPs of *BCL11A* had a favorable probability of producing more HbFs in response to HU treatment (Friedrisch et al., 2016).

A cross-sectional study of 141 patients with SCA, including 99 patients under HU treatment, followed up at the Sickle cell Disease Reference Center in Itabuna (Northeastern Brazil) was examined for the role of *HBB* haplotypes and SNPs at quantitative trait loci (QTL) associated with baseline HbF in regulating HbF response to HU (Aleluia et al., 2017). HbF measures were not performed in patients with clinical manifestations of vaso-occlusive crisis or transfusions in the last three months. Patients were genotyped for $\beta^S$-*globin* gene cluster haplotypes and SNPs of *BCL11A* (rs6732518 and rs766432), *HBS1L-MYB* (rs11759553 and rs3595442), and *OR51B5/6* (rs4910755 and rs7483122). Almost 48% of the patients received 15 mg/kg/day, while 23.8% received 20 mg/kg/day and 26.2% received 25 mg/kg/day. The only patient who received the maximum dose of 35 mg/kg/day was excluded from the analysis. Multiple linear regression analysis adjusted for gender and age were used to investigate the association of SNPs with HbF induction, and the authors concluded that homozygous subjects for the minor allele of rs766432 of *BCL11A* had higher increases in HbF (Aleluia et al., 2017).

In summary, seven studies involving patients with SCA treated with HU identified 50 SNPs of 17 different genes to be associated with HbF changes from baseline to HU (**Table 2**; **Figure 2**). Five out of the seven included studies examined SNPs of *BCL11A*, of which four (80%) found SNPs to be associated with HbF changes (Ware et al., 2011; Green et al., 2013; Friedrisch et al., 2016; Aleluia et al., 2017). These studies confirmed the associations of the *BCL11A* SNPs rs1427407 (Ware et al., 2011; Friedrisch et al., 2016), rs4671393, rs11886868 (Green et al., 2013; Friedrisch et al., 2016), rs766432 (Green et al., 2013; Aleluia et al., 2017), and rs7557939 (Green et al., 2013). In addition, two studies found associations for SNPs of *ARG1* (rs17599586, rs2781667, and rs1799586) and *ARG2* (rs2246012, rs2295644, rs10483801, and rs10483802) (Ma et al., 2007; Ware et al., 2011). Among them, only the SNP rs1799586 of *ARG1* was found to be associated with HbF changes in the two studies (Ma et al., 2007; Ware et al., 2011).

## Gene Set Enrichment Analysis and Pathway Analysis

Reactome pathways were obtained from the enrichment analysis using the set of genes that had SNPs found to be associated with changes on HbF levels in patients with SCA under HU therapy (**Figure 3**; **Supplementary Table S3**). The reactome pathways with both lowest adjusted $p$-values and highest combined scores were related to VEGF binding, namely, "VEGF ligand–receptor interactions" (R-HSA-194313; adjusted $p$-value = 0.0002847; combined score = 4,826.43) and "VEGF binds to VEGFR leading to receptor dimerization" (R-HSA-195399; adjusted $p$-value = 0.0002847; and combined score = 4,826.43). Moreover, we obtained the reactome pathway "urea cycle" (R-HSA-70635; adjusted $p$-value = 0.0003048; combined score = 3,461.84)] (**Figure 3**; **Supplementary Table S3**). The reactome pathway "nitric oxide stimulates guanylate cyclase" (R-HSA-392154; **Figure 3**) ranked fourth but with a lower combined score (200.68; $p$-value = 0.02105; **Supplementary Table S3**).

## DISCUSSION

Genetic variability in response to HU therapy is scarcely explored, despite almost 50 y of HU use and 30 y of the treatment of patients with SCA (Ware et al., 2011). Notably, the literature regarding the effects of genetic polymorphisms on HbF levels in patients with SCA (Hb SS) treated with HU is remarkably scarce. In this systematic review, only seven studies met the inclusion criteria.

Importantly, patient-specific factors, including age, concomitant diseases, diet, and genetic factors, contribute to the interindividual variability in drug efficacy and risk of adverse reactions, and genetic polymorphisms explain around 20–30% of the interindividual variability in drug response (Lauschke et al., 2017). Indeed, the knowledge of how genetic variation contributes to variation in drug response has been expanded (Gong et al., 2021), and guidelines for the clinical implementation of pharmacogenetics have been published (Relling et al., 2020).

## Single-Nucleotide Polymorphisms and HbF Modulation

As expected, some genes previously associated with baseline HbF and known for acting in the HbF regulation pathway were found

**TABLE 2** | Genes and chromosomes for the 50 different SNPs found to be associated with changes on HbF [described as delta (Δ) % HbF, Δ HbF (g/dl), maximum tolerated dose (MTD) % HbF, or maximum HbF] in response to hydroxyurea therapy in the seven cohort studies included in the systematic review. *The SNP rs17599586 of *ARG1* and three SNPs of *BCL11A* (rs1427407, rs4671393, and rs11886868) were found to be associated by two different cohort studies.

| Gene | Chromosome | SNP | HbF response | Reference |
|---|---|---|---|---|
| *ARG1* | 6 | *rs17599586 | Δ % HbF; Δ HbF (g/dL) | Ma et al. (2007) |
| *ARG1* | 6 | rs2781667 | Δ % HbF; Δ HbF (g/dL) | Ma et al. (2007) |
| *ARG1* | 6 | *rs17599586 | Δ % HbF | Ware et al. (2011) |
| *ARG2* | 14 | rs2246012 | Δ HbF (g/dl) | Ma et al. (2007) |
| *ARG2* | 14 | rs2295644 | Δ % HbF | Ware et al. (2011) |
| *ARG2* | 14 | rs10483801 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *ARG2* | 14 | rs10483802 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *ASS* | 9 | rs7860909 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *ASS* | 9 | rs10793902 | Δ % HbF | Ma et al. (2007) |
| *ASS* | 9 | rs10901080 | Δ % HbF | Ma et al. (2007) |
| *ASS* | 9 | rs543048 | Δ HbF (g/dl) | Ma et al. (2007) |
| *BCL11A* | 2 | rs766432 | Δ % HbF | Aleluia et al. (2017) |
| *BCL11A* | 2 | *rs1427407 | MTD % HbF; Δ % HbF | Friedrisch et al. (2016) |
| *BCL11A* | 2 | *rs4671393 | MTD % HbF | Friedrisch et al. (2016) |
| *BCL11A* | 2 | *rs11886868 | MTD % HbF; Δ % HbF | Friedrisch et al. (2016) |
| *BCL11A* | 2 | rs766432 | Maximum HbF | Green et al. (2013) |
| *BCL11A* | 2 | *rs11886868 | Maximum HbF | Green et al. (2013) |
| *BCL11A* | 2 | *rs4671393 | Maximum HbF | Green et al. (2013) |
| *BCL11A* | 2 | rs7557939 | Maximum HbF | Green et al. (2013) |
| *BCL11A* | 2 | *rs1427407 | MTD, mg/kg | Ware et al. (2011) |
| *FIGF* | X | rs6632521 | Δ % HbF | Ma et al. (2007) |
| *FLT1* | 13 | rs9319428 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *FLT1* | 13 | rs2182008 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *FLT1* | 13 | rs3751395 | Δ HbF (g/dl) | Ma et al. (2007) |
| *FLT1* | 13 | rs8002446 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *FLT1* | 13 | rs2387634 | Δ HbF (g/dl) | Ma et al. (2007) |
| *GLP2R* | 17 | rs12103880 | Maximum HbF | Green et al. (2013) |
| *HAO2* | 1 | rs10494225 | Δ % HbF | Ma et al. (2007) |
| *HBE* | 11 | rs7130110 | Maximum HbF; Δ % HbF | Green et al. (2013) |
| *MAP3K5* | 6 | rs9376230 | Δ % HbF | Ma et al. (2007) |
| *MAP3K5* | 6 | rs9483947 | Δ % HbF | Ma et al. (2007) |
| *NOS1* | 12 | rs7309163 | Δ HbF (g/dl) | Ma et al. (2007) |
| *NOS1* | 12 | rs816361 | Δ % HbF | Ma et al. (2007) |
| *NOS1* | 12 | rs7977109 | Δ % HbF; Δ HbF (g/dL) | Ma et al. (2007) |
| *NOS2A* | 17 | rs1137933 | Δ % HbF | Ma et al. (2007) |
| *NOS2A* | 17 | rs944725 | Δ % HbF | Ma et al. (2007) |
| *PDE7B* | 6 | rs2327669 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PDE7B* | 6 | rs11154849 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PDE7B* | 6 | rs9376173 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PDE7B* | 6 | rs1480642 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PDE7B* | 6 | rs487278 | Δ HbF (g/dl) | Ma et al. (2007) |
| *PIR* | X | rs2071182 | Δ HbF (g/dl) | Ma et al. (2007) |
| *SALL2* | 14 | rs61743453 | Δ % HbF | Sheehan et al. (2014) |
| *SAR1* | 10 | rs2310991 | Δ % HbF; Δ HbF (g/l) | Kumkhaek et al. (2008) |
| *SAR1* | 10 | rs76901216 | Δ HbF (g/dl) | Kumkhaek et al. (2008) |
| *SAR1* | 10 | rs76901220 | Δ HbF (g/dl) | Kumkhaek et al. (2008) |
| *SAR1* | 10 | rs4282891 | Δ HbF (g/dl) | Kumkhaek et al. (2008) |
| *TOX* | 8 | rs2693430 | Δ HbF (g/dl) | Ma et al. (2007) |
| *TOX* | 8 | rs826729 | Δ % HbF | Ma et al. (2007) |
| *TOX* | 8 | rs765587 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *TOX* | 8 | rs9693712 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *TOX* | 8 | rs172652 | Δ % HbF | Ma et al. (2007) |
| *TOX* | 8 | rs380620 | Δ % HbF; Δ HbF (g/dl) | Ma et al. (2007) |
| *TOX* | 8 | rs12155519 | Δ HbF (g/dl) | Ma et al. (2007) |

to be associated with HbF changes in response to HU therapy in the included studies. There is evidence that SNPs of intron 2 of *BCL11A* affect HbF changes in patients with SCA treated with HU. Five out of the seven included studies examined the role of the three main QTLs associated with baseline HbF levels: *BCL11A*, *XmnI*, and *HBS1L-MYB* intergenic region (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016; Aleluia et al., 2017). Noteworthy, BCL11A is a negative regulator of HbF expression. Subjects with variation in any of the established SNPs of *BCL11A* are known to have

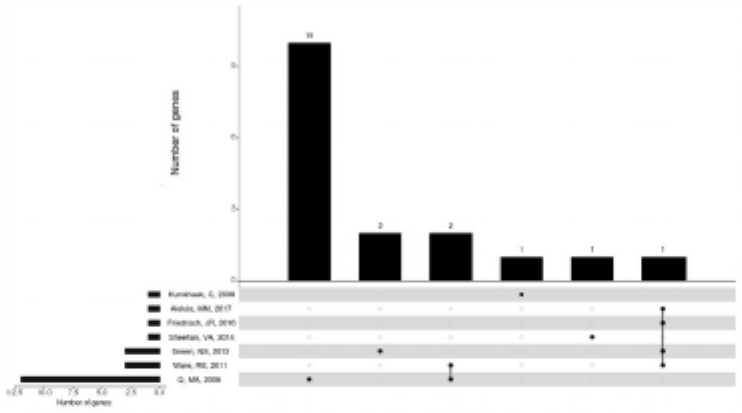


**FIGURE 2 |** Overlapping genes with SNPs associated with changes on HbF levels in the included studies involving patients with SCA (Hb SS) under HU therapy. *BCL11A*, *ARG1*, and *ARG2* genes showed overlap between included studies. Upset plot showing the total number of genes with SNPs found to be associated with changes on HbF levels identified in each study (horizontal bars), and the number of genes exclusive to one study or shared by different studies (vertical bars). Black dots below vertical bars indicate genes quantified.

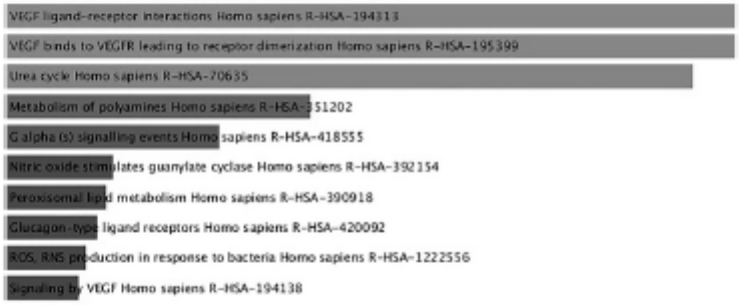


**FIGURE 3 |** Reactome pathways obtained from enrichment analysis of the set of genes with SNPs found to be associated with HbF changes in patients with SCA treated with hydroxyurea. Pathways are ordered from top to bottom according to the lowest adjusted *p*-values, and light red bars indicate adjusted *p*-values < 0.001.

decreased *BCL11A* expression, which results in increased HbF production (Lettre et al., 2008; Bauer et al., 2013). *HBS1L-MYB* genes are expressed in the erythroid precursor cells (Lettre et al., 2008; Bauer et al., 2013). *HBS1L* encodes a protein with apparent GTP-binding activity and is involved in a variety of cellular processes, while *MYB* encodes a transcription factor for erythroid differentiation in hematopoiesis (Thein et al., 2007). The *HBS1L-MYB* intergenic region is known to contain several common QTLs associated with HbF levels and a long-range erythroid enhancer that regulates *MYB* expression by chromatin looping (Stadhouders et al., 2014). Finally, the *Xmn1* restriction site at -158 position of the *HBG* gene is associated with an increased expression of γ-globin and higher HbF production (Sripichai and Fucharoen, 2016). Together, they account for approximately 20–50% of the variation in HbF levels in patients with SCA and β-thalassemia, and even in healthy adults (Galarneau et al., 2010).

Four out of five studies that examined SNPs of *BCL11A* found associations with HbF response (Ware et al., 2011; Green et al., 2013; Sheehan et al., 2014; Friedrisch et al., 2016). These SNPs (rs1427407, rs4671393, rs11886868, rs766432, and rs7557939) are located in intron 2 of *BCLL1A*, which is a region marked by functional elements. These SNPs are located nearby several DNase hypersensitive sites, which indicate a genomic region of open chromatin. Noteworthy, the critical SNP rs1427407 (G > T) falls within a +62 DNase I hypersensitive site, an erythroid enhancer of *BCL11A*, and overlaps a peak of GATA1 and TAL1 transcription factor–binding sites. Notably, the minor T-allele for the SNP rs1427407 disrupts the G nucleotide of a consensus sequence [CTG (n9)] enriched for GATA1 and TAL1 transcription factors. GATA1- and TAL1-binding sites are more frequent in the G-allele than T-allele in the primary erythroblast samples (Bauer et al., 2013). In agreement with our findings regarding the effect of SNPs of *BCL11A* on HbF response, a recent

functional *in vitro* study based on gene editing comparative analysis showed that *BCL11A* is the most clinically relevant approach focused on HbF resurgence (Lamsfus-Calle et al., 2020). This functional information supports the effect of *BCL11A* SNPs on baseline HbF, but the way it affects the response to HU remains to be elucidated. Biological network analysis integrating effects of HU on gene expression in erythroid precursors could highlight pathways involved in this process.

The *XmnI* variant in the *HBBP1* gene was also examined. One study found the SNP rs7482144 to be associated only with an increase in baseline HbF (Ware et al., 2011) but not with HbF changes to HU, while other studies excluded this SNP because it had a very low allele frequency (0.45%, only one heterozygous subject) in a Brazilian cohort (Friedrisch et al., 2016).

The SNPs rs9399137 and rs9402686 are located in the *HBS1L-MYB* intergenic region but were not found to be associated with the increase in HbF in two included studies (Sheehan et al., 2014; Friedrisch et al., 2016). *SAR1A*, a gene belonging to the small GTPase superfamily that encodes a GTP-binding protein called SAR1A, has been reported to be associated with *HBG* expression. The SNP rs2310991 of *SAR1A* was associated with the change in absolute HbF concentration (Kumkhaek et al., 2008). Conversely, other studies found no association of rs2310991 with posttreatment HbF levels (Kumkhaek et al., 2008; Green et al., 2013).

Our findings may potentially guide the selection of candidate gene regulatory sequences within these genomic regions to be validated by *in vitro* functional assays in cells treated with HU, such as luciferase reporter assays. Further studies may examine whether the variation in these SNPs would affect the activity of the gene regulatory element, such as an enhancer or a silencer. Therefore, the present findings can contribute to guide further functional studies, which may advance the research focused on genomics of HbF changes in response to HU therapy.

## Signaling Pathways Underlying HbF Changes in Response to HU Therapy

Pathway analysis using genes with SNPs found to be associated with HbF changes in patients with SCA treated with HU in the included studies revealed pathways underlying HbF changes in response to HU. For example, we found enrichment of the reactome pathway "urea cycle" (R-HAS-70635; **Figure 3**), which is directly related to arginine (Friebe and Koesling, 2003). Indeed, cytosolic arginase 1 is a canonical enzyme of the urea cycle. Arginase 2 was described to play a role in the regulation of the urea cycle arginine metabolism and in downregulation of nitric oxide synthesis (Mori, 2007). Arginase isoforms encoded by *ARG1* and *ARG2* genes were also related to the increase in HbF levels induced by HU (Ware et al., 2011). The SNPs rs175999586 and rs2295644 of *ARG1* and *ARG2* were associated with the changes in HbF, respectively. Notably, rs2295644 has been implicated in kidney disease, so it could affect the renal clearance of HU and possibly the dose of the MTD (Ware et al., 2011). Another *ARG2* SNP (rs10483801) was also associated with the absolute HbF change (Ma et al., 2007).

We also found the enrichment of the reactome pathway "nitric oxide stimulates guanylate cyclase" (R-HSA-392154; **Figure 3**). Noteworthy, HU was suggested to act as a nitric oxide donor in patients with SCA (King, 2004). Nitric oxide is synthetized from L-arginine, stimulates vasodilatation of the endothelium and disaggregation of platelet aggregates, and inhibits platelet activation, an important modulator of SCA pathophysiology (Radomski et al., 1987). Moreover, HU was shown to modulate the nitric oxide signaling pathway in erythrocytes, rheology of erythrocytes, and oxidative stress through its effects on HbF and possibly on nitric oxide bioavailability (Nader et al., 2018).

A complex regulatory environment determines the HbF concentration in the blood, as well as chromosome remodeling, transcription factors, erythropoiesis modulation, gene regulatory elements linked to the $\beta$-globin gene cluster, and the kinetics of erythroid cell differentiation and differential red cell survival (Ma et al., 2007). Therefore, there is a large opportunity for the genetic modulation of HbF production. Consistent with this complex regulation apparatus, even with the restricted number of studies, our systematic review suggests that there is huge heterogeneity in genetic elements modulating the HbF levels in response to HU treatment. Unfortunately, some genetic associations with HbF response have not been reproduced by other studies, and further investigations are needed to conclude their use in predicting HbF response to HU.

Dosing and monitoring regimens of HU have yet to be determined (Ware, 2010). The best results from treatment with HU are found when the dose is escalated to the MTD, improving laboratory variables and reducing clinical complications. The dose escalation of HU is a labored process that requires risk monitoring of cytopenias, mainly neutropenia, and the clinical response to treatment with HU may take up to six months after reaching the MTD (NIH US, 2014). Therefore, severe patients with clinical recommendation for HU might have to experience a long exposure time until deducing that the treatment with HU is ineffective. Therefore, the prediction of HbF induction in response to treatment with HU by using SNPs in the intron 2 of *BCL11A* gene may have potential clinical applicability in the management of SCA.

The induction of HbF is a powerful mechanism of action of HU. However, since several other mechanisms of actions are known, further research is needed to conclude whether such SNPs are able to predict a subgroup of patients as "responders" to HU. Noteworthy, these SNPs were previously associated with increased baseline HbF levels, milder hematological parameters, and lower risk of clinical complications (Sales et al., 2020). Interestingly, not all these associations were dependent on HbF. Therefore, future studies should evaluate if the SNPs located in intron 2 of the *BCL11A* gene are also able to distinguish patients who show a reduced rate of clinical complications when treated with HU from those patients who do not show this reduction. This evidence is of huge importance to assess the cost-effectiveness of the use of pharmacogenetic tests for these SNPs in the SCA management.

Some studies do not meet the inclusion criteria of this review due to the different genotypes of the study subjects (Borg et al., 2012; Chondrou et al., 2017; Elalfy et al., 2017). They involve other β-type hemoglobinopathies, and known differences in their hematological parameter could bias the review. However, these studies highlight specific points regarding pathways related to the HbF regulation. Two studies suggested that *KFL1* expression and the SNP rs3191333 of *KFL1* play a role in the HbF regulation and are biomarkers of HU response in β-type hemoglobinopathies. It makes biological sense, since KLF1 regulates *BCL11A* expression and the γ- to β-globin gene switching (Zhou et al., 2010). Further studies can confirm their influence in HU therapy in patients with SCA (Hb SS).

Another study suggests that the vascular endothelial growth factor (*VEGFA*) gene is a biomarker in β-type hemoglobinopathies severity and efficacy of HU therapy (Chondrou et al., 2017). These findings are in agreement with a study included in this systematic review that found SNPs in the *FLT1* gene, encoding VEGF receptor 1, associated with HbF changes by HU therapy in Hb SS patients (Ma et al., 2007). Interestingly, we found enrichment of two reactome pathways related to VEGF ligand–receptor interactions (R-HSA-194313 and R-HSA-195399; **Figure 3**). The binding of VEGF ligands to VEGFR receptors in the cell membrane triggers intracellular signaling cascades, which results in proliferation, survival, migration, and increased permeability of vascular endothelial cells (Matsumoto and Mugishima, 2006). It is important to SCA pathophysiology, since endothelial dysfunction plays a key role in sickle cell vasculopathy, as reviewed elsewhere (Wood et al., 2008).

Our systematic review highlighted the role of SNPs on HbF induction upon HU therapy. However, it is important to note that this is one of the several mechanisms underlying response to HU. Indeed, a previous systematic review reported on the molecular mechanisms of HbF induction by HU in SCD (Pule et al., 2015). The reviewed findings pointed out three main pathways: epigenetic modifications, signaling pathways involving HU-mediated response, and posttranscriptional pathways, focusing on regulation by small non-coding RNAs (miRNAs). In this context, several miRNAs were identified as differentially expressed in patients with SCD under HU treatment, most of them being functionally related to genes known to regulate HbF, including *BCL11A* (Mnika et al., 2019). Notably, an experimental study showed that downregulation of *BCL11A*, *MYB*, and *KLF1* induces *γ-globin* expression by miRNA-mediated mechanisms, and miR-26b directly interacted with the 3′-untranslated region of *MYB* (Pule et al., 2016). Since miRNAs have been associated with a multitude of regulatory mechanisms, their functions may add to the complex mechanisms underlying response to HU.

In summary, the regulation of HbF involves both *cis*- and *trans*-regulatory elements, which interact in a complex network. HU promotes the induction of HbF, and the mechanisms by which it interacts with genetic modifiers of HbF affecting drug response are not fully understood. In this context, SNPs located within gene regulatory elements can have a major effect on differences in drug response (Luizon and Ahituv, 2015). A proposed schematic diagram to HbF regulation in response to HU is shown in **Figure 4**, including the functional findings of genes found in this systematic review as candidates to modify the HbF response to HU in patients with SCA.

## Confounding Factors

The first clinical manifestations of SCA appear along with the replacement of HbS by HbF (Rumaney et al., 2014). After 10 y, age is no longer an indicator of red cell deformability in patients with SCA; instead, this hemorheological parameter is mainly affected by the level of HbF, sex, and HU treatment (Thein et al., 2007).

The level of HbF is the best predictor of clinical severity of SCA (Wonkam et al., 2014). However, there is no threshold or value that characterizes the high and low baseline HbF levels for response to HU treatment. It was established that subjects who start with baseline HbF values between 5 and 10% can have a 2- to 3-fold HbF increase, whereas subjects with very low baseline HbF can have a 10-fold increase after treatment with HU (Wonkam et al., 2014). In the MSH cohort study, the baseline HbF was not predictive to HbF response to HU. On the other hand, baseline HbF was found as a predictor of the direction of association to % HbF at MTD (Ware et al., 2011). However, the change in HbF outcome in patients with SCA treated with HU was largely heterogeneous among the seven included studies, which examined different HbF outcomes in response to HU. Changes in HbF upon HU therapy was calculated as absolute HbF (g/dl), % HbF, and F-cell count from baseline until the MTD or a defined time of therapy (about 2 y). Although the % HbF and the amount of F cells are highly correlated, some patients with high levels of HbF develop severe complications of SCA probably due to the heterogeneous distribution of HbF among erythrocytes (Khandros et al., 2020). The number of F cells with polymer-inhibiting concentrations of HbF is likely to be a more accurate predictor of clinical benefits of HU therapy than HbF levels. However, the distribution of HbF among F cells is often unavailable, mainly in health centers of least developed countries. Using HbF under the MTD to calculate delta HbF probably provides the maximum level that the patient can achieve.

Patients with SCA experience several acute clinical events involving pronounced changes in hematological parameters (Novelli and Gladwin, 2016). Moreover, they commonly receive blood transfusion for treating and avoid a range of complications, which introduces biases on evaluating the association with hematological variables, including HbF. However, three of the seven included studies did not describe whether strategies were used to deal with these established confounding factors, which introduces bias in our analyses and constitute a limitation of this systematic review.

Although clinical experience of HU therapy for patients with SCA has been related for more than 25 y, there is still much questioning about the pharmacokinetics, pharmacodynamics,

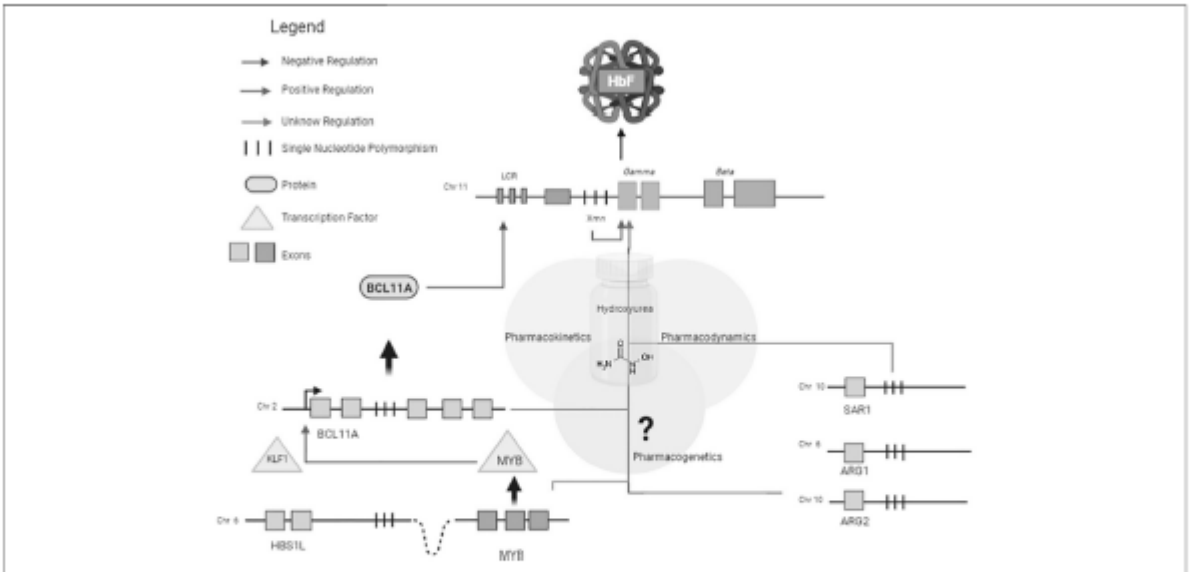


**FIGURE 4 |** Schematic diagram showing HbF regulation in response to HU, an HbF-inducing drug. *BCL11A* is a master repressor of gamma genes. The erythroid transcription factor KLF1 activates the transcription of *BCL11A* by binding to its promoter region. The expression of *KLF1* is possibly regulated by MYB. The *HBS1L-MYB* intergenic region contains several trait-associated common genetic variations and a distal enhancer of *MYB*, promoting long-range transcription regulation by chromatin looping. *SAR1A* encodes a GTP-binding protein which has been reported to be associated with γ-globin expression. Variations in the candidate genes *ARG1* and *ARG2* are associated with HbF changes. Pharmacokinetic genes can affect absorption, distribution, and metabolism of HU.

and pharmacogenetics of HU (Ware et al., 2011). To better understand the interpatient variability, polymorphisms in genes encoding drug-metabolizing enzymes and solute transporters were recently examined to learn their role in HU bioavailability and metabolism (Yahouedehou et al., 2020). The authors found evidence for the involvement of enzymes of the CYP450 family and catalases in the metabolism of HU, and the association between urea transporter-B (UTB) and response to HU in erythroid cells. SNPs in the *CYP2D6* (rs3892097), *CAT* (rs7943316 and rs1001179), and *SLC14A1* (rs2298720) genes were found to be linked to reduced metabolism or the elimination of HU, which may increase its therapeutic effects in patients with SCA (Yahouedehou et al., 2020). Unfortunately, this study did not examine the association between the SNPs with HbF response to HU, and thus, it was not included in the systematic review.

There was great heterogeneity in the patients' age in the included studies. For example, one study examined patients aged 4–54 y (mean 21 ± 14 y) (Friedrisch et al., 2016). The average age of initiation of HU was 9.6 ± 4.8 y in another study (Ware et al., 2011).

The present study has layers of complexity linked to the multifactorial characteristic of the disease. The heterogeneity of HU dose, patient age, HbF outcomes in response to HU, and candidate genes brought limitations to the search and contributed to the result being only seven included studies. These findings highlight that the pharmacogenetics of response to HU in patients with SCA is a fertile field for further investigations.

## CONCLUSION

The literature about the pharmacogenetics of response to HU therapy in patients with SCA is highly heterogeneous regarding the chosen candidate genes and SNPs examined for the possible association with changes in HbF levels, and regarding the HbF outcomes measured during HU therapy. Nevertheless, the findings of the studies included in this systematic review point out two main conclusions. First, as well as the baseline HbF, changes in HbF levels in response to HU therapy are likely to be regulated by genetic variations on multiple loci. Second, there is evidence that SNPs located in intron 2 of the *BCL11A* gene affect HbF changes in patients with SCA treated with HU. However, further studies are needed to test whether such SNPs may also predict the success of the treatment in ameliorating other hematological parameters and reducing the incidence of clinical complications.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**; further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

RS, BN, JT, KG, and ML made substantial contributions to the conception or design of the work; RS, BN, and JT acquired the data,

and all authors analyzed and interpreted the data for the work; RS, BN, and ML drafted the manuscript, and all authors revised it critically for important intellectual content; and all authors read and approved the final version of the manuscript for submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.779497/full#supplementary-material

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# Anexo E – Apresentações em eventos científicos

Semana do CONHECIMENTO
UFMG 2020

*A Inteligência Artificial e as travessias nas fronteiras do conhecimento*

## CERTIFICADO

Certificamos que o trabalho intitulado Identificação in silico de genes-alvo para microRNAs em vias moleculares relacionadas a fisiopatologia do Diabetes Mellitus Gestacional, foi apresentado na XXIX Semana de Iniciação Científica , promovida pela Pró-Reitoria de Pesquisa, no período de 05/10/2020 a 23/10/2020.

De autoria de: JOAO RAFAEL GONCALVES PEREIRA, JULIANA DE OLIVEIRA CRUZ, RENATA COALHO TEIXEIRA , JÉSSICA ABDO GONÇALVES TOSATTI, KARINA BRAGA GOMES BORGES

Orientador(a): MARCELO RIZZATTI LUIZON

Ana Maria Hermeto
Diretora de Fomento à Pesquisa

Mario Fernando Montenegro Campos
Pró-Reitor de Pesquisa

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SEMANA NACIONAL DE CIÊNCIA E TECNOLOGIA 2020

Realização UFMG

Semana do CONHECIMENTO
UFMG 2020

*A Inteligência Artificial e as travessias nas fronteiras do conhecimento*

## CERTIFICADO

Certificamos que o trabalho intitulado Perfil medicamentoso de pacientes com a Síndrome dos Ovários Policísticos, foi apresentado na XXIX Semana de Iniciação Científica , promovida pela Pró-Reitoria de Pesquisa, no período de 05/10/2020 a 23/10/2020.

De autoria de: ADRIANA FIALHO DA SILVA FONTES, JESSICA ABDO GONÇALVES TOSATTI

Orientador(a): KARINA BRAGA GOMES BORGES

Ana Maria Hermeto
Diretora de Fomento à Pesquisa

Mario Fernando Montenegro Campos
Pró-Reitor de Pesquisa

Código de autenticidade: 2d00ce98adf1abcedcf3ceeb0859343a19737f1eba978f8a36e92875063b46fd7e3a1
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SEMANA NACIONAL DE CIÊNCIA E TECNOLOGIA 2020

Realização UFMG

Estácio

# CERTIFICADO

SEPESQ
Seminário de Extensão e Pesquisa

Certificamos que JÉSSICA ABDO GONÇALVES TOSATTI apresentou o trabalho intitulado PERFIL ALIMENTAR DE COLABORADORES DE DUAS REDES DE FAST FOOD DURANTE O EXPEDIENTE no VI Seminário de Pesquisa e Extensão do Centro Universitário Estácio de Belo Horizonte, realizado no dia 21 e 22 de outubro de 2020.

Belo Horizonte - MG, 15 de março de 2021.

Mariana Cavaca Alves do Valle
Pró-Reitora de Pesquisa e Extensão

Fábio Dall Alba
Reitor

Estácio

# CERTIFICADO

SEPESQ
Seminário de Extensão e Pesquisa

Certificamos que JÉSSICA ABDO GONÇALVES TOSATTI apresentou o trabalho intitulado NÍVEL DE CONHECIMENTO DOS PAIS SOBRE ALIMENTOS ULTRAPROCESSADOS NA ALIMENTAÇÃO DOS FILHOS EM IDADE PRÉ-ESCOLAR no VI Seminário de Pesquisa e Extensão do Centro Universitário Estácio de Belo Horizonte, realizado no dia 21 e 22 de outubro de 2020.

Belo Horizonte - MG, 15 de março de 2021.

Mariana Cavaca Alves do Valle
Pró-Reitora de Pesquisa e Extensão

Fábio Dall Alba
Reitor

# Certificado

Certificamos que o trabalho intitulado **EFEITOS DA SUPLEMENTAÇÃO DE RESVERATROL NA FUNÇÃO COGNITIVA DE PACIENTES COM DIAGNÓSTICO DE DOENÇA DE ALZHEIMER: UMA META-ANÁLISE DE ENSAIOS CLÍNICOS RANDOMIZADOS**, dos autores: Jéssica Abdo Gonçalves Tosatti, Adriana Fialho Da Silva Fontes, Paulo Caramelli e Karina Braga Gomes. Foi aprovado no "I Congresso Multidisciplinar de Análises Clínicas e Toxicológicas" (COMACT) da Universidade Federal de São João del-Rei – Campus Centro-Oeste Dona Lindu, realizado nos dias 15 a 19 de março de 2021.

Divinópolis-MG, 19 de março de 2021.

Prof. Eduardo Sergio da Silva
**Diretor do Campus Centro-Oeste Dona Lindu**
**Universidade Federal de São João del-Rei**

Gabriela do Carmo Passos Nascimento
**Diretora Geral Discente do COMACT**

Farah Maria Drumond Chequer Baldoni
**Diretora Geral Docente I COMACT**

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# CERTIFICADO

HOSPITAL DE CLÍNICAS
PORTO ALEGRE RS

Certificamos que o trabalho **Influência da suplementação de ácido graxo n-3 em marcadores de inflamação e de estresse oxidativo em pacientes com Síndrome dos Ovários Policísticos: uma revisão sistemática e meta-análise de ensaios clínicos randomizados** dos autores **Jéssica A. G. Tosatti; Michelle T. Alves; Adriana F S Fontes; Ana L. Cândido; Fernando M. Reis; Vânia E. Araújo; Karina B. Gomes**, foi apresentado na modalidade E-pôster, durante 17° Encontro de Endocrinologia Feminina - Endofeminina, realizado no formato digital, nos dias 11 e 12 de junho de 2021.

Profª. Nadine Oliveira Clausell
*Diretora-Presidente do Hospital de Clínicas*

Profª. Poli Mara Spritzer
*Coordenadora do Evento*

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# CERTIFICADO

HOSPITAL DE CLÍNICAS
PORTO ALEGRE RS

Certificamos que o trabalho **Perfil de expressão de microRNAs em placentas de gestantes diagnosticadas com Diabetes Mellitus Gestacional por meio de revisão sistemática e análise de vias biológicas reguladas** dos autores **Juliana de Oliveira Cruz; Jéssica Abdo GonçalvesTosatti; Izabela Mamede Costa Andrade da Conceição; Karina Braga Gomes; Marcelo Rizzatti Luizon**, foi apresentado na modalidade E-pôster, durante 17° Encontro de Endocrinologia Feminina - Endofeminina, realizado no formato digital, nos dias 11 e 12 de junho de 2021.

Profª. Nadine Oliveira Clausell
*Diretora-Presidente do Hospital de Clínicas*

Profª. Poli Mara Spritzer
*Coordenadora do Evento*

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evento on-line

XIII·RPDA
Reunião de Pesquisadores em Doença de Alzheimer e Desordens Relacionadas
25 A 27 NOVEMBRO

COMPORTAMENTO BIOMARCADORES DEMÊNCIA DA DOENÇA DE PARKINSON HIDROCEFALIA DE PRESSÃO NORMAL DEMÊNCIA DOENÇAS PRIÔNICAS AFASIA PROGRESSIVA PRIMÁRIA DEMÊNCIA RAPIDAM PROGRES DEMÊNCIAS IMUNOM DOENÇA DE SÍNDROME CORT ALZHEIM FRONTOTEMPORAL DEMÊNCIA COM COMPROMETIMENTO

# CERTIFICADO

O Trabalho intitulado: **Effects of resveratrol supplementation on the cognitive function of patients with Alzheimer's disease: a meta-analysis of randomized controlled trials.**

Foi exposto na modalidade **e-pôster digital** na **XIII Reunião de Pesquisadores em Doença de Alzheimer e Desordens Relacionadas**, realizada pelo Departamento Científico de Neurologia Cognitiva e do Envelhecimento da Academia Brasileira de Neurologia, nos dias 25, 26 e 27 de novembro de 2021, tendo como Autor(a) Principal **Jéssica Tosatti** e Co-autores **Adriana Fontes, Paulo Caramell e Karina Gomes.**

Dra. Jerusa Smid
Presidente da XIII Reunião de Pesquisadores em Doença de Alzheimer e Desordens Relacionadas

Dr. Breno Barbosa
Comissão Organizadora da XIII Reunião de Pesquisadores em Doença de Alzheimer e Desordens Relacionadas

Dr. Lucas Schiling
Comissão Organizadora da XIII Reunião de Pesquisadores em Doença de Alzheimer e Desordens Relacionadas

**SEMANA DO CONHECIMENTO UFMG 2021**

*Transversalidade da ciência para construção de futuros*

Certificamos que o trabalho intitulado Influência do tratamento com metformina no hiperandrogenismo em mulheres com a Síndrome dos Ovários Policísticos: uma revisão sistemática e meta-análise de ensaios clínicos randomizados., foi apresentado na XXX Semana de Iniciação Científica , promovida pela Pró-Reitoria de Pesquisa, no período de 03/08/2021 a 29/10/2021.

De autoria de: ADRIANA FIALHO DA SILVA FONTES, FERNANDO MARCOS DOS REIS, ANA LUCIA CANDIDO, JESSICA ABDO GONÇALVES TOSATTI

Orientador(a): KARINA BRAGA GOMES BORGES

Ana Maria Hermeto
Diretora de Fomento à Pesquisa

Mario Fernando Montenegro Campos
Pró-Reitor de Pesquisa

Apoio

Código de autenticidade: 2d00ce98adf1abcedc[illegible]3a40786cdca3a8105fb4877c4e0f5a37c5dff23
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SEMANA NACIONAL DE CIÊNCIA E TECNOLOGIA 2021

UFMG

AE-PCOS
ANDROGEN EXCESS & PCOS SOCIETY

**19th ANNUAL MEETING OF THE ANDROGEN EXCESS & PCOS SOCIETY**

**ID** #45 Type: Basic Science

**TITLE**: INFLUENCE OF METFORMIN ON HYPERANDROGENISM IN WOMEN WITH PCOS: A META-ANALYSIS OF CLINICAL TRIALS

**AUTHORS**: Fontes AFS (1), Reis FM (2), Cândido AL (2), Gomes KB (1), Tosatti JAG (1)

Affiliation: (1) Department of Clinical and Toxicological Analysis, Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil;

(2) Department of Internal Medicine, Faculty of Medicine, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil;

OBJECTIVE: The excess of insulin observed in Polycystic Ovary Syndrome (PCOS) is directly related to the increased production of androgen hormones. Thus, the use of metformin to control hyperinsulinism could lead to a reduction of hyperandrogenism in these patients. The aim of this study was to evaluate the effects of metformin treatment on markers of hyperandrogenism in patients diagnosed with PCOS through a systematic review and meta-analysis.

METHODS: We conducted a systematic review, with meta-analysis, of randomized placebo-controlled clinical trials evaluating the effects of metformin treatment in adult patients with PCOS on the outcomes dehydroepiandrosterone (DHEAS) and total testosterone in accordance with the Cochrane Handbook for Systematic Reviews of Interventions recommendations. Searches were performed in the main electronic databases: MEDLINE via Pubmed, CENTRAL, Embase, CINAHL, Web of Science and Scopus, including studies published until June 2021. The meta-analysis was conducted based on the mean differences and standard deviations between the values of the evaluated outcomes, at the baseline (before the use of metformin) and at the end (after the use of metformin) of the study, using the random effect and the effect measure both presented as the standardized mean difference (SMD). Significant values were considered as $p<0.05$ with 95% CI. The substantial heterogeneity for the outcomes assessed by the meta-analysis was explored from the sensitivity analysis, in order to examine changes in the size of the combined effect, excluding each study successively.

RESULTS: 3,694 primary studies were selected and, after removing duplicates and analyzing titles, abstracts, and full text, considering the pre-established inclusion and exclusion criteria, eleven studies were included in the quantitative evaluation. A significant increase in DHEAS levels [SMD: 0.50 (95% CI: 0.05 to 0.94) points; I2 = 74%; p-value for heterogeneity: <0.00001] and significant reduction in total testosterone levels [SMD: -0.63 (95% CI: -1.18 to -0.09) points; I2 = 86%; p-value for heterogeneity: <0.00001] were observed in both of outcomes, in the metformin treatment group, when compared to the placebo group, after combining the results.

CONCLUSIONS: The results indicate that the use of metformin can lead to changes in androgen levels in patients with PCOS.

(Support: CNPq and CAPES).

## Anexo F - Declaração de participação como membro de Comitê Científico

Estácio

**DECLARAÇÃO**

Declaro, para os devidos fins, que **Jéssica Abdo Gonçalves Tosatti,** participou do Comitê Científico do VI Seminário de Pesquisa e Extensão do Centro Universitário Estácio de Belo Horizonte e I Seminário Regional Centro Sul de Pesquisa, ocorrido nos dias 21 e 22 de outubro de 2020, durante o período de 01 de setembro de 2020 a 22 de outubro de 2020.

Belo Horizonte, 11 de fevereiro de 2021.

**Mariana Cavaca Alves do Valle**
Pró-Reitora de Pós-Graduação, Pesquisa e Extensão
Centro Universitário Estácio de Belo Horizonte

CENTRO UNIVERSITÁRIO ESTÁCIO DE BELO HORIZONTE
Mariana Cavaca Alves do Valle - MF3967
Estácio Pró-Reitora de Pós-Graduação, Pesquisa e Extensão

## Anexo G - Declaração de orientação de Trabalho de Conclusão de Curso

DECLARAÇÃO

Declaro para os devidos fins que **Jéssica Abdo Gonçalves Tosatti** foi orientadora do Trabalho de Conclusão de Curso dos alunos Felipe de Sousa Gonçalves, Paula Aleixo Mazoni Mimessi e Renata Rodrigues de Castro Morais, intitulado "**Perfil alimentar de colaboradores de duas redes de *fast food* durante o expediente**".

Belo Horizonte, 29 de novembro de 2020

*Viviane Aparecida de Souza Lacerda*
*Coordenadora do Curso de Nutrição*
*Centro Universitário Estácio de BH – Campus Prado*

Estácio

DECLARAÇÃO

Declaro para os devidos fins que **Jéssica Abdo Gonçalves Tosatti** foi orientadora do Trabalho de Conclusão de Curso dos alunos Fabrício Augusto Meira Barbosa e Tatiana Soares Campos, intitulado "**Nível de conhecimento dos responsáveis sobre alimentos ultraprocessados na alimentação dos filhos em idade pré-escolar**".

Belo Horizonte, 29 de novembro de 2020

*Viviane Aparecida de Souza Lacerda*
*Coordenadora do Curso de Nutrição*
*Centro Universitário Estácio de BH – Campus Prado*

Estácio

# Anexo H - Certificado de menção honrosa

Certificado

Certificamos que o trabalho intitulado **EFEITOS DA SUPLEMENTAÇÃO DE RESVERATROL NA FUNÇÃO COGNITIVA DE PACIENTES COM DIAGNÓSTICO DE DOENÇA DE ALZHEIMER: UMA META-ANÁLISE DE ENSAIOS CLÍNICOS RANDOMIZADOS.** Dos autores: Jéssica Abdo Gonçalves Tosatti, Adriana Fialho Da Silva Fontes, Paulo Caramelli, Karina Braga Gomes. Recebeu **Menção Honrosa** no "I Congresso Multidisciplinar de Análises Clínicas e Toxicológicas" (COMACT) da Universidade Federal de São João del-Rei – Campus Centro-Oeste Dona Lindu, realizado nos dias 15 a 19 de março de 2021

Divinópolis-MG, 19 de março de 2021.

Prof. Eduardo Sergio da Silva
**Diretor do Campus Centro-Oeste Dona Lindu**
**Universidade Federal de São João del-Rei**

Gabriela do Carmo Passos Nascimento
**Diretora Geral Discente do COMACT**

Farah Maria Drumond Chequer Baldoni
**Diretora Geral Docente I COMACT**

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## Anexo I - Certificados de participação como membro de comissão examinadora de monografia

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UNIVERSIDADE FEDERAL DE MINAS GERAIS

CERTIFICADO

CERTIFICAMOS, PARA OS DEVIDOS FINS QUE

Karina Braga Gomes Borges – orientadora,–Michelle Teodoro Alves coorientadora, Jessica Abdo Gonçalves Tosatti e Rita Carolina Figueiredo Duarte, participaram como membros da Comissão Examinadora da Monografia de Conclusão de Curso, intitulada "Avaliação dos níveis de galectina-3 em pacientes com diabetes mellitus tipo 2 e suas complicações" apresentada pela aluna Isabella Damaris Passos de Souza.

Belo Horizonte, 04 de novembro de 2019.

Profa Dra. Cristina Mariano Ruas
Coordenadora do Colegiado de Coordenação Didática do Curso de Farmácia

FACULDADE DE FARMÁCIA DA UNIVERSIDADE FEDERAL DE MINAS GERAIS
Av. Presidente Antônio Carlos, 6627 – Campus Pampulha – CEP 31270-901 – Fone: 31 3409-6742/6743/6744
cografar@farmacia.ufmg.br www.farmacia.ufmg.br

CERTIFICADO

CERTIFICAMOS, PARA OS DEVIDOS FINS QUE

**Michelle Teodoro Alves** – orientador(a), **Karina Braga** – coorientador(a**), Jéssica Abdo Gonçalves Tosatti** e **Rahyssa Rodrigues Sales**, participaram como membros da Comissão Examinadora da Monografia de Conclusão de Curso, intitulada "Perfil Laboratorial e Clínico de Pacientes com Doença Falciforme com COVID-19: uma Revisão Sistemática" apresentada pelo(a) aluno(a) Jessica Diniz Pereira.

Belo Horizonte, 31 de janeiro de 2022.

**Prof. Dr. Marcio de Matos Coelho**
**Coordenador do Colegiado de Coordenação Didática do Curso de Farmácia**

**FACULDADE DE FARMÁCIA DA UNIVERSIDADE FEDERAL DE MINAS GERAIS**
Av. Presidente Antônio Carlos, 6627 – Campus Pampulha – CEP 31270-901 – Fone: 31 3409-6742/6743/6744
cografar@farmacia.ufmg.br www.farmacia.ufmg.br

# CERTIFICADO

Certificamos que **Cintia Tarabal Oliveira, Viviane Aparecida de Souza Lacerda e Jessica Abdo Gonçalves Tosatti**, participaram da banca examinadora do Trabalho de Conclusão de Curso intitulado "**O IMPACTO DA DIETOTERAPIA NA EVOLUÇÃO DE PACIENTES DIAGNOSTICADOS COM DOENÇA RENAL CRÔNICA: UMA REVISÃO NARRATIVA DA LITERATURA**", apresentado pela aluna CAROLINA BERNARDI ALVES MAIA, como requisito para o título de Bacharel em Nutrição do Centro Universitário Estácio BH no dia 03 de novembro de 2021.

**Beatriz Martins Bicalho**
Coordenadora do Curso de Educação Física
Centro Universitário Estácio BH

Estácio

## Anexo J - Certificado de coorientação de Monografia de Conclusão de Curso

# CERTIFICADO

**CERTIFICAMOS, PARA OS DEVIDOS FINS QUE**

**Karina Braga Gomes** – orientadora**, Jéssica Abdo Gonçalves Tosatti** – coorientadora, **Laura Machado Lara Carvalho e Luana Bernardes Xavier Costa**, participaram como membros da Comissão Examinadora da Monografia de Conclusão de Curso, intitulada "INFLUÊNCIA DA METFORMINA NOS NÍVEIS DE TESTOSTERONA TOTAL EM MULHERES COM A SÍNDROME DOS OVÁRIOS POLICÍSTICOS: UMA REVISÃO SISTEMÁTICA E METANÁLISE DE ENSAIOS CLÍNICOS RANDOMIZADOS" apresentada pela aluna Adriana Fialho da Silva Fontes.

Belo Horizonte, 13 de janeiro de 2022.

**Prof. Dr. Marcio de Matos Coelho**
Coordenador do Colegiado de Coordenação Didática do Curso de Farmácia

**FACULDADE DE FARMÁCIA DA UNIVERSIDADE FEDERAL DE MINAS GERAIS**
Av. Presidente Antônio Carlos, 6627 – Campus Pampulha – CEP 31270-901 – Fone: 31 3409-6742/6743/6744
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# APÊNDICES

## APÊNDICE A – Termo de Consentimento Livre e Esclarecido (TCLE)

**UNIVERSIDADE FEDERAL DE MINAS GERAIS**
**FACULDADE DE FARMÁCIA**
**DEPTO. ANÁLISES CLÍNICAS E TOXICOLÓGICAS**

**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

**Projeto de Pesquisa "O PAPEL DAS CITOCINAS E OS PARÂMETROS BIOQUÍMICOS NA SÍNDROME DOS OVÁRIOS POLICÍSTICOS"**

**Prezado(a) Senhor(a) ou responsável,**

A pesquisa que você está sendo convidado a participar como voluntário (a) é um estudo científico que tem o objetivo de detectar algumas alterações genéticas em citocinas relacionadas à síndrome dos ovários policísticos. O benefício que você receberá será através da possibilidade de conhecimento da presença de possíveis alterações genéticas e suas implicações, importantes para que o clínico possa planejar melhor o tratamento adequado e possa investigar também a sua família com relação a esta alteração. Os indivíduos participantes serão selecionados no Hospital das Clínicas, em Belo Horizonte. Nesta pesquisa, cada participante deve participar de uma única entrevista e responder a um questionário, que será aplicado pela equipe da pesquisa, e deve doar uma única amostra de sangue, na qual serão realizados vários exames laboratoriais gratuitos cujos resultados serão encaminhados para o seu médico. A coleta de sangue venoso inclui um pequeno risco de acidente de punção, representado, principalmente por extravasamento sanguíneo subcutâneo de pequena gravidade, que pode resultar em leve dor localizada e formação de um pequeno hematoma. Para minimizar este risco, a coleta de sangue será realizada no próprio Hospital das Clínicas, por um profissional treinado, com capacidade técnica e experiência que estará atento e tomará todas as providências necessárias. Na coleta de 16 mL de sangue será utilizado material descartável de boa qualidade (agulhas e tubos a vácuo).

O nome do participante e, também, os resultados dos exames serão mantidos em segredo e privacidade, sob a responsabilidade da equipe de pesquisadores.

Caso você não queira participar da pesquisa, não haverá qualquer prejuízo no seu tratamento ou na assistência recebida pelo seu médico. Para qualquer dúvida sobre esta pesquisa você deverá entrar em contato, por telefone, com as pessoas responsáveis pela mesma, cujos nomes estão relacionados a seguir.

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Agradecemos a sua valiosa participação!

### PARTICIPANTE

Como participante deste projeto de pesquisa, declaro estar de acordo com os objetivos propostos no mesmo, bem como doar uma amostra de sangue para a realização dos exames laboratoriais.
Nome: _____

Documento de Identificação: _____ ou responsável: _____ Data: ____/_____/__