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MINIREVIEWS

Molecular and serology methods in the diagnosis of COVID-19: An overview

Marcel Silva Luz, Ronaldo Teixeira da Silva Júnior, Gabriella Almeida Santos de Santana, Gabriela Santos Rodrigues, Henrique de Lima Crivellaro, Mariana Santos Calmon, Clara Faria Souza Mendes dos Santos, Luis Guilherme de Oliveira Silva, Qesya Rodrigues Ferreira, Guilherme Rabelo Mota, Heloísa Heim, Filipe Antônio França da Silva, Breno Bittencourt de Brito, Fabrício Freire de Melo

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Abstract

Coronavirus disease-19 (COVID-19) has become a pandemic, being a global health concern since December 2019 when the first cases were reported. Severe acute respiratory syndrome coronavirus 2, the COVID-19 causal agent, is a β-coronavirus that has on its surface the spike protein, which helps in its virulence and pathogenicity towards the host. Thus, effective and applicable diagnostic methods to this disease come as an important tool for the management of the patients. The use of the molecular technique PCR, which allows the detection of the viral RNA through nasopharyngeal swabs, is considered the gold standard test for the diagnosis of COVID-19. Moreover, serological methods, such as enzyme-linked immunosorbent assays and rapid tests, are able to detect severe acute respiratory syndrome coronavirus 2-specific immunoglobulin A, immunoglobulin M, and immunoglobulin G in positive patients, being important alternative techniques for the diagnostic establishment and epidemiological surveillance. On the other hand, reverse transcription loop-mediated isothermal amplification also proved to be a useful diagnostic method for the infection, mainly because it does not require a sophisticated laboratory apparatus and has similar specificity and sensitivity to PCR. Complementarily, imaging exams provide findings of typical pneumonia, such as the ground-glass opacity

radiological pattern on chest computed tomography scanning, which along with laboratory tests assist in the diagnosis of COVID-19.

Key Words: COVID-19; Pandemic; Diagnosis; Polymerase chain reaction; Molecular biology; Serology

Core Tip: Severe acute respiratory syndrome coronavirus 2 is primarily detected by PCR, which is the gold standard diagnostic method to detect viral RNA. On the other hand, techniques such as serology with detection of immunoglobulin M and immunoglobulin G antibodies, imaging, and laboratory tests also assist in the diagnosis of severe acute respiratory syndrome coronavirus 2 infection. Moreover, the reverse transcription loop-mediated isothermal amplification has similar specificity and sensitivity to PCR. In this review, we discuss the main diagnostic methods and their uses in the current pandemic.

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INTRODUCTION

In December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as responsible for severe cases of pneumonia in Wuhan, China, which culminated in the description of a new disease: coronavirus disease-19 (COVID-19)[1]. As a result of the large number of affected countries and high potential of viral infection, the World Health Organization declared COVID-19 as a pandemic in March 2020. Up to December 2020, 66243918 cases and 1528984 global deaths were officially confirmed[2]. SARS-CoV-2 is a single-stranded RNA, enveloped virus, which has the ability to attach to the angiotensin-converting enzyme 2 cell receptor due to the expression of the spike (S) protein on the viral envelope and then enter the host tissues[3]. The *Coronaviridae* family is made up of viruses historically known to cause diseases in animals and humans. The SARS and Middle East Respiratory Syndrome outbreak in 2002 and 2012, respectively, granted them wider visibility in the scientific community[4]. Furthermore, the dissemination potential of SARS-COV-2 is far higher than the others, due to structural differences in the S protein[5].

The most common signs and symptoms of COVID-19 include fever, dry cough, shortness of breath, myalgia, ageusia, anosmia, headache, rhinorrhea, nausea, vomiting, and diarrhea[6], and most patients showing severe symptoms are often affected by chronic disease. In addition, disturbed immune status, increased age, and obesity are strongly correlated to higher mortality rates[7]. Reverse transcription (RT)-PCR is considered the gold standard test for the diagnosis of COVID-19, due to its highly widespread and reliable technique performed in laboratories worldwide[8]. In addition, immunoenzymatic and immunochromatographic assays as well as reverse transcription loop-mediated isothermal amplification (RT-LAMP) are other diagnostic methods that have been applied in this field. Of note, clinical and epidemiological analysis, chest radiography and tomography, and laboratorial findings are crucial tools for an accurate diagnosis and appropriate evaluation of patients[9]. This article aimed to review the main aspects of COVID-19 diagnostic methods, providing updated information with an emphasis on molecular biology techniques and serology tests used in the detection of SARS-CoV-2 infection.

RT-PCR

RT-PCR is considered as the gold standard method in COVID-19 diagnosis, due to rapid detection with an average of 3-4 h and high sensibility and specificity[10]. The sample is usually taken through nasopharyngeal swab[11]. However, a systematic review and meta-analysis with 7 studies showed that bronchoalveolar lavage fluid had a higher positivity rate in the detection of SARS-CoV-2[12].

The test analysis usually starts from a sample collected from nasal and oropharyngeal swabs[13]. It is then divided in several steps that occur in different preset temperatures in order to provide RT and nucleic acid amplification[14]. The result is thus analyzed through probes marked with fluorescent dyes that enhance the sensitivity of the test[15]. The analysis of fecal samples, especially in children[16], may

be used as well, as the virus can remain viable for approximately 5 wk after patient respiratory samples are negative for SARS-CoV-2 RNA[17].

RT-PCR is considered the actual main method as a result of its fastness, reproducibility, and mitigation of false-positive results[18]. The test shows good sensitivity and specificity, such as 94% and 100%, respectively (Table 1)[19-28]. Studies have also pointed to a level of detection that can vary from 3.8 to 23.0 copies/mL of viral RNA and showed no cross-reactivity with circulating respiratory viruses [20].

However, in a study with 610 patients from Wuhan, China, 18 patients had a positive RT-PCR result after two consecutive negative results, which might be owing to insufficient viral material, test handling error, or incorrect collection processing[21]. This is an alert to the need for a pattern in sampling procedures and alignment between the test and the patient's clinical manifestation in order to achieve a higher diagnostic accuracy[22].

RT-LAMP

LAMP is a DNA amplification technique under isothermal conditions in a sample with 4 or 6 primers, which in contrast to RT-qPCR does not require a sophisticated laboratory apparatus, although it has similar specificity and sensitivity rates[29]. The visualization through pH-sensitive dyes, without the need of expensive instrumentation is also an advantage of this test[30]. As SARS-CoV-2 is an RNA virus, the test is therefore called RT-LAMP, due to the need for RTase to amplify RNA sequences[29].

The RT-LAMP is a fast test, providing results within 30 min[31]; moreover, unpurified samples can be directly used[32]. Studies also show that when the template has more than 200 copies of viral RNA, amplification curves appear within 15 min[33], which means an even quicker diagnosis. In addition, studies describe a level of detection of 2 copies of viral RNA in a 25 μL reaction[34], sensitivity rates that varies from 80%[26] to 97.5%[27], concomitantly with no cross-reactivity with other respiratory pathogens (high specificity)[33,35] and lower cost, all of which endorses that the diagnosis of COVID-19 through LAMP needs to be considered[36,37].

However, a meta-analysis including 138 articles showed that RT-LAMP sensitivity (86.3%) is lower than that of RT-PCR (96.2%)[38]. Furthermore, carry-over contamination, which can lead to false positive results, are common in LAMP reactions, probably as a consequence of aerosol formed from the products of the test[34]. This phenomenon highlights the need of laboratories with good practice of molecular biology and separate spaces to deal with the components of the test as well as more studies about the efficacy of all types of genes and primers used in this test.

SEROLOGICAL TESTS

Serological tests have become even more available during the COVID-19 pandemic. Consequently, research on their role as auxiliary diagnostic methods for SARS-CoV-2 infection has experienced exponential growth[39]. Thereby, these tests may support the COVID-19 diagnosis, especially when there is a longer period of symptoms with negative RT-PCR assays in a patient with a suspected infection by SARS-CoV-2[40]. Moreover, its use allied to RT-PCR greatly increases the diagnostic sensitivity[41].

Among the serological tests commonly used for diagnosis of COVID-19, the ELISA, the chemiluminescence immunoassay (CLIA), and the lateral flow immunochromatographic assay (LFA) stand out. The ease, agility, and point-of-care testing are great advantages associated with the use of these tests[42]. However, they may often show low sensitivity, require specialized equipment[39,42], or have cross-reactivity with other pathogens, such as SARS-CoV-1[43]. A study also showed a cross-reactivity of 26% in serological tests for COVID-19 during acute Zika virus infection[44].

The sensitivity of the test is strictly related to the elapsed time from the beginning of symptoms, being more useful 15 d after the onset of clinical manifestations, especially regarding the detection of isolated immunoglobulin (Ig) G[45]. In that context, a meta-analysis including 40 studies evaluated the presence of anti-SARS-CoV-2 IgG during the first symptomatic week, and the rates of false-negative diagnoses ranged from 44% to 87%[46]. Therefore, simultaneous analysis of IgM and IgG antibodies, as they have different emergence times, may increase the serological test sensitivity[39,47]. Although some studies compare IgG with other antibodies, such as IgA, to analyze any increase in the effectiveness of serological surveillance, the results are not promising[48].

The overall specificity for all types of antibodies was higher than 98%. The average sensitivity for IgG detection ranges from 80% to 85%, with CLIA being the most sensitive, followed by ELISA, and with much lower performance the LFA test[42,45]. IgM evaluation showed a sensitivity of 80.9% for CLIA, 84.5% for ELISA, and 51.4% with LFA. This study also demonstrated that in the use of combined IgM/IgG tests, the CLIA performance was higher than ELISA and LFA, with results of 97.3%, 90.5% and 85.8%, respectively[45].

Table 1 Coronavirus disease 2019 main diagnostic methods characteristics

Ref.	Diagnostic method	Sensitivity	Specificity	Time to result
Liu *et al*[23]	ELISA (IgM/IgG)	57.9%-90.7%	No cross-reactivity observed	About 100 min
Cai *et al*[24]	CLIA (IgM/IgG/IgM and IgG)	57.2%-81.5%	No cross-reactivity observed	ND
Montesinos *et al*[25]	LFA (IgM/IgG/IgM and IgG)	~70.0%	95.8%-100%	About 10 min
Scohy *et al*[52]	Antigen detection	30.2%	100%	About 15 min
Porte *et al*[54]	Antigen detection	93.9%	100%	About 15 min
Suo *et al*[19]	RT-PCR	94.0%	100%	ND
Österdahl *et al*[26]	RT-LAMP	80.0%	73%–100%	About 25 min
Dao Thi *et al*[27]	RT-LAMP	97.5%	99.7%	About 30 min
Ai *et al*[28]	Chest CT	97.0%	25%	ND

ELISA: Enzyme-linked immunosorbent assay; IgA: Immunoglobulin A; IgM: Immunoglobulin M; IgG: Immunoglobulin G; LFA: Lateral flow assay; CLIA: Chemiluminescence immunoassay; RT-PCR: Reverse transcription-polymerase chain reaction; RT-LAMP: Reverse transcription loop-mediated isothermal amplification; CT: Computed tomography; ND: Not described.

Sensitivity and specificity differences according to the viral protein analyzed are also documented: S protein is more specific, but the nucleocapsid and receptor-binding domain proteins are more sensitive in patients with mild infection[47,49]. Therefore, research on antibodies against different antigens may be useful in order to improve diagnostic methods, avoid false-negatives, and reach a higher diagnostic accuracy.

ANTIGEN DETECTION METHODS

Viral antigen is a molecule with immunogenic potential that can be targeted by diagnostic tests through a reaction with monoclonal antibodies. Several antigen detection tests have been developed as alternatives for the rapid diagnosis of the COVID-19[50,51]. The results of the test with nasopharyngeal secretions are ready within 15 min[52], and it can be performed either through immunochromatography, with rapid detection, or ELISA with better sensitivity[50].

The average sensitivity of antigen detection tests is around 50%–70%, and they are 100% specific[51,53]. Of note, the performance of those tests may be influenced by higher or lower viral loads as well as by the specific antigen used. In that context, studies have shown different results when this method was evaluated, and the sensitivity values varied from 30.2%[52] to 93.9%[54] Overall, higher rates of accurate diagnosis in antigen tests were greatly correlated with early infection, when the viral load of the upper respiratory tract is higher[55].

Therefore, although the COVID-19 diagnosis through antigen detection has a high specificity and is faster and cheaper than RT-PCR, the precise time of usage of this test is crucial for proper detection of the virus antigens[52]. That said, the current gold standard diagnostic test for COVID-19 is still more reliable because its use is associated with lower rates of false negative results[56]. Nevertheless, utilization of antigen detection tests, with additional research, could turn into a viable option in the current pandemic context.

COMPLEMENTARY DIAGNOSTIC METHODS

Chest computed tomography findings

Chest computed tomography (CT) has been used as an alternative and complementary method for COVID-19 diagnosis since CTs can detect pulmonary abnormalities even when RT-PCR results turn negative[57] for highly suspect cases with clinical symptoms[58] in the early days of infection[59].

The chest CT diagnosis works through analysis of the variation in imaging findings that occur according to the disease progression and severity[60]. The pulmonary imaging abnormalities start to appear around 4 d after the first symptoms, and their findings are more visible following the second week of clinical manifestations[58].

Accordingly, the most predominant COVID-19 pneumonia imaging changes are ground-glass opacity lesions with or without consolidations, peripheral and bilateral lung distribution of the disease, and multilobar lung involvement, predominantly in the lower lobes[61,62]. Some less common CT manifest-

ations are the crazy-paving pattern, ground-glass opacity with consolidation, interlobular septal thickening, and pleural effusion[63]. Chest CT scans are highly sensitive to COVID-19 lung abnormalities[64], mainly in high-risk symptomatic cases[65].

However, these imaging findings have low specificity[63]. A study performed with 1014 patients showed an average specificity of 25%[28], probably due to other viral pneumonias leading to similar imaging alterations, a fact that limits the use of this method in areas with high prevalence of other respiratory tract infections[64]. Moreover, chest CT exams can detect no abnormalities in some asymptomatic or mild symptomatic cases[63], making CT scans more of a complementary diagnostic test than a definitive one. Table 1 summarizes the sensitivity, specificity, time to result, and limitations of the diagnostic methods discussed in this review so far.

Laboratory findings

Patient reports from Wuhan showed recurrent cases of lymphocytopenia since the beginning of the infections in China[66]. Besides that, studies also show a relevant frequency of patients with leukocytosis during SARS-CoV-2 infection[67]. A meta-analysis showed that non-surviving patients had an expressive increase in leukocyte count, total bilirubin, serum ferritin, and interleukin 6 as well as a reduced lymphocyte count[68]. Thus, leukocyte series elevation can represent worse prognostic and high risk of unfavorable outcomes.

Increases in the levels of lactate dehydrogenase and C-reactive protein were also highlighted and associated with pulmonary and myocardial lesions, especially in severe patients[69]. Low serum albumin rates and high levels of alanine aminotransferase and aspartate aminotransferase points to possible liver complications, which is very common in acute phases of the disease in patients with a severe infection[70,71]. Furthermore, the association of these points with elevation in renal biomarkers (for example, creatinine), coagulation measures, and heart and muscle injury scores suggest potential progression to multiple organ failure in severe patients[68]. Thereby, elevated levels of D-dimer, fibrin degradation products, and fibrinogen can be observed during the course of the disease, with the D-dimer alterations being the most common[72]. A study related that levels of these coagulation parameters were observed in severe patients with worse prognosis, while mild disease or early stage patients had normal ranges[73,74]. In addition, thrombocytopenia is also a possible laboratory finding in COVID-19. A meta-analysis reported that platelet count was minor in severe disease and even smaller in non-surviving patients[74]. These findings might be indicative of disease progression and coagulation disorders, which means that the tracking of these signs is very important while managing patients[74,75].

Moreover, possible coinfections of SARS-CoV-2 and bacterial infections might cause neutrophilia and leukocytosis, associated with lymphocytopenia, without increasing inflammatory factors, such as D-dimer and C-reactive protein[76]. Therefore, laboratory findings have proven to be a helpful option as a complementary diagnosis. It is also suitable in the visualization of possible comorbidities in patients with COVID-19 and as an indicator of disease severity.

Several studies are being carried out to test the efficacy of drugs, foods, and mineral supplements against COVID-19. Lymecycline and famotidine, for example, are being studied as a potential treatment for COVID-19[77,78], due to a possible ability to bind some SARS-CoV-2 structures (M^{pro}, S protein, RdRp, and furin) and have an anti-inflammatory action[79], respectively. However, there is, up to this moment, not enough evidence and controlled clinical trials to affirm its efficacy against the disease. In addition, mineral supplements such as zinc apparently have some antiviral properties that could be used against SARS-CoV-2 infection, such as a capability of modulating the host's immune response and attenuating the cytokine storm caused by COVID-19[80]. However, a randomized clinical trial of 214 patients showed that zinc supplementation had no significant benefits[81].

CONCLUSION

Notably, the use of serology and antigen detection tests have important limitations since false negative results are common. Nonetheless, in a pandemic context, these methods are crucial for epidemiological surveillance. RT-PCR remains the gold standard test and should be preferred to diagnose COVID-19. However, the high potential of RT-LAMP, given that it is a fast and affordable test, should be considered in diagnostic propedeutics. In addition, the laboratory and imaging findings play important roles as complementary diagnostic tools aiding in patient management.

FOOTNOTES

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MINIREVIEWS

Network meta-analyses: Methodological prerequisites and clinical usefulness

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Abstract

It is an undeniable fact that systematic reviews play a crucial role in informing clinical practice; however, conventional head-to-head meta-analyses do have limitations. In particular, studies can only be compared in a pair-wise fashion, and conclusions can only be drawn in the light of direct evidence. In contrast, network meta-analyses can not only compare multiple interventions but also utilize indirect evidence which increases their precision. On top of that, they can also rank competing interventions. In this mini-review, we have aimed to elaborate on the principles and techniques governing network meta-analyses to achieve a methodologically sound synthesis, thus enabling safe conclusions to be drawn in clinical practice. We have emphasized the prerequisites of a well-conducted Network Meta-Analysis (NMA), the value of selecting appropriate outcomes according to guidelines for transparent reporting, and the clarity achieved *via*

sophisticated graphical tools. What is more, we have addressed the importance of incorporating the level of evidence into the results and interpreting the findings according to validated appraisal systems (*i.e.*, the Grade of Recommendations, Assessment, Development, and Evaluation system - GRADE). Lastly, we have addressed the possibility of planning future research *via* NMAs. Thus, we can conclude that NMAs could be of great value to clinical practice.

Key Words: Network meta-analysis; Quality of evidence; Evidence-based medicine; Systematic reviews

Core Tip: Systematic reviews with or without meta-analyses provide the highest quality of evidence, thus lying on the top of evidence-based medicine hierarchy. However, pair-wise meta-analyses present the inherent limitation of exclusively comparing direct evidence. By contrast, Network Meta-Analyses (NMAs) also consider indirect evidence, thereby offering additional useful information. Conducting an NMA, however, has certain requirements such as assuming that transitivity across the included studies exists. What is more, maintaining sufficient statistical power in the analyses is crucial. In addition, performing head-to-head statistical comparisons before setting up networks of interventions is a prerequisite for a methodologically sound NMA, and selecting not only positive but also negative outcomes is required. Lastly, implementing quality appraisal systems to grade the level of evidence is highly recommended. Should all the above criteria be fulfilled, then accurate clinical conclusions can be drawn from an NMA.

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INTRODUCTION

Due to the plethora of different interventions for various clinical entities[1] identifying the most efficient and safe treatment is among the prime interests of a researcher[2-4]. In the case of conventional meta-analyses, only two interventions can be compared at a time, and only those evaluated in head-to-head trials[5-7]. What is more, intervention effect estimates can only be calculated from direct evidence[2]. In contrast to pair-wise meta-analyses, network meta-analyses (NMA) enable not only simultaneous direct comparisons of multiple interventions but also indirect comparisons provided a common comparator is shared between interventions[2]. This is even possible in the case of two interventions that have never been directly compared[2]. In addition, interventions may also be ranked utilizing the surface under the cumulative ranking (SUCRA) curves, thus allowing for judgments such as which treatment presents the highest probability of being the most effective[2]. It is underlined that identifying more than one highly efficacious treatment in an NMA is a common phenomenon given the subtle differences in treatment rankings of the modalities lying on the top of ranking probabilitiy tables. Overall, incorporating the results from network meta-analyses into clinical practice guidelines could help clinicians select the best available intervention to improve healthcare.

PREREQUISITES FOR A WELL-CONDUCTED NMA - THE ASSUMPTION OF TRAN SITIVITY AND HETEROGENEITY

For a systematic review of randomized evidence to qualify as a network meta-analysis, the assumption of transitivity must be fulfilled. To elaborate further, transitivity implies that it is possible to conclude hypothetical comparisons through a common comparator[6]. However, this is only possible in the absence of systematic differences between studies[8] with some degree of heterogeneity being permitted [6]. To illustrate further, heterogeneity is defined as a form of inter-study discrepancy due to differences that cause deviations in the observed effects other than sampling error[9]. However, when the discrepancy between studies exceeds that explained by clinical diversity, effects sizes cannot be safely estimated based on direct and indirect evidence and the distribution of effect modifiers needs to be examined[6].

PREREQUISITES FOR A WELL-CONDUCTED NMA- STATISTICAL POWER

It is worthy of note that the statistical power of a network of interventions should be sufficient to enable safe clinical conclusions to be drawn. To be more specific, the ratio between the number of included papers relative to the number of the competing interventions should be satisfactory. On top of that, the sample size per intervention arm as depicted by the size of the nodes in a network meta-analysis plot should also be robust enough (Figure 1). Lastly, prospective registration with systems such as the grade of recommendations, assessment, development, and evaluation system (GRADE) is valuable in assessing the heterogeneity and additional characteristics such as publication bias, indirectness, imprecision, the study limitations, and inconsistency[5].

CONDUCTING PAIR-WISE META-ANALYSIS PRIOR TO NMA

Of additional note, for a given dataset, researchers must conduct not only NMA but also traditional pair-wise meta-analyses. To be more precise, one can take advantage of early exploration of the results of conventional pair-wise meta-analyses before setting up networks of interventions. Authors should then proceed with the network meta-analysis to take advantage of indirect evidence synthesis for them to supplement their study results.

PREREQUISITES FOR A WELL-CONDUCTED NMA- SELECTING APPROPRIATE OUTCOMES

In determining primary and secondary outcomes, both positive and negative results should be considered. Outcomes of primary interest should be prioritized over outcomes of secondary clinical importance to ensure that the findings will be clinically relevant. For instance, laboratory tests are not routinely considered as primary endpoints as they tend to not directly inform decisions. However, they may play an explanatory and/or adjuvant role in explaining the intervention outcome[10].

FOLLOWING GUIDELINES FOR TRANSPARENT REPORTING

The PRISMA guidelines represent a checklist of 27 items that may be used when reporting a systematic review of health interventions with or without meta-anlysis[11]. Hutton *et al*[12], in 2015, has expanded the original list by including 5 additional items that apply to network meta-analyses. Firstly, the geometry and summary of the intervention networks have been incorporated in the methods, including a diagrammatic representation and a brief description. What is more, the findings of inconsistency assessment can be included in addition to the presentation of the networks' structure.

It should be noted that prospective registration (*e.g.* with PROSPERO database) of all NMAs is encouraged. By doing so, transparency is promoted and bias is prevented by avoiding unintended duplicate reviews[13]. It is also highlighted that adherence to a pre-existing protocol plays a crucial role in preventing selective outcome reporting[10,14,15]. In other words, registration of a systematic review in advance of study commencement precludes data manipulation and/or unethical reporting. Last but not least, prospective registration may enable researchers to assess whether the topic they intend to investigate has already been addressed by earlier authors, thus avoiding unnecessary research repetition.

SOPHISTICATED GRAPHICAL TOOLS IN NMA - DO WE NEED THEM?

Despite NMAs gaining popularity, a lot of criticism exists given their complex methodology discouraging clinicians from getting involved in this type of research[16]. This is due to the increased level of statistical and computational knowledge required. To tackle this issue, introducing graphical tools into the manuscript results in a significant increase in clarity and reproducibility[16].

What is more, competing interventions can be ranked from the most to the least effective *via* the use of SUCRA curves[2]. On the other hand, league tables enable a structured presentation of the result of each pair of comparisons with its corresponding 95% confidence intervals (Figure 2).

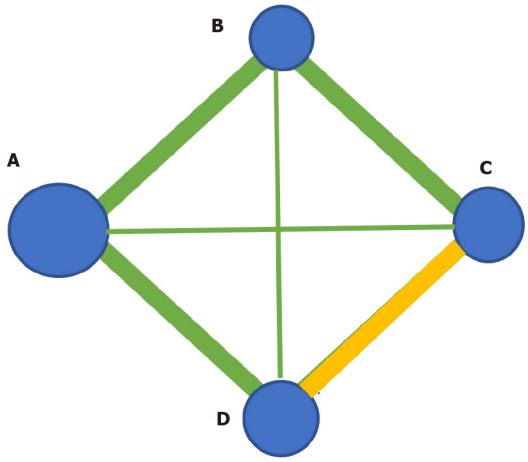

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Figure 1 A Network-Meta-Analysis plot example. Network meta-analysis plot including four competing interventions (*i.e.* A, B, C, and D). The nodes represent the included interventions with their size being proportional to sample size. The thickness of the edges connecting the nodes is reflected in the number of trials included in the given comparison. The edges depicted in green and yellow denote that the involved comparisons are at low and moderate risk of bias, respectively.

A				
-0.17 (-1.60, 1.25)	**B**			
-1.34 (-2.68, 0.01)	-1.16 (-2.99, 0.66)	**C**		
0.93 (-0.82, 2.68)	1.11 (-1.15, 3.36)	2.27 (0.16, 4.48)	**D**	
3.05 (1.41, 4.68)	3.22 (1.17, 5.27)	4.38 (2.39, 6.38)	2.11 (-0.28, 4.51)	**E**

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Figure 2 Hypothetical league table demonstrating standardized mean differences, from a network meta-analysis of five competing interventions, that is A-E. Statistically significant values are depicted in TextTitle.

PUBLICATION BIAS IN NMA AND ITS IMPACT ON CLINICAL ESTIMATES

It has been evidenced that detection of publication bias (that is typically reporting positive more often than negative results) in NMA is not uncommon. Inevitably, introducing this kind of bias in meta-analysis threatens the validity of the results of the study as an "overly rosy picture" may be painted. To elaborate further, the evaluation of small study effects acts as a proxy for the assessment of publication bias. For the above assessment, a sophisticated statistical tool namely a comparison-adjusted funnel plot can be implemented. Apart from funnel plots, researchers can also employ Egger's test to statistically evaluate the presence of small-study effects[16-18].

QUALITY APPRAISAL SYSTEMS AND TRANSITION TO CLINICAL PRACTICE

The GRADE system features 6 components[5], that are study limitations, heterogeneity, inconsistency, indirectness, imprecision, and publication bias[5,19]. The quality of evidence may be high, moderate, low, or very low. As a rule of thumb, randomized trials yield high-quality evidence, whereas observational studies more often than not offer a low quality of evidence with the risk of bias potentially affecting clinical judgment[20].

Potential limitations of randomized trials include failure to conceal allocation, failure to blind, loss to follow-up, and failure to appropriately consider the intention[20]. Guyatt *et al*[20], in 2011, also mentioned terminating a study early for apparent benefit, and selective reporting of outcomes according to the results. The indirectness may be due to patients deviating from those of interest, when the treatments have not been compared in head-to-head trials, and when there are different outcomes from those being expected from the study[21]. Furthermore, the contributions of biological and social factors to the magnitude of effect in the outcomes represents indirectness[21]. On the other hand, inconsistency is defined as a disagreement between direct and indirect evidence in NMA[19]. In addition, Salanti *et al* [19], have suggested the adoption of a quantitative approach to assess the risk of bias.

INVESTIGATING CLINICAL DIVERSITY IN NMA

It is an undeniable fact that a great many confounding factors can be encountered in a broad systematic review of randomized trials. Thus, conducting sensitivity analysis to delineate the impact of clinical heterogeneity factors is strongly recommended. For instance, the effect of low-quality trials, variation in intervention characteristics as well as differences due to variable outcome measurement tools needs to be considered in those secondary analyses. From a technical point of view, the researcher needs to improve the trial(s) with the above characteristics from the analysis, repeat the statistical tests and subsequently compare the new results with the findings of the primary analysis[22].

PLANNING FUTURE RESEARCH WITH NMA- IS IT POSSIBLE?

Directing the design of future studies based on NMA results appears to be of significant importance as mismanagement of resources can be overcome[23-25]. For a researcher to provide an estimate of whether the results of a subsequent trial are likely to change in the future, an interval plot should be considered. By visually inspecting an interval plot, an investigator can enable predictions on the efficacy of a particular intervention in a future trial[16,26,27].

IMPROVING INTERPRETATION OF NMA FINDINGS

To improve interpretability and clarity of the results of an NMA, researchers are encouraged to back-transform their data in a manner that interpretation of their results is improved. For instance, when it comes to Patient-Reported Outcome Measures, investigators can back-transform Standard Mean Differences to Mean Differences and subsequently assess their findings against the established minimal clinically important difference for a particular questionnaire[28].

CONCLUSION

Overall, NMAs play a crucial role in the decision-making process. As long as common methodological mistakes are avoided, researchers can produce reliable and accurate clinical conclusions.

FOOTNOTES

Author contributions: Karthavapu V and Tsikopoulos K were involved in the conceptualization of the study; Tsikopoulos A and Sidiropoulos K conducted the literature research and extracted relevant information; Tsikopoulos K and Kitridis D assessed the quality of the included studies; Christofilos SI and Stoikos PN were involved in the generation of tables and the writing of the paper; Karthavapu V supervised and revised the paper accordingly; Throughout the study, Christofilos SI was an intern of Professor Maniatis's group at University College London; all authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Clinical and Translational Research

COVID-19 and thyroid disease: An infodemiological pilot study

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Abstract

BACKGROUND

Google Trends searches for symptoms and/or diseases may reflect actual disease epidemiology. Recently, Google Trends searches for coronavirus disease 2019 (COVID-19)-associated terms have been linked to the epidemiology of COVID-19. Some studies have linked COVID-19 with thyroid disease.

AIM

To assess COVID-19 cases *per se vs* COVID-19-associated Google Trends searches and thyroid-associated Google Trends searches.

METHODS

We collected data on worldwide weekly Google Trends searches regarding "COVID-19", "severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)", "coronavirus", "smell", "taste", "cough", "thyroid", "thyroiditis", and "subacute thyroiditis" for 92 wk and worldwide weekly COVID-19 cases' statistics in the same time period. The study period was split in half (approximately corresponding to the preponderance of different SARS-COV-2 virus variants) and in each time period we performed cross-correlation analysis and mediation analysis.

RESULTS

Significant positive cross-correlation function values were noted in both time periods. More in detail, COVID-19 cases *per se* were found to be associated with no lag with Google Trends searches for COVID-19 symptoms in the first time period and in the second time period to lead searches for symptoms, COVID-19 terms, and thyroid terms. COVID-19 cases *per se* were associated with thyroid-related searches in both time periods. In the second time period, the effect of "COVID-19" searches on "thyroid' searches was significantly mediated by COVID-19 cases (P = 0.048).

CONCLUSION

Searches for a non-specific symptom or COVID-19 search terms mostly lead Google Trends thyroid-related searches, in the second time period. This time

frame/sequence particularly in the second time period (noted by the preponderance of the SARS-COV-2 delta variant) lends some credence to associations of COVID-19 cases *per se* with (apparent) thyroid disease (*via* searches for them).

Key Words: Data collection; Epidemiology; Thyroid; Medical informatics; Methods; Trends

Core Tip: Google Trends searches for coronavirus disease 2019 (COVID-19)-associated terms have been linked to the epidemiology of COVID-19. In this study we aimed to assess worldwide COVID-19 cases *per se vs* COVID-19-associated Google Trends searches and thyroid-associated Google Trends searches for 92 wk. The study period was split in half and in each time period we performed cross-correlation analysis and mediation analysis. Significant cross correlation function factors for "COVID-19" and "severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)" were mostly found in the second time period, whereas COVID-19 cases *per se* were associated with "thyroid" searches in both time periods. In the second time period, which was characterized by the spread of SARS-CoV-2 delta variant, the effect of "COVID-19" searches on "thyroid" searches was significantly mediated by COVID-19 cases ($P = 0.048$). The observed time frame/sequence lends some credence to associations of COVID-19 cases *per se* with (apparent) thyroid disease.

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INTRODUCTION

Digital epidemiology uses digital data which was not generated with the primary goal of serving epidemiological research[1]; such data are within the domain of "infodemiology"[2]. Google Trends (available at https://trends.google.com) searches may - according to some researchers - accurately reflect the epidemiology of infectious, acute, or chronic diseases, including, among others, coronary or thyroid disease[2-10]. Recently, Google Trends searches for COVID-19-associated terms have been tentatively linked to the epidemiology of COVID-19[11-17]. Some - but not all – clinical studies have linked COVID-19 with thyroid function abnormalities and more particularly with a form of subacute-like thyroiditis[18-22]. Since the use of Google Trends to study a wide range of medical topics is becoming more widespread and the available research on COVID-19-related thyroid disease is conflicting, with this work we aimed to look at the issue of COVID-19-related thyroid disease from a different angle, namely, that of digital epidemiology, since the latter may be a useful adjunct to classical epidemiology.

MATERIALS AND METHODS

Data and data collection

We collected data on worldwide weekly Google Trends searches, by means of their "relative search volumes" (RSVs). The latter is normalized internet search volume values over a given time period, with a minimum of 0 and a maximum of 100 (see also https://support.google.com/trends/). More in detail, we used the worldwide RSVs of the search terms in the English language for "COVID-19", "SARS-CoV-2", "coronavirus", "smell", "taste", "cough", "thyroid", "thyroiditis", and "subacute thyroiditis" for 92 wk, from January 26, 2020 to October 24, 2021. The search terms were chosen because of their ubiquity and uniformity in lay and medical terms. For the same time period, worldwide weekly COVID-19 cases' statistics, as provided by the Johns Hopkins University Coronavirus Resource Center (available at https://coronavirus.jhu.edu/map.html), were collected[23]. The study period was split in half: The first half corresponded to the time period with preponderance of the SARS-CoV-2 alpha variant and the second to the time period with preponderance of the delta variant (Figure 1).

Statistical analysis

In each of the aforementioned time periods, we performed cross-correlation analysis. The threshold for statistical significance of each cross-correlation factor value at the $P = 0.05$ level was set according to lag,

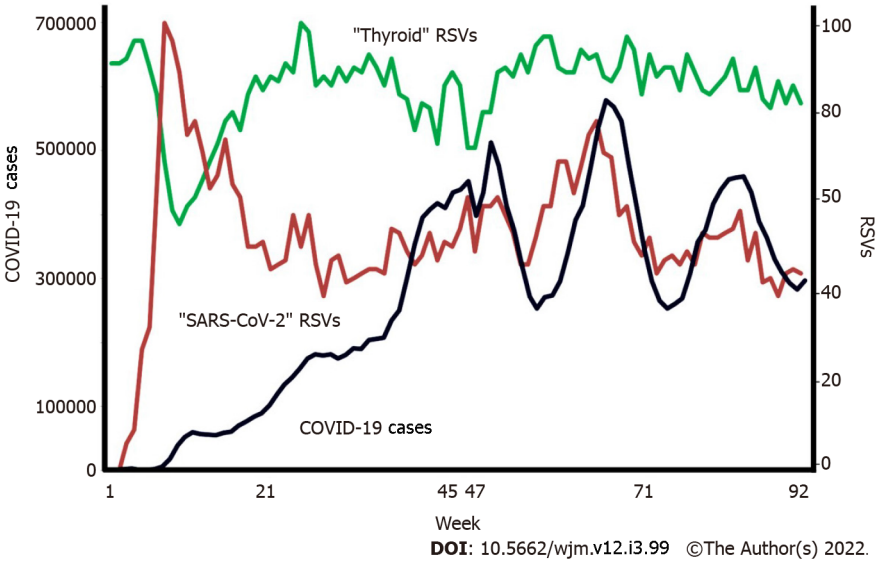

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Figure 1 Time series plot of selected study data: Worldwide coronavirus disease 2019 weekly cases and Google Trends relative search volumes for "severe acute respiratory syndrome coronavirus 2" and "thyroid" during the study period. Note the differences in magnitude, particularly during the second half of the study period. COVID-19: Coronavirus disease 2019; RSVs: Relative search volumes; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

thus the cross-correlation factor had to be higher than 0.290 at lag = 0 and 0.324 at lag = 8. A lag = 0 indicates contemporaneous correlation, a negative lag indicates that the first variable leads within a set time frame the second variable, and a positive lag indicates that the first variable follows (lags) within a set time frame the second variable. After the calculation of cross-correlation factor values, further evaluation among the variables was done with mediation analysis, implementing Sobel's test. Statistical analyses were done with Minitab v.17.1 (Minitab Inc, State College, PA, United States, 2010) and JASP v0.15 (JASP Team, University of Amsterdam, NL, 2021).

RESULTS

Worldwide, COVID-19 weekly cases *per se* gradually increased over time and showed wide fluctuations during the second half of the study period (Figure 1). The RSVs of the studied search terms also showed fluctuations (Figure 1 and Supplemental Figures 1 and 2). Significant positive cross-correlation factor values were noted in both time periods. More in detail, significant cross-correlation factors for "COVID-19" and "SARS-COV-s" were mostly found in the second time period (Table 1), whereas COVID-19 cases *per se* were associated with "thyroid" searches in both time periods. In the second time period, the effect of "COVID-19" searches on "thyroid" searches was significantly mediated by COVID-19 cases (Sobel test statistic $P = 0.048$).

DISCUSSION

COVID-19 cases *per se* were found to be associated with no lag with Google Trends searches for COVID-19 symptoms in the first time period and in the second time period to lead searches for symptoms, COVID-19 terms, and thyroid terms. Searches for a non-specific symptom or COVID-19 search terms mostly led Google Trends "thyroid" searches, in the second time period. This time frame/sequence particularly in the second time period, which was noted by the preponderance of the SARS-CoV-2 delta variant, lends some credence to associations of COVID-19 cases *per se* with (apparent) thyroid disease (*via* searches for them). Moreover, this finding, points to a possible higher probability of thyroid disease with SARS-CoV-2 delta variant compared to the alpha variant (and may also explain discrepancies regarding COVID-19 *vs* thyroid disease among previous relevant studies).

Digital health is in the spotlight as the COVID-19 crisis progresses[24,25]. At the same time, digital epidemiology is emerging at a very fast pace[25]. More and more of what we do and say - including epidemiologically relevant behaviors - is stored electronically, often in an accessible form. Internet data mining has a revolutionary impact on the way we monitor global health and health behaviors. Infectious and chronic disease data can be collected and disseminated in almost real time through a number of online sources. Google Trends provides a powerful measure of public interest in a topic,

Table 1 Positive cross correlation function values between variables; only significant values are presented (please see text for details)

		1st time period	2nd time period
COVID-19 cases *vs*	"Smell"	CCF: +0.644; Lag: 0	CCF: +0.540; Lag: 0
	"Taste"	CCF: +0.604; Lag: 0	CCF: +0.433 to +0.368; Lag: -2 to 0
	"COVID-19"	--	CCF: +0.412 to +0.315; Lag: -3 to 0
	"SARS-CoV-2"	--	CCF: +0.677 to +0.589; Lag: -2 to 0
	"Thyroid"	CCF: +0.323 to +0.315; Lag: -8 to -7	CCF: +0.412 to +0.343; Lag: -8 to -7
"COVID-19" *vs*	"Thyroid"	--	CCF: +0.374; Lag: -5
"SARS-CoV-2" *vs*		--	CCF: +0.323; Lag: -7

COVID-19: Coronavirus disease 2019; CCF: Cross correlation function; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

being a proxy of internet searches for it. The frequency of internet searches for disease terms may not reflect directly the epidemiological characteristics of a given disease, which is related and/or described by such search terms. Media coverage may skew subsequent internet searches. Nevertheless, the frequency of internet queries for various diseases' symptoms are correlated to a degree with physician visits for these diseases[26,27]. Google Trends has been used – despite its shortcomings – to monitor the yearly influenza epidemics[24,28]. Another source that has provided health data is Twitter. A smartphone application can be used to assess COVID-19 symptoms and may indicate future disease hotspots within 5-7 d[29]. The collection and classification of data ranging from the detection of suspected cases to the monitoring and assessment of pandemic risk is crucial. However, as this is a very evolving field, validation of digital health measures vis-à-vis input data, tentative associations, or predictive models is still needed. Regarding COVID-19, the influence of media on Google Trends RSVs has been studied and was found to be maximal after a week[30], whereas the effect of COVID-19 cases on "COVID-19" searches has been studied[31], and has been found to be most notable after 11.5 d[32]. Thus, with the lags in the observed cross-correlation factors, we believe that the Google Trends searches for COVID-19 and/or thyroid-related items may reflect personal interest fuelled by probable real disease (COVID-19 or thyroid disease).

Receptors for the SARS-CoV-2 virus are found in tissues beyond the respiratory system, such as the thyroid, thus an effect of COVID-19 on the thyroid is plausible[33]. Indeed, there is some evidence of thyroid dysfunction in patients with COVID-19, characterized by changes in hormone levels (low triiodothyronine or low thyrotropin levels) or laboratory results compatible with the presence of subacute thyroiditis[20,34]. Italian researchers observed that in the spring of 2020, 15% of COVID-19 patients (n = 93) admitted to the intensive care unit (ICU) at a hospital in Milan had changes in thyroid hormones. By comparison, only 1% of patients in the same period of 2019 (n = 101) had changes in thyroid hormones [19]. Considering the fact that viral infections can cause thyroiditis, the researchers began a monitoring program to look at thyroid function 3 mo after COVID-19 treatment. The researchers found that thyroiditis, in patients with moderate to severe COVID-19, was different from common subacute thyroiditis: Many patients had mild dysfunction and the rate of thyroid disease was higher in men. Thyroid dysfunction appeared to be associated with more severe COVID-19 disease. After 3 mo, thyroid function was normal in all followed patients (n = 53), with persistence of ultrasound findings of thyroiditis in one third of them[35]. Another study from Greece was based on the premise that the interpretation of thyroid tests in ill patients is hampered by changes that ensue in the context of non-thyroidal illness syndrome and studied thyroid function in cohorts of COVID-19 positive (n = 102, 46 in the ICU) and COVID-19 negative patients (n = 94, 41 in the ICU)[18]. The researchers noted a non-thyroidal illness syndrome pattern in 60% of ICU and 36% of ward patients (with no significant differences between COVID-19 positive and negative patients)[18]. The thyroid laboratory work-up was compatible with thyrotoxicosis in 14.6% of SARS-CoV-2 positive ICU patients *vs* 7.7% in SARS-CoV-2 negative ICU patients (P = NS) and, overall in 8.8% of SARS-CoV-2 positive *vs* 7.4% of negative patients. Thus, the authors concluded that a non-thyroidal illness syndrome pattern is common in COVID-19 but it relates to the severity of disease rather than SARS-CoV-2 infection, whereas a thyrotoxicosis pattern was less frequently observed and was not different between patients with and without COVID-19[18].

Our study has several limitations and its caveats have to be considered. We collected only Google Trends data for English-language searches; however, we have shown in an older study that searches in this language dwarf searches in all other languages[4]. Additionally, Northern hemisphere internet searches dwarf Southern hemisphere searches[4]. Analyses were done on a weekly worldwide basis since Google Trends searches for extended time periods are provided as such. From the literature, worldwide and weekly or monthly Google Trends data are considered to be more reliable than country-wide and daily data[36,37]. No periodicity in the data was assessed since the total time duration of data collection was rather short. As stated above, the datasets were split in half given the vast differences in

COVID-19 epidemiology in 2020-2021 due to the preponderance of different SARS-CoV-2 variants. Finally, we have to bear in mind the fact that Google Trends searches are limited to internet-literate persons, who are easily influenced by media items, although few (medical) research articles are reported by news outlets (targeting diverse audiences) and generate public interest[38].

CONCLUSION

Given the relatively recent onset of SARS-CoV-2 virus infection, the available monitoring data are limited in time and therefore long-term studies are needed to evaluate even longer-term effects on the endocrine glands. Research into the virus continues to grow, shedding more light on the real health risks posed by COVID-19. Ideally, it would be interesting to assess time and localization-delimited Google Trends searches with the corresponding thyroid disease incidence, as reported by physicians or as recorded in healthcare databases, to verify the associations observed. Understanding the nature of a pandemic of this magnitude means saving human lives and proper knowledge of ways to prevent further infection.

ARTICLE HIGHLIGHTS

Research background

Google Trends searches for symptoms and/or diseases may reflect actual disease epidemiology. Recently, Google Trends searches for coronavirus disease 2019 (COVID-19)-associated terms have been linked to the epidemiology of COVID-19. Some studies have linked COVID-19 with thyroid disease.

Research motivation

Since the use of Google Trends to study a wide range of medical topics is becoming more widespread and the available research on COVID-19-related thyroid disease is conflicting, with this work we aimed to look at the issue of COVID-19-related thyroid disease from a different angle, namely, that of digital epidemiology, since the latter may be a useful adjunct to classical epidemiology.

Research objectives

We assessed worldwide COVID-19 cases *per se vs* COVID-19-associated Google Trends searches and thyroid-associated Google Trends searches for 92 wk.

Research methods

We collected data on worldwide weekly GT searches regarding "COVID-19", "severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)", "coronavirus", "smell", "taste", "cough", "thyroid", "thyroiditis", and "subacute thyroiditis" for 92 wk and worldwide weekly COVID-19 cases' statistics in the same time period. The study period was split in half (approximately corresponding to the preponderance of different SARS-COV-2 virus variants) and in each time period we performed cross-correlation analysis and mediation analysis.

Research results

Significant positive cross-correlation function values were noted in both time periods. More in detail, COVID-19 cases *per se* were found to be associated with no lag with Google Trends searches for COVID-19 symptoms in the first time period and in the second time period to lead searches for symptoms, COVID-19 terms, and thyroid terms.

Research conclusions

Searches for a non-specific symptom or COVID-19 search terms mostly led Google Trends thyroid-related searches, in the second time period. This time frame/sequence particularly in the second time period (noted by the preponderance of the SARS-COV-2 delta variant), lends some credence to associations of COVID-19 cases *per se* with (apparent) thyroid disease (*via* searches for them).

Research perspectives

Given the relatively recent onset of SARS-CoV-2 virus infection, the available monitoring data are limited in time and therefore long-term studies are needed to evaluate even longer-term effects on the endocrine glands. Research into the virus continues to grow, shedding more light on the real health risks posed by COVID-19. Ideally, it would be interesting to assess time and localization-delimited Google Trends searches with the corresponding thyroid disease incidence, as reported by "sentinel" physicians or as recorded in healthcare databases, to verify the associations observed. Understanding the nature of a pandemic of this magnitude means saving human lives and proper knowledge of ways

to prevent further infection.

FOOTNOTES

Author contributions: All authors conceived this work, searched the literature, analyzed the data, performed the analyses, and wrote this manuscript.

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ORIGINAL ARTICLE

Observational Study

Lutetium in prostate cancer: Reconstruction of patient-level data from published trials and generation of a multi-trial Kaplan-Meier curve

Andrea Messori

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Abstract

BACKGROUND

Lutetium has been shown to be an important potential innovation in pre-treated metastatic castration-resistant prostate cancer. Two clinical trials have evaluated lutetium thus far (therap and vision with 99 and 385 patients, respectively), but their results are discordant.

AIM

To synthetize the available evidence on the effectiveness of lutetium in pre-treated metastatic castration-resistant prostate cancer; and to test the application of a new artificial intelligence technique that synthetizes effectiveness based on reconstructed patient-level data.

METHODS

We employed a new artificial intelligence method (shiny method) to pool the survival data of these two trials and evaluate to what extent the lutetium cohorts differed from one another. The shiny technique employs an original reconstruction of individual patient data from the Kaplan-Meier curves. The progression-free survival graphs of the two lutetium cohorts were analyzed and compared.

RESULTS

The hazard ratio estimated was in favor of the vision trial; the difference was statistically significant ($P < 0.001$). These results indicate that further studies on lutetium are needed because the survival data of the two trials published thus far are conflicting.

CONCLUSION

Our study confirms the feasibility of reconstructing patient-level data from

survival graphs in order to generate a survival statistics.

Key Words: Survival analysis; Individual patient data reconstruction; Kaplan-Meier curves; Meta-analysis; Prostate Cancer; Lutetium

Core Tip: This paper describes the application of a new technique of individual-patient data reconstruction to the progression-free survival curves published in two trials evaluating lutetium in metastatic prostate cancer. Our analysis interpreted these survival data and showed discordant results between the two trials, that need to be addressed by further clinical research.

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INTRODUCTION

Lutetium has been shown to be an important potential innovation in pre-treated metastatic prostate cancer, but the extent to which outcomes are improved by this treatment still needs to a fully investigated. Three studies have evaluated lutetium in this disease condition. One was phase II (therap trial[1]), the second was phase III (vision trial[2]); the third, which was an observational real-world study[3], differed from the first two because lutetium was given after radium-223.

In recent times, techniques that reconstruct individual patient data from the graphs of Kaplan-Meier curves have considerably improved in terms of performance and easy applicability[4]. One advantage is that the availability of these techniques permits to combine multiple survival curves published in different trials without using any meta-analytical statistics. An example of this approach is presented herein. Our objective was two-fold: 1) to quantify the gain in progression-free survival determined by lutetium: 2) to demonstrate the applicability of techniques of patient-level data reconstruction in addressing specific questions based on time-to-event endpoints without the need to employ any meta-analytic statistics.

MATERIALS AND METHODS

We applied the shiny technique of individual patient data reconstruction[4] to the Kaplan-Meier graphs of progression-free survival reported in the therap phase-II trial[1] and in the vision phase III trial[2]. Both trials were conducted in patients with metastatic castration-resistant prostate cancer previously treated for their metastatic disease. In the therap trial, the treatment group received Lu-PSMA-617 (6·0–8·5 GBq intravenously every 6 wk for up to six cycles) while the controls were given cabazitaxel (20 mg/m^2 intravenously every 3 wk for up to ten cycles). In the vision trial, the treatment group received ^{177}Lu-PSMA-617 (7.4 GBq every 6 wk for four to six cycles) plus protocol-permitted standard care while the controls received standard care alone. In the therap trial, progression-free survival was defined as the interval from randomisation to first evidence of pupil-size artefact progression defined by an increase of at least 25% and at least 2 ng/mL after 12 wk (as per PCWG316), radiographic progression using locally reported computed tomography and bone scanning [Response Evaluation Criteria In Solid Tumors (RECIST) 1.1 and PCWG3 criteria for bone lesions], commencement of non-protocol anticancer treatment, or death from any cause. In the vision trial, the end-point was image based progression free survival.

The progression-free survival graphs of the two lutenium cohorts by Hofman *et al*[1] for the therap trial (99 patients; follow-up of 18 mo; 90 events) and Sartor *et al*[2] for the vision trial (385 patients; follow-up of 30 mo; 254 events). For each of these two Kaplan-Meier curves, the graph was digitalized and converted into x-y data pairs using Webplotdigitizer (version 4.5, https://apps.automeris.io/wpd/). Then, the shiny package (version: 1.2.2.0; subprogram "Reconstruct Individual Patient Data"; https://www.trialdesign.org/one-page-shell.html#IPDfromKM, see reference[4]) was used to reconstruct patient-level data on the basis of x-y data pairs, total number of enrolled patients, and total number of events. Finally, the pooled survival curves were generated from the reconstructed patient-level data and analyzed through standard Cox statistics. For this purpose we used three packages

("coxph", "survfit", and "ggsurvplot") under the R-platform. The hazard ratio (HR) was estimated.

RESULTS

The shiny procedure combined with standard Kaplan-Meier statistics allowed us to compare the 99 patients given lutetium in the therap trial with the 385 patients given lutetium in the vision trial.

Figure 1 shows the two Kaplan-Meier curves generated from reconstructed patient-level data. The HR estimated from these curves favored the patients of the vision trial and was 0.59 (95%CI, 0.46 to 0.75). The difference was statistically significant ($P < 0.001$).

DISCUSSION

When two or more randomised trials are available on a therapeutic issue and the clinical end-point is the form of time-to-event, synthetising the clinical evidence is a complex issue, and there is presently no consensus on which methodological approach should be preferred[5,6]. Pooling the values of HR is certainly the method most commonly used, but its important limitations have been widely recognised for many years (*e.g.* the inability to account for the length of follow-up, the inability to model variations of risk over time, the dimensionless nature of HR as opposed to the greater informative value of absolute parameters such as medians, *etc.*)[8]. The development of the restricted mean survival time has represented an advancement in this field[8,9], but the use of this parameter unfortunately remains low.

In this context, the marked improvement in performance of techniques that reconstruct individual-patient data[4] represents an important innovation, the role of which still needs to be fully evaluated. On the one hand, reconstructing individual-patient data is a mandatory pre-requisite to determine the RMST, and this explains the increased use of these reconstruction techniques when a single trial needs to be analysed[7]. On the other hand, another potential use of these techniques is being recognised when multiple trials are available: in such cases, these techniques offer a new methodological alternative to standard meta-analytic methods[5,6] and also to the more recent approaches where meta-analysis is based on the use of RMSTs[8,9].

The various parameters mentioned above (especially HR, RMST, and median) have been investigated for many years to identify their respective advantages and disadvantages, and the literature on this issue is wide[7]. In contrast, the literature on the use of reconstructed survival curves is still in its early stages[4,6], and this holds true particularly when multiple trials are analysed and pooled together.

The experience described herein offers a limited but useful contribution to the development of meta-analysis-like methods based on reconstructed survival curves.

The two control groups of the two trials differed in the treatment they received, and so were not included in our analysis, which was focused only on the two lutetium groups of the two trials. In comparing these two group with one another, our results raise the need to explain the statistically different outcomes shown by the HR and presented in Figure 1.

The inclusion criteria of the therap and vision trials were very similar, and so they likely had no substantial role in determining this difference. In fact, in the therap trial, patients had metastatic castration-resistant cancer and PET eligibility criteria for the trial were PSMA-positive disease, and no sites of metastatic disease with discordant FDG-positive and PSMA-negative findings; previous treatment with androgen receptor-directed therapy was allowed. In the vision trial, patients had metastatic castration-resistant prostate cancer previously treated with at least one androgen-receptor–pathway inhibitor and one or two taxane regimens and had PSMA-positive gallium-68 (68Ga)–labeled PSMA-11 and PET scans. While these differences in the inclusion criteria do not seem to suggest a better prognosis for patients included in either trial, a number of factors (*e.g.* environmental and lifestyle factors, tissue biomarkers, molecular pathological epidemiology, the microbiota, *etc.*) might have influenced tumor development and response to therapy. Hence, the discrepancies observed across the two trials included in our analysis might be explained by these factors. As regards innovative treatments such as lutetium, it should be stressed that molecular pathological epidemiology research has a growing role and is increasingly recognized to be a promising strategy to improve prediction of response to therapy.

In summary, the main strength of our analysis lies in the originality of the methodological approach that reflects the recent availability of very efficient patient data reconstruction techniques. The main limitation is represented by the indirect nature of the comparison between the two lutetium cohorts.

CONCLUSION

Our study indicates that further studies on Lu-PSMA-617 are needed because the survival data of the two trials published thus far demonstrate quite conflicting results. The example described in this paper

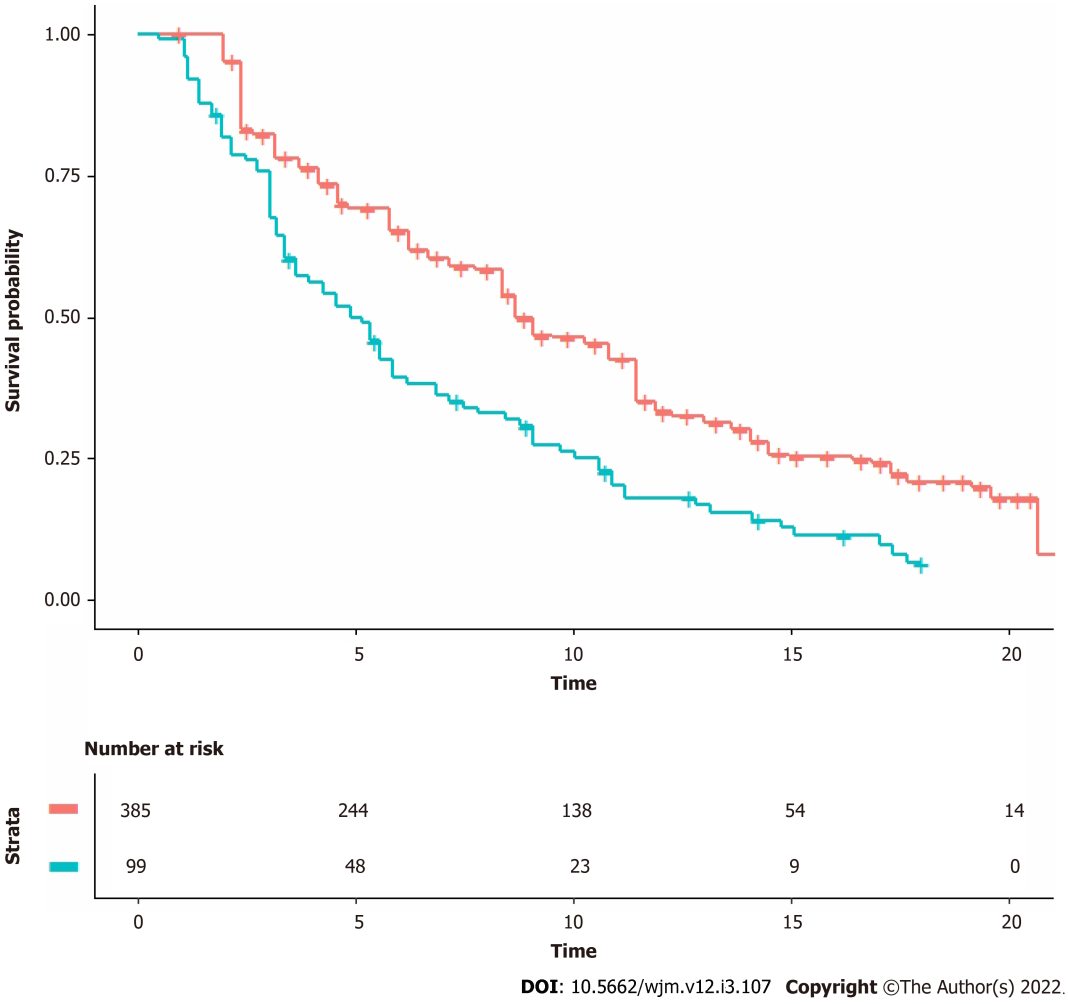

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Figure 1 Kaplan-Meier curves from reconstructed patient-level data. Pooled Kaplan-Meier survival curves obtained by reconstruction of individual patient data from two trials (therap[1] and vision[2]). Vision trial in red, therap trial in blue; time expressed in months. See text for details.

confirms the feasibility of reconstructing patient-level data from survival graphs in order to generate a survival statistics from these reconstructed data. To evaluate the advantages and disadvantages of this new methodological approach, further analyses will be needed.

ARTICLE HIGHLIGHTS

Research background

Two trials have been published to assess the effectiveness of lutetium in metastatic prostate cancer. The need to convert these effectiveness data into a pooled estimate represents a useful opportunity to test an innovative technique of individual patient reconstruction based on the analysis of Kaplan-Meier curves (shiny method).

Research motivation

The main motivation was to test the performance of the shiny method based on a real data-set.

Research objectives

Clarifying the effectiveness of lutetium in metastatic prostate cancer and confirm the reliability of the shiny method as a tool for reconstructing individual patient data.

Research methods

The clinical trials that have thus far evaluated lutetium in metastatic prostate cancer have been identified by standard literature search. A pooled survival curve has been generated from these trials by using the shiny technique of individual patient data reconstruction.

Research results

Two clinical trials were identified. A pooled Kaplan-Meier survival curve was generated that synthesizes the current evidence on the effectiveness of this treatment in this disease condition.

Research conclusions

A two-fold conclusion: First, lutetium is effective in metastatic prostate cancer; second, the Shiny technique can successfully be used to pool survival data from two trials without employing any meta-analytical method.

Research perspectives

The shiny technique has been confirmed to be a useful new tool for analyzing survival data from multiple trials and therefore deserves to be further applied in the analysis of clinical evidence.

FOOTNOTES

Author contributions: Messori A is the sole author, read and approved the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Airway management training program for nurses *via* online course in COVID-19 preparedness

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Abstract

BACKGROUND

Nursing officers are an integral component of any medical team. They participate in taking care of basic airway management and assist in advanced airway management, specifically amidst the current coronavirus disease 2019 (COVID-19) pandemic.

AIM

To assess the efficacy of a standardized web-based training module for nurses in preparedness to fight against COVID-19.

METHODS

The training was held in three sessions of 1 h each, consisting of live audio-visual lectures, case scenarios, and skill demonstrations. The sequence of airway equipment, drug preparation, airway examination, and plans of airway management was demonstrated through mannequin-based video-clips.

RESULTS

Pre- and post-test scores as well as objective structured clinical examination scores were analyzed using Student's *t*-test and the Likert scale was used for feedback assessment. It was found that the mean score out of the total score of 20 was 8.47 ± 4.2 in the pre-test, while in the post-test it was 17.4 ± 1.8 (P value < 0.001). The participants also felt self-reliant in executing the roles of airway assistant (63.3%) and drug assistant (74.3%). Fear of self-infection with COVID-19 was also high, as 66% of participants feared working with the patient's airway.

CONCLUSION

Amidst this COVID-19 emergency, when the health care systems are being

persistently challenged, training of nursing staff in the safe conduct of airway management can ensure delivery of life-saving treatment.

Key Words: COVID-19; Nursing; Airway management; Online; Training; Preparedness

Core Tip: The health care response systems are being persistently challenged by coronavirus disease 2019 (COVID-19). Nurses are actively involved in various tasks of airway management like preparation of airway equipment, drugs, and basic airway management. This study demonstrated a gross lack of knowledge regarding airway management despite receiving basic life support training. The participants felt more self-reliant and confident in executing the roles of airway assistant and drug assistant after the session. There is a need to train nursing staff from different subsets of practice in the safe conduct of airway management and simulation based online training program for health professionals can be employed for preparedness against COVID-19.

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INTRODUCTION

Coronavirus infection is a public health emergency of international concern[1]. The frontline health care workers are at a heightened risk of catching the disease[2]. Nearly 15% of coronavirus disease 2019 (COVID-19) patients require hospitalization and oxygen support and 5% require definitive airway management. As it primarily involves the respiratory system, the caring medical team should acquire airway management skills[3]. Nursing officers are an integral component of such team, taking care of basic airway management and assisting in advanced airway management[4]. The World Health Organization (WHO)'s prescribed norm is one doctor and three nurses for 1000 people. A wide disparity, however, prevails in the health care professional to population ratio, with developing nations having poorer statistics. The nurse/population ratio is 2.1 in India, as per the latest available WHO's global health workforce statistics[5]. This highlights our currently overloaded staff and also the necessity to keep our health care workers safe while dealing with COVID-19 patients as we cannot afford to lose the already worn-down workforce[6]. Acquiring adequate skills for airway management demands both technical proficiency and clinical knowledge. A lapse in judgment can contribute to increased morbidity and mortality in critically ill patients. Due to the threat of viral contamination during face-to-face training, online teaching is rising as the new norm of education. Thus, with the safety of health care workers as our chief priority, we designed an interactive online airway course to increase the ability of nursing officers in airway management of critically sick patients. The aim of the study was to assess the efficacy of a standardized web-based training module in preparedness to fight against COVID-19 and enhance Emergency Airway Response Team, knowledge, team dynamics, and personnel confidence.

MATERIALS AND METHODS

Study design

After institutional ethical approval (study registration No. AIIMS/IEC/20/283), we conducted a prospective, observational study over a period of 4 mo at the Advanced Center of Continuous Professional Development (CPD) Department through a dedicated online course conducted thrice weekly in our tertiary care institute. Our study was designed following the STROBE guidelines. A list of 30 participants (nursing officers) per session was prepared and we ensured a uniform representation from each department. Inability to attend the course due to prior commitment or network issues led to exclusion from that session and such participants were subsequently included in next scheduled course. Course content was diligently constructed to cover information regarding pandemic preparation, COVID-19 spread, risk alleviation, education about personnel protective equipment (PPE), protection required during airway procedures, signs of respiratory distress, indications of intubation, airway assessment, difficult airway predictors, airway management guidelines and sequence of plan, catalogue

of airway equipment and COVID-19 intubation kit, drugs, procedure of rapid sequence induction, mask ventilation using vice grip, steps of video-laryngoscopy, intubation, supraglottic airway placement, and front of neck access (FONA).

Through the online portal of "Google meet", the training was held in three sessions of 1 h each, consisting of live audio-visual relay of lectures, case scenarios, presentations, and skill station. The sequence of personal protection, airway equipment and drug preparation, designated COVID-19 isolation area for airway management, clinical airway examination with difficult airway assessment using MACHOCHA score, and plans of airway management (Plans A, B, C, and D) were demonstrated through simulator mannequin-based video-clips. The skill stations consisted of 1 h and included demonstration of preparation of appropriate equipment and drugs required for induction in a trolley, designation of negative pressure isolation room for intubation, team dynamics, plans of airway management, use of airway adjuncts, intubation using video-laryngoscope (Plan A), choosing appropriate size of supraglottic airway device and its insertion (Plan B), bag-mask ventilation using vice grip (Plan C), and equipment required (Plan D)-surgical scalpel cricothyroidotomy/FONA by the instructors *via* videos and skill stations. Participants could clarify their doubts by speaking through the microphone or writing it in the common chat window. To ensure an active participation, interaction of participants with instructors in the language that they were most comfortable with was encouraged. Each scenario was followed by a debriefing session, after which the participants were encouraged to enlist their achievements and shortcomings from the session.

Data analysis

The participants were provided with Google form links of "pre- and post-test questionnaire". Both the questionnaire forms were identical and consisted of 20 multiple-choice questions (1 mark each), which included specific theoretical questions related to airway management. The participants also had to answer 10 objective structured clinical examinations (OSCE), each consisting of one mark each. Each participant's performance during the skill stations was independently evaluated by two experienced instructors based on OSCE response. For a successful completion of training program, it was necessary for the participants to obtain 70% of marks in the post-test and more than 80% in OSCE assessment. A feedback form was filled at the end of the session, consisting of eight assertions on a 5-point rating Likert scale. The score of "5" indicated "strong agreement" with the statement while a score of "1" indicated that participants were in "strong disagreement" with it. Two faculty members, experts in airway management, validated the questionnaire and survey form at an independent level. An investigator who was blinded to the study protocols collected and then analyzed the outcome data. The basis for sample size estimates was convenience sampling.

Statistical analysis

The Statistical Package for the Social Sciences version 23.0 software (SPSS, IBM Corp. Armonk, NY, United States) was utilized to perform statistical analyses. A pre- and post-test questionnaire, specifically developed for this course, was analyzed as the primary outcome. The secondary outcome was evaluated as OSCE based assessment. The results are summarized as descriptive statistics and presented as the mean ± SD or mean ± SE. The Student's *t*-test was employed to analyze the data for intra- and inter-group comparisons. To assess the survey form, a mean Likert score was averaged to the total number of items. A P value < 0.05 was considered statistically significant.

RESULTS

A total of 1055 nursing officers were trained during the program. One hundred and nine participants who could not complete either the pre- or post-test were excluded from the analysis. Nine hundred and forty-six nursing officers were able to complete the pre- and post-test and thus included in the final analysis (Figure 1). The mean years of work experience of the participants was 4.01 ± 3.16 (mean ± SD). On analyzing the questionnaire, it was found that the mean score out of the total score of 20 was 8.47 ± 4.2 in the pre-test, while in the post-test it was 17.4 ± 1.8 (Figure 2); the difference was statistically significant (P < 0.001). Although 68% of our participants were trained in basic life support (BLS), questions in the pre-test, based on the specific knowledge of airway and plans for airway management, were frequently missed. The concept of team dynamics and role allocation was also alien to the majority of nursing officers. The overall knowledge and cognizance regarding airway management of COVID-19 patients improved significantly following the session (P < 0.001). Approximately 92% of the participants accurately responded to specific questions related to airway management in the post-test. There was improvement in OSCE based assessment and all participants could score above 80% in OSCE.

Participants were asked to provide feedback at the end of session. Amongst the various questions asked in feedback, one was pertaining to the part of training which they found most helpful. The video demonstration of airway procedures, preparation of airway trolley, and medications was the most cherished by the nursing officers. After attending the program, 79% of participants felt that they were familiar with airway management techniques and protocols for COVID-19 patients. The participants

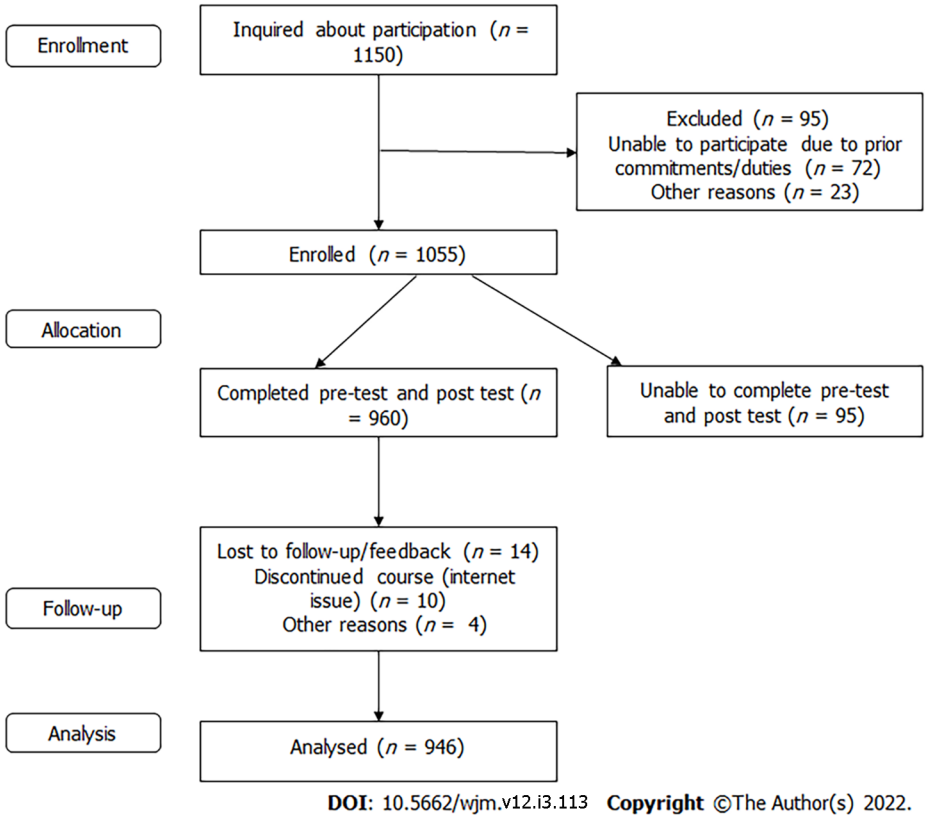

Figure 1 Flow diagram for participant enrollment and analysis.

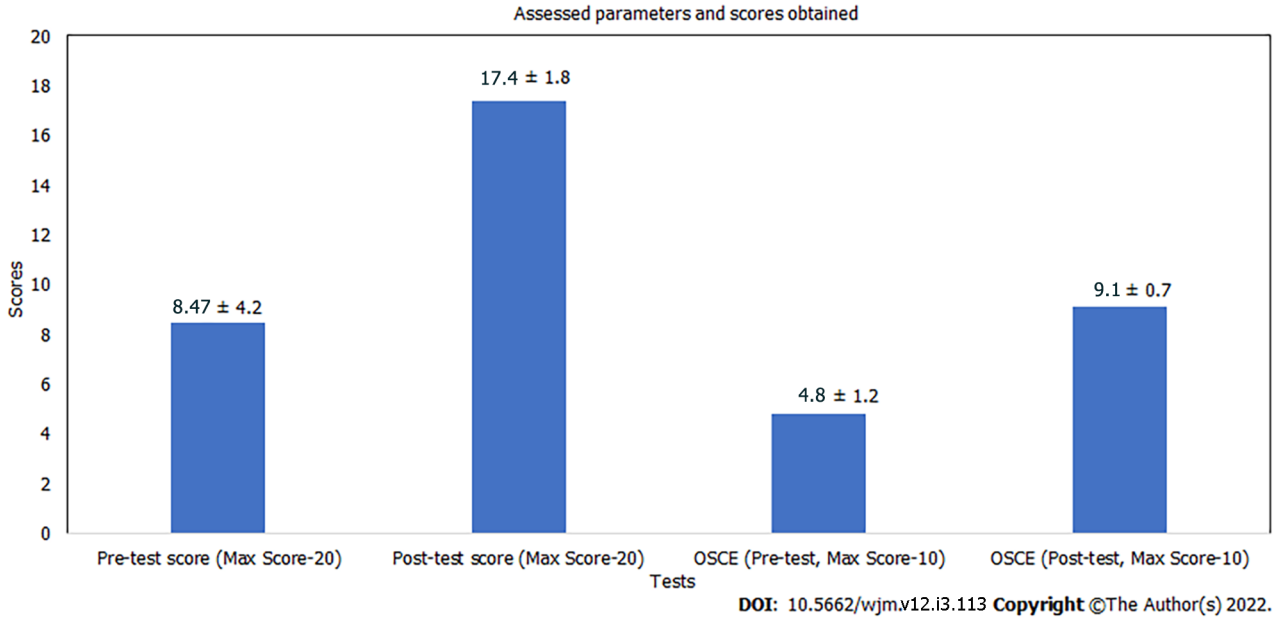

Figure 2 Scores obtained in pre-test, post-test, and objective structured clinical examinations.

also felt self-reliant in executing the roles of airway assistant (63.3%) and drug assistant (74.3%). An increase in level of self-confidence was reflected in other parameters like performing laryngoscopy, supraglottic airway device (SGA) insertion, and arrangement of necessary equipment as well (Table 1). Fear of self-infection with COVID-19 was also high, as 66% of participants feared working with the patient's airway (Table 2). This short online training module for airway management in COVID-19 patients was liked by majority of our participants and they strongly believed that it helped in improving their clinical acumen and skills.

Table 1 Level of confidence in various roles amongst study participants (scores 1 to 5, with 1 meaning strongly disagree and 5 meaning strongly agree)

S. No.	Role/procedure	Percentage of participants with a score ≥ 4	Percentage of participants with a score of 3	Percentage of participants with a score ≤ 2
A Pre-training assessment				
1	Airway assistant	35	31	34
2	Drug assistant	41	45	14
3	Laryngoscopy	9	15	76
4	SGA insertion	14	28	58
5	Familiarity with airway management plan	29	24	47
B Post-training assessment				
1	Airway assistant	63.3	21.6	15.1
2	Drug assistant	74.3	23	2.7
3	Laryngoscopy	58	13	29
4	SGA insertion	78	19	3
5	Familiarity with airway management plan	79	14	7

SGA: Supraglottic airway devices.

Table 2 Reasons for fear regarding management of coronavirus disease 2019 patients

S. No.	Reason for fear	Percentage of participants
1	Breach in PPE	49
2	High aerosol generation	34
3	Lack of airway experience	38
4	Cross-infection	57
5	Difficult airway situation	23
6	No fear	18

PPE: Personnel protective equipment.

DISCUSSION

The role of nursing staff in any health care service is indispensable. They form a pivotal part in the patient care in wards, emergency area, outpatient department (OPD), operation theatres, high dependency units, and intensive care units (ICU). They are actively involved in various tasks of airway management like preparation of airway equipment and drugs, checking for adequate resources like oxygen, airway suctioning, and basic airway management[7]. There is a high probability that the first responder to any patient with respiratory urgency is a nurse who might have to manage airway till a physician help arrives[8]. In such a scenario, the lack of knowledge and experience in airway management can not only jeopardize patient care but also result in a heightened risk of infection transmission[9]. The goals for the airway rescuer in COVID-19 patients is to rapidly secure an airway, preferably in first attempt, with clear backup contingencies, while reducing the aerosol generation and preventing redundant contamination[10-12]. European Society guidelines for management of airway in COVID-19 patients, recommend endotracheal intubation using rapid sequence intubation (RSI) for Plan A; in the advent of failure of plan A, SGA placement as Plan B; face mask ventilation as Plan C; and finally FONA as Plan D[13]. Our prime expectation from this training module was to equip the nursing officers with adequate information, clearing their queries and fears related to COVID-19 patient care such that they could efficiently work in a high performing airway rescue team without compromising personal safety. Although the pretest score was as low as 8.47 ± 2.4, by the end of our session, the respondents were clear with these features and achieved a high score of 17.4 ± 1.8. It was evidenced by

their poor performance in the pre-test questionnaire regarding airway assessment and difficult airway predictors like modified Mallampati grade and MACOCHA score. The reason for this could be accredited to the mixed population from different practice areas (general wards, OPDs, operation theatres, emergency, and ICU) and the years of experience. We succeeded in educating them about airway assessment sufficiently enough to perform significantly better in the post-session analysis with the same set of questions.

Although the regular curriculum of nursing does impart education about basic airway support, it is not emphasized enough. The proportion of respondents (58%) who were already trained in BLS had a better understanding of basic airway care as compared to those who had not completed BLS course. This was evident by their fair knowledge about identification of respiratory distress, indications for intubation, and basic equipment in airway management. The majority of participants (71%), however, showed gross deficit in information with respect to the plans for airway management, drugs required for RSI, advanced airway equipment like video laryngoscope and procedure of intubation, supraglottic airway device insertion, and FONA. This revealed the necessity to train them in both basic and advanced airway care so that in the crisis, they can play the role of competent assistants in airway management.

Continuing medical education programs, workshops, and seminars comprise an efficient approach to achieve proficient teaching and learning[14,15]. Conforming to the principle of social distancing amidst this highly infectious health emergency, simulation-based medical education has an important role in learning. Various international recommendations include airway training simulation as a part, which has shown to be beneficial with respect to behavior changing process, acquisition of skills, and trainee satisfaction[16].

A wide range of airway complications and increased viral transmission may occur if nurses who are involved in such teams have no experience in emergency airway management[17,18]. Cook *et al*[19] documented that permanent harm or death due to airway related complications was mainly due to inadequate access to properly skilled staff or equipment, inability to identify at-risk patients, poor planning, and lack of structured strategies for tackling predictable airway complications.

It is noteworthy that registered nurses, even those working in ICU, may spend a larger fraction of working hours in patient care, without the requirement to manage respiratory emergencies on an everyday basis[20]. This in itself reveals the state of experience of nurses working in non-ICU environment with respect to airway care. Kelleher *et al*[21] conducted a study to investigate the endotracheal care practices amongst critical care nurses and found a wide variety in their techniques, with non-adherence to best practice recommendations and resultant lower-quality care. Another descriptive analytic study showed that the knowledge and performance of intensive care nurses regarding endotracheal suctioning and care was good (71.6 ± 10.91) and medium (41.22 ± 7.91), respectively[22].

The key to effective airway management is proper assessment and anticipation of any associated difficulties[23]. The foundation of any high performing team is a strong understanding of team dynamics. Ranging from deploying of the scarce available resources or employing the latest evidence-based guidelines to building a firm groundwork of healthy teamwork with good and clear communication can provide a strategic lead in tackling this pandemic. Our aim was to emphasize on the clear role allocation, closed loop communication, and cross monitoring (checking for cross- contamination) while working in the airway rescue team. There was a statistically significant improvement in terms of knowledge and confidence in competent role execution as airway team members in the post survey analysis as compared to their pre-test evaluation.

The feedback submitted by the participants highlighted the truth that this global crisis has fostered fear among all healthcare workers. The majority of the participants admitted that the fear was mainly based on risk of breach in PPE, aerosol spread, lack of proper training in airway prior to actual patient handling, and fear of contracting infection and carrying the infection back home amongst others. These responses go in line with a study done in healthcare workers working with COVID-19 patients that revealed higher anxiety, depression, and apprehension due to similar factors among 71.5%, 44.6%, and 50.4% of the respondents, respectively[24,25].

This training module highlighted the need to put more emphasis on airway training of the nursing staff and contributed to fill up the lacunae in the realm of airway care while giving due weightage to occupational safety and health. We believe that by using simulation based online training program for nurses, we successfully educated them and simultaneously strengthened our workforce in airway management, if and when the need arises.

Strengths

Our study adds to the theoretical development of efficacy of online simulation-based training of health care professionals in inevitable situations like the COVID-19 pandemic. To the best of our knowledge, this is a novel study to train nurses for airway management of COVID-19 patients through an online platform and gives evidence of statistically significant improvement in knowledge, attitude, and confidence regarding the same. We took extra care to reach up to individual level participation and trained them in the language that they understood well. Free will to attend the training program as many times needed was the additional advantage of our course.

Limitations

We completely acknowledge that the chief and inevitable limitation in our study was an inability to conduct the skill station training in person. The heterogeneous study population with diverse levels of exposure to airway care was another limitation. Although the majority of participants passed the post session evaluation, 140 of them had to repeat this course once due to sub-par scoring. The infrequent issues with internet connectivity, first time online course learning, difficulty to comprehend, and language disturbance were responsible for inefficiency in understanding, leading to poor response in post-test analysis and hence the need for repetition of course.

CONCLUSION

Amidst this COVID-19 public health emergency, when the health care response systems are being persistently challenged, training of nursing staff from different subsets of practice in the safe practice of airway management can play a substantial role in ensuring access of life-saving treatment to COVID-19 patients, without compromising the safety of health care professionals. Our study in its unique aspect has the potential to pave way for further large-scale research while confirming to incorporate similar training regimes aimed at improving the preparedness and skill of various health professionals to tackle this crisis efficiently.

ARTICLE HIGHLIGHTS

Research background

The nursing officers are an integral part of medical team. They contribute in basic airway management and as an assistant in advanced airway management, which holds great significance in the coronavirus disease 2019 (COVID-19) pandemic.

Research motivation

The pandemic has resulted in over-burdened medical staff with lack of adequate skills for airway management to handle this respiratory disease pandemic.

Research objectives

The primary research objective was to create an interactive online airway course to increase the ability of nursing officers in airway management of critically sick patients.

Research methods

The training was conducted through live audio-visual lectures, case scenarios, and skill demonstrations through mannequin-based videos. The demonstrations for airway equipment, preparation of drugs, airway examination, and plans of airway management were done.

Research results

The mean score out of the total score of 20 was 8.47 ± 4.2 in the pre-test, while it was 17.4 ± 1.8 in the post-test ($P < 0.001$). After attending the program, 79% of participants felt that they were familiar with airway management techniques and protocols for COVID-19 patients. An increase in level of self-confidence was reflected in other parameters like performing laryngoscopy, Supraglottic airway insertion, and arrangement of necessary equipment as well.

Research conclusions

The training of nursing staff from different subsets of practice in the safe practice of airway management can play a substantial role in ensuring access of life-saving treatment to COVID-19 patients, without compromising the safety of health care professionals.

Research perspectives

This research has the potential to pave way for further large-scale research while confirming to incorporate similar training regimes aimed at improving the preparedness and skill of various health professionals to tackle this crisis efficiently.

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FOOTNOTES

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SYSTEMATIC REVIEWS

Single-use duodenoscopes for the prevention of endoscopic retrograde cholangiopancreatography -related cross-infection – from bench studies to clinical evidence

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Abstract

BACKGROUND

Several strategies have been implemented to reduce or abolish the life-threatening risk of endoscopic retrograde cholangiopancreatography (ERCP)-related multidrug-resistant infections due to duodenoscopes contaminations; among those strategies, serial microbiologic tests, thorough reprocessing schedules, and use of removable scope cap have been adopted, but the potential cross-infection risk was not eliminated.

AIM

To review available evidence in the field of single-use duodenoscopes (SUD) use for ERCP.

METHODS

An overview on ongoing clinical studies was also performed to delineate which data will become available in the next future.

RESULTS

One bench comparative study and four clinical trials performed with EXALT model-D (Boston Scientific Corp., United States) have been identified. Of them, one is a randomized controlled trial, while the other three studies are prospective single-arm, cross-over studies. Pooled technical success rate (4 studies, 368 patients) was 92.9% [95% confidence interval (CI): 89.9-95.5; I^2: 11.8%]. Pooled serious adverse event (4 studies, 381 patients) rate was 5.9% [3.7%-8.5%; I^2: 0.0%].

CONCLUSION
Although few clinical trials are available, evidence is concordant in identifying an absolute feasibility and safety and feasibility for SUD use for ERCP. The expertise and quality of evidence in this field are going to be improved by further large clinical trials; data on cost-effectiveness and environmental impact will be needed for a worldwide spread of SUD use for ERCP.

Key Words: Multidrug; Resistance; Contamination; Infection; Reprocessing; Guidelines

Core Tip: Endoscopic retrograde cholangiopancreatography (ERCP) has significantly changed the management and natural history of patients with biliary and pancreatic diseases. While in the past decades ERCP procedure were considered safe and bearing low-risk for exogenous pathogens transmission, the risk of duodenoscopes contaminations and related cross-infection was recently demonstrated and quantified. To overcome this issue, two different single-use duodenoscopes (SUD) have been developed and are commercially available. The sterile packaging and the disposable intent guarantee to avoid exogenous patient-to-patient cross-infections. A systematic review of all available clinical evidence on the use of SUD for ERCP was performed, demonstrating an overall pooled safety and efficacy. Although few clinical trials are available, evidence is concordant in identifying an absolute feasibility and safety and feasibility for single-use duodenoscopes (SUD) use for ERCP. Future large clinical trials are ongoing to increase the knowledge and quality of evidence in the field; data on cost-effectiveness and environmental impact will be needed for a worldwide spread of SUD use for ERCP.

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) has significantly changed the management and natural history of patients with biliary and pancreatic diseases[1-3].

Millions of ERCP procedures have been performed annually and this worldwide amount is going to constantly increase due to epidemiological trends of main indications (*i.e.*, biliary stone disease, malignant biliary obstruction), aging of population, and increasing therapeutic applications[4,5].

In recent years, several outbreaks of multi-drug resistant ERCP-related infections have been reported; main risk factors for ERCP-related infections are patients' immunocompromised status and interventional procedures, such as biliary stenting for intrahepatic strictures[6,7]. Contamination of the biliary tract from endogenous gut microbiota bacteria is responsible for the vast majority of post-ERCP infections. However, several issues related to multidrug resistant infections related to duodenoscope contaminations have been reported (*i.e. P. aeruginosa* and carbapenem-resistant enterobacteriaceae)[8,9].

While in the past decades ERCP procedure were considered safe and bearing low-risk for exogenous pathogens transmission, the risk of duodenoscopes contaminations and related cross-infection was recently demonstrated and quantified[10-12].

Food and Drug Administration alerted physicians' community about duodenoscope-related infections in 2015. The peculiar design of these side-viewing instruments was identified as the potential sources of contamination. Indeed, in the tip of the scope is allocated the elevator mechanism with his dedicated cable passing through the scope body; this complex mechanism, despite adequate procedures, is difficult to accurately clean making reprocessing more challenging due to the possible formation of bacteria-containing biofilm[13].

Post-market studies conducted by main manufacturers demonstrated an unexpected higher rate of duodenoscope contamination. A recent meta-analysis tried to overcome the lack of data and quantify the risk of cross-infection in ready-to-use duodenoscopes. A pooled contamination rates up to 15% was identified and none of the available standard reprocessing protocols are able to correctly clean these instruments[14,15].

Several strategies have been proposed to overcome duodenoscope-related infections, such as deep bacterial coltures, improved protocol for reprocessing, and avoiding the use of scopes with fixed cap to allow decontamination. Unfortunately, duodenoscope contaminations could not be avoided with these

strategies[16-19].

Four reusable duodenoscopes with detachable cap are available, from three manufacturers. For a detailed focus on this field, a recent American Society for Gastrointestinal Endoscopy (ASGE) practice guideline was published[4,20].

Two different single-use duodenoscopes (SUD) are commercially available in the US. The sterile single-use package allow the avoidance of exogenous contaminations[4].

The aim of this study was to perform a systematic review of all available clinical evidence on the use of SUD for ERCP.

MATERIALS AND METHODS

Study selection

A systematic literature research was performed through MEDLINE using Pubmed, Google Scholar, and Embase interfaces at the end of November 2021. The search queries were ("duodenoscope"[all fields] OR "single-use"[all fields] OR "disposable"[all fields]) AND "ERCP"[all fields]). Institutional Review Board evaluation for this purpose was not required. Relevant studies were independently analyzed by two authors (AL, RMZ).

Inclusion criteria were: (1) Population: All adult individuals who underwent ERCP; (2) Interventions: SUD use for ERCP; (3) Objectives: Technical success (amount of successfully-completed procedures with SUD among all procedures); and (4) safety: Incidence of ERCP-related complications.

Statistical analysis

Technical success rate and other aims were pooled through a random-effects model based on DerSimonian and Laird test. Heterogeneity was estimated using I^2 tests: I^2 less than 30% was considered low, while $I^2 > 30\%$ but $< 60\%$ was considered weak. Funnel plots inspection was used to assess possible publication bias.

Main objective was the technical success, (completed ERCP using SUD among the entire amount conducted). Secondary objectives were adverse events (AEs).

Statistical analysis was performed with MedCalc package v20 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021).

RESULTS

Development of single use duodenoscopes

In 2017, several animal studies on porcine and canine models have been conducted with ERCP experts, to evaluate duodenoscope prototypes. Simulated ERCP procedures have been tested with the SUD prototype (Boston Scientific, United States) and a reusable duodenoscope. Involved physicians were asked to rate specific endoscopes tasks qualitatively and quantitatively. These pre-clinical tests allowed the development of the EXALT model-D by Boston Scientific[21].

Bench model comparison

In 2019, ERCP experts from United States completed the first comparative study on two simulators. Three reusable duodenoscopes from the major companies in the field (Olympus Corp., Japan, Pentax Corp., Japan and Fujifilm Holdings Corp., Japan) were compared to EXALT-D using a 0-10 score in four different tasks (and 14 sub-tasks): Guidewire locking with elevator, plastic and metal stents placement and removal, and Dormia Basket passage. The technical success rate for each task and time to achieve the completion was recorded and compared.

The results of this bench study showed that EXALT-D SUD showed similar overall performance, task completion times, tip control and guidewire locking to three different reusable duodenoscopes. Moreover, mechanical scope navigation and image quality was considered excellent (≥ 8 on a scale of 10)[21].

Clinical studies

Four studies have been conducted since the introduction of SUD (study flow diagram according to PRISMA 2009 guidelines is shown in Figure 1); studies are summarized in Table 1.

Muthusamy *et al*[22] conducted in April-May 2019 a multicenter study involving ten US centers and seven ERCP experts; 73 consecutive patients undergoing ERCP have been enrolled. Thirteen patients entered a running "first-in-men study" evaluating the feasibility of ERCP maneuvers. All these "roll-in" procedures (100%) have been successfully completed and the operators stated that they feel confident to extremely-confident in performing ERCP with the SUD.

Table 1 Characteristics of studies assessing the performance of single-use duodenoscope for endoscopic retrograde cholangiopancreatography

Ref.	Region, Study design	Population (no.); male gender (%)	Age (yr, SD)	Naïve papilla (%)	ASGE complexity 3-4 (%)	Technical success (%)	Serious AEs (%)	Note
Muthusamy *et al*[22], 2020	United States, Case-series	No. 60, Male 61.7%	64.4 ± 14.1	26.70%	45.00%	96.70%	6.70%	The study included a roll-in phase with 13 patients
Bang JY *et al* [23], 2020	United States, RCT	No. 48, Male 54.2%	67.2 ± 14.4	100%	16.70%	SUD: 95.8%; Reusable: 100%	4.20%	Primary outcome was no. attempts to achieve cannulation (SUD median 2; reusable 5; P = 0.013)
Napoléon *et al* [24], 2022	France, Prospective	No. 60, Male 43.3%	65.5 ± 13.6	53.30%	40.00%	95%	1.70%	96.7% of cases with optimal operators' satisfaction
Slivka *et al*[25], 2021	United States, Prospective	No. 200, Male 48.5%	62.6 ± 14.0	45.50%	40.50%	90.50%	6.50%	Included 14 expert and 5 "non-expert" ERCP operators with similar outcomes

ASGE: American society for gastrointestinal endoscopy; AEs: Adverse events; SD: Standard deviation; SUD: Single-use duodenoscope; RCT: Randomized controlled trial. ASGE complexity score refers to Cotton PB, Eisen G, Romagnuolo J, Vargo J, Baron T, Tarnasky P, Schutz S, Jacobson B, Bott C, Petersen B. Grading the complexity of endoscopic procedures: results of an ASGE working party. *Gastrointest Endosc* 2011; 73: 868-874 [PMID: 21377673 DOI: 10.1016/j.gie.2010.12.036].

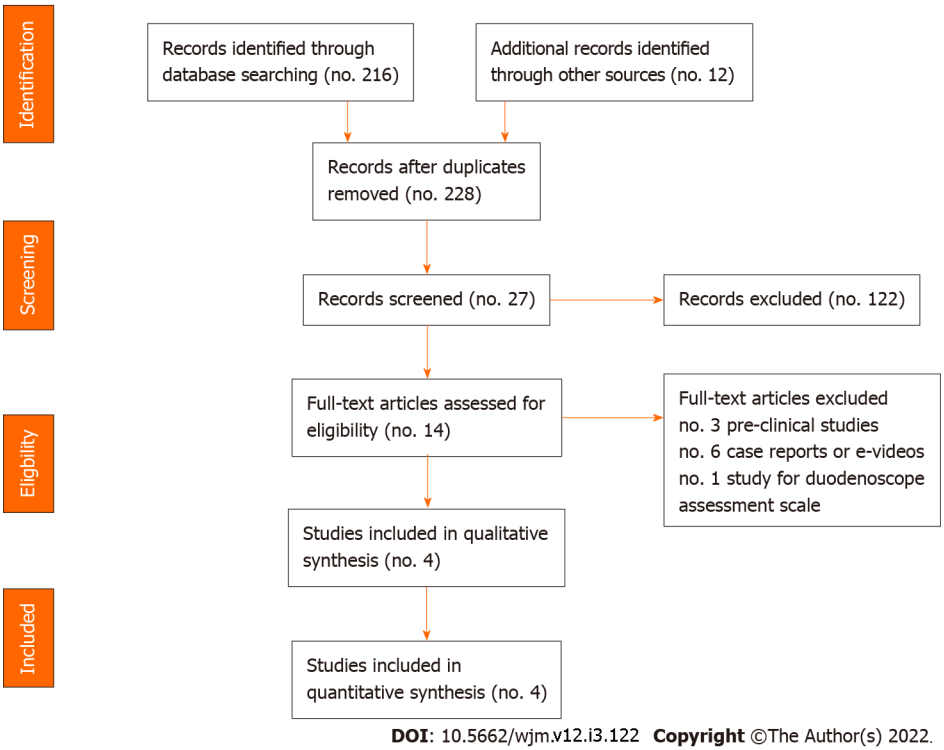

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Figure 1 Study flow diagram according to PRISMA 2009 guidelines.

Sixty consecutive patients have been subsequently enrolled in the study. Most patients (61.7%) were male with a mean age of 64.4 ± 14.1 years. Most cases (73.3%) had a medical history of previous ERCP. In two cases (3.3%), cross-over to a reusable duodenoscope was required due to ERCP technical failure. In one case (tight intrahepatic stricture dilation in a patient with sclerosing cholangitis) the use of a reusable duodenoscope allowed a successful ERCP completion; in one case, papilla showed neoplastic infiltration.

Between January and March 2020, Bang *et al*[23] randomized 98 patients to underwent ERCP with a reusable duodenoscope (TJF-180, Olympus America Inc., United States) or a SUD (EXALT-D, Boston Scientific, United States). Forty-eight patients (54.2% male, 67.2 ± 14.1-year-old) were allocated to the SUD arm and compared to 50 patients (46.0% male, 60.8 ± 18.2-year-old) of the reusable duodenoscope arm; no patient had previous ERCP or bilio-pancreatic intervention. The Authors observed comparable selective cannulation rate (95.8% *vs* 100%), with similar time to reach the papilla (20 *vs* 20 sec); the authors observed that the number of attempt (2 *vs* 5) and time to achieve selective biliary cannulation (35 *vs* 99 sec) were significantly lower in the SUD group.

Napoléon *et al*[24] have recently published the first study conducted outside the US. In this French multicenter study involving six centres, 60 patients (43.3% male, median 65.5 [55-76] year-old) were prospectively enrolled. 95% of the procedures were successfully completed with the SUD, while in three cases the Authors switched to a reusable duodenoscope. In these 3 cases, ERCP could not be completed even with the use of a reusable duodenoscope because of a complete duodenal stricture, a neoplastic infiltration of the ampullary region and a complete biliary stricture; these patients were treated with surgery, EUS- hepaticogastrostomy and percutaneous trans-hepatic drainage, respectively. In this study, 46.7% of patients had previous ERCP. Among the remaining cases, selective biliary cannulation was achieved in 93.8% of cases, after a median of 1 minute and 1.5 guidewire attempts[24].

The results of a large prospective study, conducted in United States, have been published by Slivka *et al*[25]. The Authors enrolled 200 patients undergoing ERCP for various indications; in fact, 40.5% of ERCP procedures presented high complexity (ASGE 3-4). The Authors reported an overall 90.5% technical success rate. Interestingly, this is the first study that included not only expert operators (defined as > 2000 lifetime ERCP performed), but also five "non-expert" operators. The Authors observed that the crossover to a reusable duodenoscope rate (2.5% *vs* 11.3%), the ERCP completion rate (97.5% *vs* 96.3%) and procedure time (28.5 *vs* 25.0 min) were similar among expert and non-expert groups[25].

Safety profile

Muthusamy reported a case of post-ERCP pancreatitis in 1 out of 13 patients involved in the roll-in study. Moreover, they reported 2 post-ERCP pancreatitis (3.3%), one post-sphincterotomy bleeding (1.7%) and one infection of a walled-off pancreatic necrosis in the 60 patients included in the main study. The overall serious adverse event rate was 6.7%[22].

No difference was observed in term of adverse event (AE) and mortality, when ERCPs performed with the SUD were compared to those performed with a reusable duodenoscope. The authors observed two adverse events in the SUD arm (4.2%) compared to 8% adverse event rate in the control group. The Authors reported an ERCP-related mortality of 2.1% and 2%in the two groups, respectively[23].

Napoléon *et al*[24] reported 3 ERCP-related adverse events (5.0%). Of them, two cases were mild (biliary pain and one mild pancreatitis), while one patient (1.7%) presented worsening of underlying condition due to pancreatic cancer and died one week after the procedure[24].

Slivka *et al*[25] reported 13 serious adverse events (6.5%); of them, 5 bleeding, 3 post-ERCP pancreatitis and 2 cholangitis. The incidence of adverse events was similar in expert and non-expert groups (5.0% *vs* 6.9%) and was independent by ASGE complexity grade (low – ASGE 1-2: 7.0% *vs* high – ASGE 3-4: 6.2%).

All studies reported no SUD-related adverse event.

No data on specific SUD contamination after ERCP has been provided in the included studies.

Operators' satisfaction.

Muthusamy reported a median overall satisfaction with the SUD of 9 (range, 1-10). In 4 cases (6.7%) the Authors observed a poor satisfaction (4 or less), due to difficulty of stent insertion, low image quality, and technical issue with the device (turning off during the procedure)[22].

Bang *et al*[23] observed that the SUD present lower image quality and stability comparing to a reusable duodenoscope. From a mechanical point of view, the Authors reported a lower ease to pass into the stomach and frequent dysfunction of air-water valve.

Napoléon *et al*[24] reported a median overall satisfaction of 9 on a scale of 10. In two cases (3.3%), the operator reported a low satisfaction (less than 5) due to malfunction of the insufflation valve leading to irrigation water in the lumen, limiting the visibility. Among 22 different tasks, the authors considered the SUD clinically-satisfactory in 100% and comparable to a reusable duodenoscope in 97.9%of cases.

The recently published study by Slivka *et al*[25] confirmed an optimal overall satisfaction with the SUD [median 8 (range VAS 1-10)]. Among 23 evaluated maneuvers, all obtained a median of at least 4 (range 1 to 5).

Pooled safety ed efficacy

Our study group recently conducted a meta-analysis including all clinical studies assessing the safety and efficacy of SUD use for ERCP, identifying 4 studies (368 patients) [26]. We observed a 92.9% [89.9 – 95.5; I^2 11.8%] overall success rate and 5.9% [3.7 – 8.5; I^2 0%] overall incidence of serious AEs. Overall incidence of pancreatitis (2.5%), infections (1.8%) and bleeding (1.8%) was very low, in line with

suggested threshold and confirming the optimal safety[26].

Study pipeline

Five clinical studies on the use of SUD for ERCP are ongoing (no. 2) or ready to start recruitment (no. 3); these studies and contact information are summarized in Table 2.

Two of them are planned to be conducted in US, while the remaining two studies in Europe (Italy and UK) and one in China.

Following the feasibility and safety studies performed in high-volume centers by extremely experienced operators, one study (NCT04103749) will include large real-life experience with operators with various expertise.

Interestingly, an Italian study is going to assess the performance of SUD in combination with single-use digital cholangioscope in a tertiary referral center.

Finally, a large multicenter study is testing the performance of another SUD, namely the aScope™ Duodeno, manufactured by Ambu A/S (Denmark) on 550 patients undergoing ERCP. In less than 12 mo, the knowledge and quality of evidence in the field of SUD use for ERCP is going to be strongly expanded. The introduction of a validated tool for duodenoscope assessment will allow physician to utilize a reproducible and reliable tool for the assessment of technical performance of duodenoscopes [27].

Cost-effectiveness

A recently published study, based on a "Montecarlo model" assessed the cost-effectiveness of different approaches adopted for the reduction of duodenoscope-related cross-infections[28]. The cost for each ERCP procedure, based on United States data, performed with SUD has been estimated in $2991. The analysis, based on an estimated < 1% risk of duodenoscope-related cross-infections did not identified routinely SUD use as a cost-effective strategy. The Authors acknowledged that these results should be contextualized based on duodenoscope-related cross-infection rate, local ERCP volume, quality adjusted life years, post-ERCP lifespan and environmental costs[28,29].

Limitations

The lack of a reliable quantification of the impact of duodenoscope contamination-related infections does not allow to correctly evaluate the benefit of the systematic use of a SUD.

Indeed, all the published studies have been designed to compare SUD to standard reusable duodenoscopes with a non-inferiority purpose, in terms of technical and clinical success rate. Since the estimated rate of duodenoscope-related cross-infection was < 8% published studies are underpowered to detect any clinical difference.

Environmental sustainability

Another point of critical discussion will be the ecological impact of production and wasting of a single-use endoscope.

A recent international named "Green Endoscopy" (Twitter account @GreenEndoscopy) wrote an inspiring editorial on this issue. The Authors estimated a mean 1.5 kg of waste for each single endoscopic procedure, with very-low amount of recyclable materials.

The disposal SUD is equivalent up to 400 g of household waste and this weight should be added to this waste. The Authors considered "unthinkable" that each ERCP could be performed with SUD based both on cost and environmental burdens.

A comparative study on two different approaches adopted with bronchoscopes [http://ambu.co.uk/pulmonology/environmental-impact] has reported that single-use endoscopy does not much differ since the cost of disposing plastic endoscopes should be balanced with sterilization process, disinfecting equipment and consumable costs.

On the other hand, SUDs are made from recycled plastic and are claimed to be recyclable through third party companies, even if material from these duodenoscopes will not be used for production of medical devices[29].

DISCUSSION

In conclusion, the recent identification of several cluster of exogenous multidrug-resistant bacterial infection caused by duodenoscope cross-contamination necessitated the implementation of various strategies for at least prevention or abolition of that life-threatening risk. Among those strategies, the introduction of sterile, disposable duodenoscopes is able to completely abolish the contamination and cross-transmission of bacteria.

Although there are only few clinical trials available, evidence is concordant in identifying an absolute safety and feasibility. Indeed, no SUD-related adverse event is still reported, and overall risk of adverse events and mortality is comparable to ERCP performed with reusable duodenoscopes. Moreover, the pooled technical success rate in expert hands stands at optimal values, with no significant heterogeneity

Table 2 Summary of ongoing registered studies assessing the performance of single-use duodenoscope for American society for gastrointestinal endoscopy, registered on clinicaltrial.gov portal

Title, reference	Region	Investigators	Design, population, Duodenoscope	Primary outcome	Status
Single Use ERCP -SURE Study (SURE). NCT04671095	Nottingham, United Kingdom	Dr. Suresh Vasan Venkatachalapathy; suresh.venkatachalapathy@nuh.nhs.uk	Prospective, 50 patients, EXALT-D[1]	Technical success (ERCP completion)	Not yet recruiting
International Study to Evaluate Outcomes and Safety of Patients Undergoing ERCP Using a Single-use Cholangioscope and Single-use Duodenoscope (MESE). NCT04712253	Rozzano (MI), Italy	Prof. Alessandro Repici alessandro.repici@hunimed.eu; Dr. Andrea Anderloni andrea.anderloni@humanitas.it	Retrospective, 50 patients, EXALT-D[1]	Technical success, clinical outcomes	Recruiting
Global Prospective Case Series Using a Single-Use Duodenoscope. NCT04103749	United States	Gregory Tirrell; gregory.tirrell@bsci.com; Pooja Goswamy; pooja.goswamy@bsci.com	Prospective, 1000 patients, EXALT-D[1]	Technical success (ERCP completion)	Not yet recruiting
Exalt D Single-use Duodenoscope in ERCP Procedures in China (ExaltDScope). NCT04687774	China	Zhiwei Guzhiwei.gu@bsci.com; Jingjing Gu	Observational, 30 patients, EXALT-D[1]	Technical success (ERCP completion)	Not yet recruiting
A Single-Use Duodenoscope in a Real-World Setting. NCT04628949	United States	Elizabeth Smith; elsm@ambu.comTrine; Højgaard Tølbøll; trht@ambu.com	Prospective, 550 patients, aScope	Technical success (ERCP completion)	Recruiting

[1]EXALT-D is manufactured by Boston Scientific (MA, United States). The aScope™ Duodeno is manufactured by Ambu A/S (Denmark). ASGE: American Society for Gastrointestinal Endoscopy.

among studies.

Future studies will deepen the knowledge in this field; data on cost-effectiveness and environmental impact will be needed for a worldwide spread of SUD use for ERCP.

CONCLUSION

In conclusion, the recent identification of several cluster of exogenous multidrug-resistant bacterial infection caused by duodenoscope cross-contamination necessitated the implementation of various strategies for at least prevention or abolition of that life-threatening risk. Among those strategies, the introduction of sterile, disposable duodenoscopes is able to completely abolish the contamination and cross-transmission of bacteria.

Although there are only few clinical trials available, evidence is concordant in identifying an absolute safety and feasibility. Indeed, no SUD-related adverse event is still reported, and overall risk of adverse events and mortality is comparable to ERCP performed with reusable duodenoscopes. Moreover, the pooled technical success rate in expert hands stands at optimal values, with no significant heterogeneity among studies.

However, further studies are needed to provide high-quality data, in terms of cost-effectiveness and environmental impact, potentially allowing a worldwide spread of SUD use for ERCP.

ARTICLE HIGHLIGHTS

Research background

Single-use duodenoscope use has been proposed as an effective strategy to avoid the risk of duodenoscope-related cross-infections in patients undergoing endoscopic retrograde cholangiopancreatography (ERCP).

Research motivation

Recentaly, several manuscript have been published reporting the ouctomes of clinical studies on single-use duodenoscope use for ERCP.

Research objectives

To perform a systematic review of the literature and report qualitative and quantitative results in terms

of technical success rate, clinical success, and safety.

Research methods
Systematic review and quantitative analysis.

Research results
Five original articles have been identified. One bench comparative study and four clinical trials performed with EXALT model-D (Boston Scientific Corp., United States) have been identified. Of them, one is a randomized controlled trial, while the other three studies are prospective single-arm, cross-over studies. Pooled technical success rate (4 studies, 368 patients) was 92.9% [95% confidence interval (CI): 89.9-95.5; I^2: 11.8%]. Pooled serious adverse event (4 studies, 381 patients) rate was 5.9% [3.7%-8.5%; I^2: 0.0%].

Research conclusions
Although few clinical trials are available, evidence is concordant in identifying an absolute feasibility and safety and feasibility for single-use duodenoscopes (SUD) use for ERCP. Data on cost-effectiveness and environmental impact will be needed for a worldwide spread of SUD use for ERCP.

Research perspectives
Future perspective and study pipelines should assess the use of other models of single-use duodenoscope, cost-effectiveness of single-use duodenoscope use for ERCP and environmental sustainability.

FOOTNOTES

Author contributions: Lisotti A and Fusaroli P wrote the paper; Napoléon B, Cominardi A and Zagari RM performed data collection for systematic review; all authors reviewed manuscript for pivotal intellectual content.

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SYSTEMATIC REVIEWS

Nature and mechanism of immune boosting by Ayurvedic medicine: A systematic review of randomized controlled trials

B N Vallish, Dimple Dang, Amit Dang

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Abstract

BACKGROUND

Many Ayurvedic preparations are claimed to have immune-boosting properties, as suggested in various published randomized clinical trials (RCTs)

AIM

To compile evidence on the nature and mechanism of immune system enhancement by Ayurvedic preparations in healthy and sick individuals.

METHODS

After prospectively registering study protocol with PROSPERO, we searched PubMed, DOAJ, Google Scholar, three dedicated Ayurveda research portals, two specialty Ayurveda journals, and reference lists for relevant records published until February 6, 2021 using appropriate search strategies. Baseline features and data pertaining to the nature and mechanism of immune system function were extracted from all eligible records. Methodological quality was assessed using the Cochrane RoB-2 tool.

RESULTS

Of 12554 articles screened, 19 studies reporting 20 RCTs (17 parallel group design, three crossover design) with 1661 unique patients were included; 11/19 studies had Indian first authors. Healthy population was included in nine studies, of which one study included pregnant women and two included pediatric population; remaining studies included patients with different health conditions, including one study with coronavirus disease 2019 patients. A total of 21 Ayurvedic interventions were studied, out of which five were composite

mixtures. The predominant route of administration was oral; dose and frequency of administration of the intervention varied across the studies. The results reported with five RCTs exploring five Ayurvedic interventions were incomplete, ambiguous, or confusing. Of the remaining 16 interventions, indirect evidence of immune enhancement was reported with four interventions, while lack of the same was reported with two interventions. Enhancement of T helper cells and natural killer cells was reported with three and four interventions, respectively, while the pooled results did not clearly point toward enhancement of other components of the immune system, including cytotoxic T cells, B lymphocytes, immunoglobulins, cytokines, complement components, leucocyte counts, and other components. Nine of the 20 RCTs had a high risk of bias, and the remaining 11 RCTs had some concerns according to RoB-2.

CONCLUSION

Various Ayurvedic preparations appear to enhance the immune system, particularly *via* enhancements in natural killer cells and T helper cells.

Key Words: Immune enhancement; Ayurveda; Immune system; Healthy volunteers; Composite preparations; NK cells

Core Tip: Ayurvedic preparations have been anecdotally associated with immune boosting effect in both healthy and sick individuals. Through this systematic review, we explored the nature and mechanism behind this effect by scrutinizing 20 randomized controlled trials reported in 19 articles. While we could find indirect evidence for immune enhancement (by means of reduced illness duration and severity) with some Ayurvedic preparations, the evidence was insufficient to conclude about the exact mechanisms contributing to this phenomenon, although available evidence suggests that enhancements in natural killer cells and T helper cell number and function might contribute.

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INTRODUCTION

As of September 2021, the ongoing coronavirus disease 2019 (COVID-19) pandemic has seen at least two waves in almost all regions of the world, including India, with many predictions hinting at a global third wave due to new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants[1]. The remarkable trail of destruction left by the pandemic, coupled with the non-availability of a drug-of-choice to treat the condition[2], has resulted in an almost complete dependence on the development of safe and efficacious vaccines that can protect individuals against existing and potential virus variants. Despite developments in vaccinology, it is common knowledge that a complete protection against all future variants of the virus cannot be absolutely guaranteed by even the most advanced vaccines. At the same time, non-neutralizing antibodies that cause antibody-dependent enhancement of the immune system are reported to exacerbate paradoxically SARS-CoV-2 infection, leading to worsened organ damage[3]. This has led to the realization that therapies that produce a sufficiently strong, but not hyperactivated, immune system can lead to a healthier society and can contribute significantly to the fight of mankind against not only the COVID-19 pandemic but also future healthcare challenges of infectious disease origin. Consequently, healthcare systems in many parts of the world, especially India, have turned their attention toward potential therapies that can produce a non-specific and unaided enhancement of the immune system, as a complementary step to vaccine development[4,5].

Ayurveda is the Indian traditional system of medicine and has a history since the 2nd century BC. The core pathophysiological principles of Ayurveda surround the concept of loss of balance with respect to the three humors (*Tridoshas*), five elements (*Pancha mahabhootas*), and seven tissues (*Saptadhatus*) of the human body. Various Ayurvedic treatments are described for diseases affecting different systems of the body, and most Ayurvedic medicinal items are sourced from natural ingredients. Classical texts of Ayurveda also have mentions about management of epidemics[6,7] and has defined 'immunity (*Bala*)' as the 'ability of the body to prevent and arrest the progression of disease for maintaining homeostasis' [4]. Few facts, such as the classification of immune system in Ayurveda into *Sahaja* (innate), *Kalaja*

(chronobiologic), and *Yuktikrut* (acquired) closely resembling the modern medical classification of immune system[4] and Ayurvedic immune-boosting preparations such as *cyavanaprasa* having stood the test of time, are testaments to the near-accurate understanding of our body's immune system by the ancient Ayurveda pioneers. Thus, it is not surprising that the Ministry of AYUSH (Ayurveda, Yoga, Unani, Siddha, and Homoeopathy) of the Indian Government released an advisory recommending various natural therapies to develop immunity against COVID-19[8]. Many components named in the said recommendation have been reported to produce immune enhancement through modulation of multiple immune system pathways[8] and also through psychoneuroimmunological mechanisms[5]. However, since most of these observations are through pre-clinical and non-human studies, we were interested to know if these pre-clinical observations hold good when the Ayurvedic preparations are studied after human administration.

With this background, we performed the present systematic literature review (SLR) with an objective for gathering evidence towards the nature and mechanism of enhancement of human immune system by the administration of Ayurvedic preparations, from published randomized clinical trials (RCTs). The research question that we were looking forward to find an answer through this SLR was: 'what is known from published RCTs about the nature and mechanism of the impact of consuming ayurvedic medicine on enhancing the immune system activity of healthy or sick humans?'

MATERIALS AND METHODS

Study protocol

The SLR protocol was drafted following the guidelines given in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement and was subsequently refined through internal discussion. The final version of the protocol was prospectively registered with PROSPERO on July 10, 2020, with ID CRD42020191289.

Eligibility criteria

To identify potentially relevant articles that answered our research question, the following eligibility criteria were drafted: 'Population' was all studies involving healthy or sick adults with any form of illness (acute or chronic). The protocol was subsequently amended to include humans of all age groups, including pediatric and geriatric individuals. We did not restrict the population with respect to the age or gender of the individuals, presence of any form of disease, presence of comorbidities, or presence of special situations such as pregnancy. 'Intervention' included any type of Ayurvedic medicine, given as a combination or single drug. Studies that did not explicitly mention whether the intervention was an 'Ayurvedic medicine' or not were included in the review if the intervention was found in the work 'Indian Medicinal Plants: an Illustrated Dictionary' by Khare[9]. We also used the same resource to compile the Ayurvedic name of various interventions. Papers that included interventions only belonging to other systems of medicine (including modern/Western medicine, traditional medicines of other regions/countries, and other systems of medicine including Unani, Siddha, Homoeopathy, Yoga, Naturopathy, Osteopathy, and Chiropractic) were excluded. We did not restrict studies based on the 'Comparator' and included all studies regardless of whether or not there was a comparator. 'Outcomes' of interest included any description of enhancement, augmentation, stimulation, increasing, or strengthening of the immune system, either directly (through observation on the effect of component/s of the immune system) or indirectly (through observation of effect of said enhancement of immune system, by means of surrogate markers such as relief from illness, quickness of recovery, *etc.*). Studies that described immunomodulation instead of immune enhancement and studies that did not describe any immune system effect were excluded. 'Study design' included only RCTs. Papers not describing primary data (such as narrative or systematic reviews, letters to editors, opinion pieces, commentaries, editorials, brief communications, news items, *etc.*), case studies, case reports, and studies describing non-human experiments (including *in vitro* studies, *in vivo* studies, *in silico* experimentation, *etc*), and studies not in English language were excluded from our review.

Literature search, data extraction, and quality assessment

Following the eligibility criteria described above, a PubMed literature search strategy was drafted and modified through internal discussion. Using the refined search strategy, a systematic literature search was performed in PubMed/MEDLINE, from their inception till June 2, 2021. Recognizing that many research articles on Ayurveda are published in journals not indexed in PubMed, and following the recommendations by Aggithaya *et al*[10] (2015), we performed an additional literature search in Directory of Open Access Journals[11], AYUSH research portal[12], Digital Helpline for Ayurveda Research Articles (DHARA)[13], Annotated Bibliography of Indian Medicine (ABIM)[14], and Google Scholar. Furthermore, we also performed targeted journal search in two Ayurveda specialty journals (Ayu and Journal of Ayurveda and Integrative Medicine) using a combination of search terms and Boolean operators such as "Ayurveda", "Ayurvedic medicine", "Immune", "Immune stimulating", "Immune enhancing", "Immunomodulatory", and "Randomized clinical trials". The detailed PubMed

search strategy is presented as Supplementary Table 1, and a brief account of searches performed in each of the portals mentioned above is provided in Supplementary Table 2. Finally, we also scanned reference lists of relevant studies to identify potentially eligible records that were missed through database search.

After pooling all the eligible records, we identified the most eligible articles for our review and extracted relevant data from these papers after thoroughly studying the full texts of each article. Extracted data, such as data related to the study details (year of publication, country of the first author, study design, and details of the intervention and comparator), participant details (participant profile, number, age, and sex), details of the intervention and comparator (Ayurvedic, commercial, and scientific names, composition, dose, frequency, duration, and route of administration), and outcome details in terms of improvement from baseline, were entered into a predefined data entry grid. All outcomes related to the immune system (or *Ojas*) were considered for this study and included direct variables [such as leukocyte subtypes including T lymphocytes, B lymphocytes, natural killer (NK) cells, and various types of myelocytes, immunoglobulins, complement components, and cytokines] and indirect variables (such as absenteeism, number of healthy days and sick days, number of doctor visits, number of symptoms, *Ojas* score, *etc*.). The methodological quality of the included studies was assessed using the Cochrane RoB-2 tool[15]. We used the official Microsoft Excel tool provided by the Cochrane Foundation for implementing RoB-2, and we used separate tools for parallel group RCTs and crossover RCTs[16].

Subsequent to executing the literature search, two authors (VBN and AD) independently screened the pooled articles for their inclusion in the study, extracted data, and assessed the articles for risk of bias; any disagreements were resolved through discussion and reconciliation that was moderated by another author (DD).

Statistical analysis and data availability

Study selection and data extraction were done electronically in Microsoft Excel. To assess the inter-rater reliability (IRR) of the study inclusion and the methodological quality assessment of the included articles, Cohen's kappa value was calculated using SPSS version 20 (Armonk, NY, United States). The cut-off points for the kappa statistic were interpreted as ≤ 0.20 = slight agreement; 0.21-0.40 = fair agreement; 0.41-0.60 = moderate agreement; 0.61-0.80 = substantial agreement; 0.81-0.99 = near-perfect agreement; and 1.00 = perfect agreement[17]. Statistical review of the study was performed by an author who is a biomedical statistician (VBN). All datasets used to derive conclusions in this study are available with the corresponding author on reasonable request.

RESULTS

Study selection, baseline characteristics

From an initial pool of 12554 potentially eligible records, 19 studies were included for data extraction and review. Figure 1 depicts the study selection process.

The 19 included studies were published from 2005 to 2021. A total of 20 RCTs were reported in the 19 included papers: 16 articles described parallel group RCTs, two described crossover trials, and one article reported two RCTs: One parallel group RCT, and one crossover RCT. The first authors of the 19 papers were from six different countries, with India being the most frequent country of affiliation (11 studies), followed by Japan and United States (two studies each); there was one study each with first authors from Germany, Iran, Romania, and Turkey. The participant profile ranged from healthy participants (six studies, healthy adults; two studies, healthy pediatric population; one study, healthy pregnant women), patients with cancer (three studies, gastrointestinal cancer; one study, head and neck cancer), patients with diabetes and human immunodeficiency virus (HIV) (two studies each), and patients with COVID-19 and allergic rhinitis (one study each). Among the 17 studies that included adult participants, two included only females, one study included only males, and the remaining 14 studies included participants of either gender. The sample size of the studies ranged from 5 to 627, with a total of 1661 unique participants across the 19 articles. The complete baseline characteristics of the included studies are summarized in Table 1.

Two of the included studies explored two interventions, and the remaining 17 studies explored one intervention each, leading to a total of 21 interventions. The predominant route of administration of the intervention was oral (n = 17), with one study each delivering the intervention through subcutaneous injection, intravenous infusion, and oil dripping on the forehead; details of route of administration of the intervention were missing in one study. Among the 17 orally administered interventions, the formulations were capsules in five studies, and *Rasayana* in three studies; one study each used other formulations including tablet, tea, *Cyavanaprasa*, *Kasaya*, *Bhasma*, *Ghrita*, *Churna*/*Kalka*, and tincture, and one study did not provide information about formulation. Details of dosing and duration of administration of the intervention varied across the studies. Placebo was used as the comparator in six studies. The complete details of the interventions and comparators among the included studies are summarized in Table 2. The detailed composition of the different composite preparations used as interventions in

Table 1 Baseline study characteristics, and summary of risk of bias assessment

No	Ref.	Country of 1st author	Type of randomized controlled trial	Patient profile	Intervention[1]			Comparator[1]			Overall risk of bias as per RoB-2 tool[2]
					Sample size	Mean age	Male (N, %)	Sample size	Mean age	Male (N, %)	
1	Enesel *et al* [18], 2005	Romania	Parallel group	Patients undergoing surgery for GIT cancer	40	62 (range 37-87)	23 (57.5)	30	NA	NA	-
2	Ishikawa *et al* [21], 2006	Japan	Parallel group	Patients with inoperable colon/liver/pancreatic cancer	25	63.6 ± 8.3	21 (84)	25	65.8 ± 6.3	18 (72%)	?
3	Brush *et al* [22], 2006	United States	Parallel group	Healthy adult volunteers	3	NA	NA	2	NA	NA	-
4	Schink *et al* [27], 2007	Germany	Parallel group	Primary/locally relapsed colorectal carcinoma patients undergoing open (complete/partial) tumour resection	11	72 ± 8.2	7 (63.6)	11	69 ± 10.4	5 (45.5)	?
5	Purandare *et al*[33], 2007	India	Parallel group	Patients with diabetic foot ulcer	23	56.26	17 (73.9)	22	56.32	19 (86.4)	?
6	Uebaba *et al* [29], 2008	Japan	Crossover	Healthy adult female volunteers	16	39 ± 9	0 (0)	16	39 ± 9	0 (0)	?
7	Bhat *et al* [28], 2010	India	Parallel group (I)	Healthy adult volunteers	13	NA	NA	13	NA	NA	?
			Crossover (II)	Healthy adult volunteers	110	NA	NA	110	NA	NA	-
8	Işik *et al*[23], 2010	Turkey	Parallel group	Allergic rhinitis patients with house dust mite sensitivity	12	NA	NA	12	NA	NA	-
9	Kianbakht *et al*[30], 2011	Iran	Parallel group	Healthy adult male volunteers	45	22.5 ± 0.6	45 (100)	44	21.1 ± 0.5	44 (100)	?
10	Mondal *et al* [24], 2011	India	Crossover	Healthy adult volunteers	22	NA	NA	22	NA	NA	?
11	Nantz *et al* [26], 2012	United States	Parallel group	Healthy adult volunteers	56	25.4 ± 5.7	23 (41.1)	56	26.9 ± 7.1	26 (46.4)	?
12	Suprabha *et al*[32], 2017	India	Parallel group	Uncomplicated pregnant women in 20-24 wk of pregnancy	15	NA	NA	15	NA	NA	?
13	Gupta *et al* [34], 2017	India	Parallel group	Healthy children aged 5-12 yr	313	7.3 ± 1.8	161 (51.4)	314	7.4 ± 1.8	164 (52.2)	?
14	Rais *et al*[36], 2021	India	Parallel group	25-60 yr, asymptomatic/ uncomplicated COVID-19 RTPCR +ve, mild symptoms	80	NA	57 (71.3)	40	NA	30 (75)	-
15	Bhaskaran *et al*[31], 2019	India	Parallel group	Healthy full-term infants (< 12 mo age), > 2.5 kg birth weight, with normal growth and development	47	NA	NA	34	NA	NA	-
16	Kumar *et al* [35], 2014	India	Parallel group	Adult patients with T2DM of any stage	56	NA	NA	28	NA	NA	-
17	Ravindran *et al*[25], 2014	India	Parallel group	Patients with head & neck cancer in complete remission following primary treatment	37	NA	NA	38	NA	NA	?
18	Somarathna *et al*[20], 2010	India	Parallel group	HIV +ve patients without AIDS surveillance signs as per WHO, and no concurrent illness	21	NA	NA	6	NA	NA	-
19	Gupta *et al* [19], 2010	India	Parallel group	New HIV +ve patients with CD4 count not < 150/microliter and no complications or comorbidities	12	NA	NA	8	NA	NA	-

[1]Not all studies had reported demographics of participants including age and sex.
[2]Separate versions of RoB-2 were used for RCTs with parallel group design and crossover design. Interpretation: ?: Some concerns of bias; -: High risk of bias. Detailed risk of bias assessment results: Supplementary Table 4. HIV: Human immunodeficiency virus; NA: Not available; GIT: Gastrointestinal tract; COVID-19: Coronavirus disease 2019; RTPCR: Reverse transcription polymerase chain reaction.

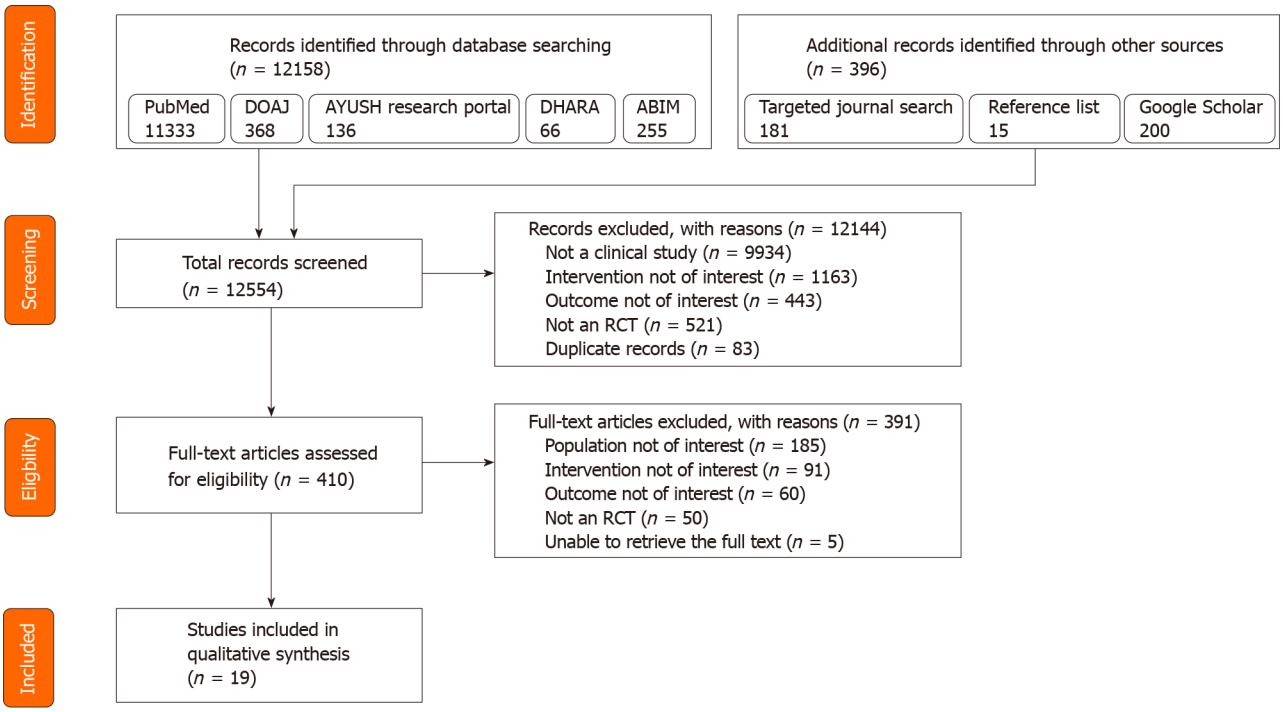

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Figure 1 Study selection process. DOAJ: Directory of Open Access Journals; AYUSH research portal: Ayurveda, Yoga, Unani, Siddha, and Homoeopathy research portal; DHARA: Digital Helpline for Ayurveda Research Articles; ABIM: Annotated Bibliography of Indian Medicine.

five of the studies is provided in Supplementary Table 3.

Of the 19 included studies, 15 studies provided information about the impact of the intervention on at least one component of the immune system (direct evidence), three studies provided information about the impact of the intervention on the overall health status of the participant (indirect evidence), and one study provided both direct and indirect evidence. The description of the results was not uniform across various studies, with different units of measurement used to express similar outcomes; this prevented us from performing a meta-analysis of the results.

Immune enhancement: Direct evidence

Impact on T lymphocyte subsets (excluding NK cells): CD4 lymphocyte counts were found to be significantly enhanced after perioperative mistletoe administration for 14 d among patients undergoing surgery for gastrointestinal (GI) cancer[18]. Among patients with HIV, CD4 lymphocyte counts were found to be enhanced after a 90-d consumption of *Shilajatu Rasayana*[19] and *Ranahamsa Rasayana*[20], as compared to standard care. However, the increase in CD4 lymphocyte count caused by the consumption of aged garlic extract for 12 wk was not statistically significant among patients with GI cancer and was less than that observed with matching placebo[21]. The results reported in three papers were ambiguous: Although *Glycyrrhizia glabra* tincture increased CD4 lymphocytes by 8.26% in 24 h in healthy individuals, the statistical significance of this finding was not reported, and the sample size was only 5 patients[22]. Significant enhancement of CD4 lymphocyte count was also not observed with *Nigella sativa* seed supplementation, and the data were not presented with clarity[23]. *Tulsi* was reported to significantly increase CD4 lymphocytes in 4 wk compared to placebo among healthy individuals, but this article did not provide any numbers to support this claim[24].

CD8 lymphocyte counts were significantly decreased with perioperative mistletoe administration for 14 d[18]. Aged garlic extract brought about a non-significant increase in CD8 lymphocyte count over 12 wk, which was also seen with matching placebo[21]. The remaining three papers presented results with less clarity: although *Glycyrrhizia glabra* tincture increased CD8 lymphocyte by 2.89% in 24 h in healthy individuals, the statistical significance of this finding was not reported, and the sample size was only 5 patients[22]. Significant CD8 lymphocyte count enhancement was observed with *Nigella sativa* seed

Table 2 Details of intervention and comparators

No	Ref.	Description	Intervention: Ayurvedic name[1]	Intervention: Scientific name	Intervention: Route; dose, frequency, duration[2]	Comparator: Description; route, dose, frequency, duration[2]
1	Enesel *et al* [18], 2005	Isorel® (Mistletoe, firtree)	Bandaaka, Suvarna-bandaaka *etc.*	*Viscum album*	Subcutaneous; 2 wk pre-operatively, 2 wk post-operatively; dose details not clear	Standard care
2	Ishikawa *et al* [21], 2006	Aged garlic extract	Lashuna, Rasona *etc.*	*Allium sativum*	Oral; 125 mg, 4 capsules daily, 12 wk	Matching placebo
3	Brush *et al* [22], 2006	*Glycyrrhizia glabra* tincture	Yashtimadhu, Madhuyashtyaahvaa *etc.*	*Glycyrrhiza glabra*	Oral; 0.44 g/7.5 mL, twice daily, 7 d	Matching placebo tincture
4	Schink *et al* [27], 2007	Standard care + Iscador® (Standardized mistletoe extract)	Bandaaka, Suvarna-bandaaka *etc.*	*Viscum album*	IV infusion; 5 mg, single dose	Standard care
5	Purandare *et al*[33], 2007	Standard care + Tinospora cordifolia	Guduuchi, Guduuchikaa *etc.*	*Tinospora cordifolia*	Details not available	Standard care + matching Placebo
6	Uebaba *et al* [29], 2008	Shirodhara oil-dripping treatment using sesame oil	Shirodhara; Tila, Snehphala	*Sesamum indicum*	Oil dripping on forehead; single sitting	Control supine position; single sitting
7	Bhat *et al* [28], 2010	Fortified tea with *Withania somnifera, Glycyrrhzia glabra, Zingiber officinale, Ocimum sanctum* & *Elettaria cardamomum*	Ashwagandha; Yashtimadhu; Aardraka, Shunthi; Tulasi; Elaa	*Withania somnifera, Glycyrrhzia glabra, Zingiber officinale, Ocimum sanctum, Elettaria cardamomum*	Oral; 2.06 g, thrice daily, 2 mo	Regular tea; Oral; 2 g, thrice daily, 2 mo
8	Işik *et al*[23], 2010	Specific immunotherapy + *Nigella sativa*	Kaalaajaaji, Kalikaa *etc.*	*Nigella sativa*	Oral; 2 g daily, 1 mo	Specific immuno-therapy alone
9	Kianbakht *et al*[30], 2011	Saffron tablet	Kumkuma, Rudhira, Kaashmiraka *etc.*	*Crocus sativus*	Oral; 100 mg daily, 6 wk	Matching placebo
10	Mondal *et al* [24], 2011	Tulsi capsules	Tulasi, Surasa, Suravalli *etc.*	*Ocimum sanctum*	Oral; 300 mg daily, 4 wk	Matching placebo
11	Nantz *et al* [26], 2012	Aged garlic extract	Lashuna, Rasona *etc.*	*Allium sativum*	Oral; 640 mg, 4 capsules daily, 90 d	Matching placebo
12	Suprabha *et al*[32], 2017	Rasayana Avaleha with milk	Composite[4]	Composite[4]	Oral; 12 g, twice daily, 2 mo	Calcium carbonate (500 mg) with ferrous sulfate (200 mg); Oral; daily, 2 mo
13	Gupta *et al* [34], 2017	Cyavanaprasa (Dabur) with milk	Composite[4]	Composite[4]	Oral; 6 g, twice daily, 6 mo	Milk; Oral, 100-200 mL, twice daily, 6 mo
14	Rais *et al*[36], 2021	**Intervention 1:** Vyaghryadi Kashaya (50 mL) + Pippali (250 mg) + Samshamani vati (500 mg)	Vyaghryadi Kashaya: Kantakari, Shunthi, Guduchi; Pippali; Samshamani vati: Guduchi	*Solanum xanthocarpum, Zingiber officinale, Tinospora cordifolia, Piper longum*	Oral; twice daily, 10 d	Vitamin C (500 mg) twice daily, Paracetamol (500 mg) as needed
		Intervention 2: Shunthi churna (2 g) + Rasona kalka (1 g)	Shunthi; Rasona, Lasuna	*Zingiber officinale, Allium sativum*	Oral; Shunti churna: Twice daily, Rasona: once daily; 10 d	
15	Bhaskaran *et al*[31], 2019	Swarna Bhasma (Calcined powder of gold), honey, ghrita	Swarna prashana, madhu, ghrita	*NA*	Oral; Swarna bhasma: 0.2-2.4 mg[3]; once daily, 4 wk	Oral Honey + ghrita; dose details not available
16	Kumar *et al* [35], 2014	**Intervention 1:** Mamajjaka capsules	Maamajjaka, Naagjhvaa *etc.*	*Enicostemma littorale*	Oral; 500 mg, twice daily, 3 mo	Control; no details available
		Intervention 2: Shilajatu capsules	Shilajatu	*Asphaltum punjabinum*	Oral; 500 mg, twice daily, 3 mo	
17	Ravindran *et al*[25], 2014	Varunadi Ghrita + Standard care	Composite[4]	Composite[4]	Oral; 5 g, twice daily, 1 y	Standard care
18	Somarathna *et al*[20], 2010	Ranahamsa Rasayana	Composite[4]	Composite[4]	Oral; 5 g, twice daily, 90 d	Standard care
19	Gupta *et al* [19], 2010	Shilajatu Rasayana	Composite[4]	Composite[4]	Oral; 95 g over first 15 d, later 6 g per day for	Standard care

	75 d	

[1]Out of various Ayurvedic names, only two are mentioned.
[2]d: Day; wk: Week; m: Month; yr: Year.
[3]Dosage varied based on age of infant.
[4]Detailed composition: Supplementary Table 3. IV: Intravenous.

supplementation among allergic rhinitis patients, but the presented results lacked clarity[23]. *Tulsi* did not have significant effect on CD8 lymphocyte counts among healthy individuals, but this paper did not provide any numbers to back this claim[24].

The impact of Ayurvedic preparations on other types/subtypes of T cells was presented in four papers. Perioperative mistletoe administration was associated with a significant increase in T lymphocyte count, CD3 lymphocyte count, and CD4/CD8 ratio among patients with GI cancer[18], and *Varunadi Ghrita* consumption for 1 year was found to significantly increase CD3 lymphocyte count among patients with head and neck cancer after complete remission[25]. Although aged garlic extract consumption for 45 d was reported to have a significantly higher γδ-T cell proliferation index among healthy adults compared to placebo, numbers to support this claim were not reported in this paper[26]. Finally, *Nigella sativa* combined with specific immunotherapy was not found to significantly alter CD3 lymphocytes among patients with allergic rhinitis, but this article did not present the results with clarity [23].

Impact on NK cell count and NK cell activity: NK cell counts were found to be significantly enhanced by perioperative administration of mistletoe for 14 d among patients undergoing surgery for GI cancer [18] and also by consumption of aged garlic extract capsules for 12 wk among patients with inoperable GI cancer[21]. Administration of *Varunadi Ghrita* for 1 year was associated with a marginal but significant increase in NK cell counts among patients with head and neck cancer[25]. *Tulsi* produced a significant increase in NK cell activity over 4 wk among healthy volunteers, but this article did not present the complete results to strengthen its claims[24]. No significant changes in NK cell counts were observed with either *Glycyrrhizia glabra* consumption[22] or *Nigella sativa* seed supplementation[23], but results presented in both these articles were incomplete.

NK cell activity was found to be significantly enhanced after 7 d of surgery with a single-dose mistletoe extract intravenous infusion perioperatively among patients undergoing colorectal carcinoma resection, compared to standard care[27]. Among patients with inoperable GI cancer, aged garlic extract administered for 12 wk was associated with a significant increase in mean NK cell activity percentage, but a non-significant reduction in mean NK cell activity per 100 cells[21]. Regular consumption of tea fortified with several Ayurvedic herbs over 2 mo by healthy individuals was associated with a significant enhancement in NK cell activity compared to regular tea; however, the crossover design of the trial did not have a sufficient wash-out period, and there were inconsistencies with the numbers in the results[28]. Next, among healthy females, a 30-min *Shirodhara* treatment with sesame oil was associated with a significant enhancement in NK cell activity compared to the control supine position for 30 min[29], and among healthy volunteers, the consumption of aged garlic extract for 45 d was associated with a significant enhancement of activated state of NK cells as well as NK cell proliferation index compared to placebo[26]; however, both these papers did not provide numbers to substantiate fully these claims.

Impact on B lymphocytes and immunoglobulins: While B lymphocyte counts were significantly enhanced by the consumption of *Varunadi Ghrita* for 1 year among patients with head and neck cancer remission[25], a significant change was not reported with consumption of mistletoe extract[18], *Glycyrrhizia*[22], *Nigella*[23], and *Tulsi*[24]. It is however worthwhile to note that the last three studies had methodological or reporting issues. Next, perioperative consumption of mistletoe extract was associated with a significant increase in serum levels of immunoglobulin (Ig)A and IgM, and also of IgG (significance not mentioned)[18]; however, a significant impact was not found on serum levels of IgG, IgM, and IgA by saffron consumption[30], on serum IgG levels of infants by calcined gold powder consumption[31], or on cord blood IgG levels by consumption of *Rasayana Avaleha* by the pregnant mother[32]. Interestingly, the last study concluded that *Rasayana Avaleha* enhanced fetal immunity level, despite absence of strong evidence pointing towards the same.

Impact on complement components and cytokines: While serum levels of complement C3 and C4 proteins were found to increase significantly after 14 d of perioperative administration of mistletoe extract among patients undergoing surgery for GI cancer[18], no significant changes in the serum levels of these proteins were observed after daily consumption of saffron tablets for 6 wk among healthy individuals[30]. Among healthy adult volunteers, the consumption of aged garlic extract for 45 d was associated with non-significantly reduced serum levels of tumor necrosis factor α and interferon γ levels compared to placebo; this 'decrease in cytokine levels' was interpreted by the authors to suggest that consumption of aged garlic extract resulted in 'enhancement of the immune system' in the sense that,

after the consumption of aged garlic extract, 'eradication of pathogens causing flu-like illnesses' could be achieved by lower levels of these cytokines[26]. By contrast, consumption of *Tulsi* capsules for 4 wk was found to increase significantly the levels of interferon gamma and interleukin-4 in healthy adult volunteers, and this 'increase in cytokine levels' was also interpreted as supporting the observation that *Tulsi* resulted in mounting 'an effective immune response'[24]. The latter paper also did not provide complete numbers to substantiate the claims.

Impact on white blood cell counts and granulocytes: Even though total white blood cell (WBC) counts were enhanced by perioperative mistletoe administration after 14 d, the enhancement seen with standard care was found to be numerically higher[18]. Total WBC counts were not found to be significantly enhanced by administration of aged garlic extract[21], saffron[30], or calcined gold powder [31].

Total lymphocyte counts were significantly enhanced by perioperative mistletoe administration among patients with GI cancer[18]. While calcined gold powder was found to enhance significantly lymphocyte counts among infants < 1 mo but not in older infants, this paper did not provide numbers to back their claims[31]. Saffron was not associated with a significant change in total lymphocyte counts as well[30].

While significant changes in absolute neutrophil counts were not reported with the use of saffron[30] and calcined gold powder[31], absolute neutrophil counts were significantly reduced by administration of *Ranahamsa Rasayana* for 90 d among patients with HIV[20]. Phagocytic function by neutrophils was found to be significantly enhanced after a 1-mo treatment with *Tinospora cordifolia* (among patients with diabetic foot ulcer)[33] and *Nigella sativa* (among patients with allergic rhinitis)[23].

No significant alterations in eosinophil or basophil levels were reported with saffron administration [30]. Absolute eosinophil count was found to decrease significantly after a 4-wk administration of calcined gold powder among infants aged < 1 mo but not among older infants; there were no significant changes in monocyte counts in either age group. However, this paper did not provide complete results to strengthen these claims[31].

Impact on other immune system variables: Perioperative subcutaneous mistletoe administration over 14 d was found to increase significantly the counts of CD2 lymphocytes (comprising of T lymphocytes and NK cells) after 14 d among GI cancer patients undergoing surgery[18]. Perioperative intravenous single-dose mistletoe infusion among GI cancer patients undergoing surgery was associated with a significant lowering of human leukocyte antigen - DR isotype expression, which was also seen among patients who received standard care[27].

Immune enhancement: Indirect evidence

Four studies reported indirect evidence of immune system enhancement following various Ayurvedic treatments.

Healthy adults who consumed capsules containing aged garlic extract for 90 d were found to have a reduced severity of cold and flu in terms of a significantly lower number of symptoms, significantly lower decrease in activity and days of reduced activity due to illness, and significantly lower number of work days missed due to illness compared to volunteers taking matched placebo capsules. However, a significant difference between these two groups was not found with respect to illness incidence, number of days with symptoms, number of symptoms per illness, and number of doctor visits. These observations were attributed to the enhancement of activities of NK cells and γδ-T cells by aged garlic extract[26].

Consumption of *Cyavanaprasa* with milk by healthy children aged 5-12 years for 6 mo was found to be associated with improved health compared to control in terms of significantly lower number of episodes of infection/allergy (overall number of episodes, and episodes of mild and moderate severity), significantly shorter duration of illness (overall duration of illness and illnesses of mild and moderate severity), significantly more children without infection/allergy, and significantly lower absenteeism due to illness, both in terms of children reporting absenteeism as well as the number of days of absenteeism due to illness. Other findings not reaching statistical significance included a lower number and shorter duration of severe episodes of infection/allergy and a lower number of children with infection/allergy [34].

The '*Ojas* score', which signifies immune function, was found to be significantly improved after 3 mo of administration of both the Ayurvedic interventions studied (*Mamajjaka* and *Shilajatu*) as well as the control treatment, and the improvement caused by both the Ayurvedic interventions was found to be significantly higher than that seen with the control treatment[35]. Finally, the number of COVID-19 patients with positive reverse transcription polymerase chain reaction results was zero, after 10 d of treatment with either *Vyagradhi Kashaya*, ginger-garlic, or Vitamin C[36].

The complete details of the main findings of all the included studies are summarized in Table 3.

Safety

Nine of the 19 included studies did not report the safety profile of the Ayurvedic intervention, and eight studies reported not finding any new safety signals of concern. Rais *et al*[36] reported that 2/40 patients

Table 3 Results from the included studies: Impact of Ayurvedic medication on components of the immune system

No	Ref.	Intervention	T Helper cells	T cytotoxic cells	T cells: Other results	NK cell count/ activity	B lympho-cyte count	Immuno-globulins	Comple-ment	Cyto-kines	WBC count/ activity	Other variables	Indirect evidence
1	Enesel *et al*, 2005	Isorel® (Mistletoe, firtree)	+	-	+	+	-	+	+	NA	+	+	NA
2	Ishikawa *et al*, 2006	Aged garlic extract	-	-	NA	+	NA	NA	NA	NA	-	NA	NA
3	Brush *et al*, 2006[1]	*Glycyrrhizia glabra* tincture	+/-	+/-	NA	+/-	+/-	NA	NA	NA	NA	NA	NA
4	Schink *et al*, 2007	Iscador® (Standardized mistletoe extract)	NA	NA	NA	+	NA	NA	NA	NA	NA	-	NA
5	Purandare *et al*, 2007	Tinospora cordifolia	NA	NA	NA	NA	NA	NA	NA	NA	+	NA	NA
6	Uebaba *et al*, 2008[2]	Shirodhara oil-dripping (sesame oil)	NA	NA	NA	+/-	NA	NA	NA	NA	NA	NA	NA
7	Bhat *et al*, 2010[3]	Fortified tea with multiple Ayurvedic ingredients	NA	NA	NA	+/-	NA	NA	NA	NA	NA	NA	NA
8	Işik *et al*, 2010	Specific immunotherapy + *Nigella sativa*	+/-	+/-	+/-	+/-	+/-	NA	NA	NA	+	NA	NA
9	Kianbakht *et al*, 2011	Saffron tablet	NA	NA	NA	NA	NA	-	-	NA	-	NA	NA
10	Mondal *et al*, 2011[4]	Tulsi capsules	+/-	+/-	NA	+/-	+/-	NA	NA	+/-	NA	NA	NA
11	Nantz *et al*, 2012[5]	Aged garlic extract	NA	NA	+/-	+/-	NA	NA	NA	+/-	NA	NA	+
12	Suprabha *et al*, 2017	Rasayana Avaleha with milk	NA	NA	NA	NA	NA	-	NA	NA	NA	NA	NA
13	Gupta *et al*, 2017	Cyavanaprasa (Dabur) with milk	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+
14	Rais *et al*, 2021	**Intervention 1:** Vyaghryadi Kashaya + Pippali + Samshamani vati	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-
		Intervention 2: Shunthi churna + Rasona kalka	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-
15	Bhaskaran *et al*, 2019[6]	Swarna Bhasma, honey, ghrita	NA	NA	NA	NA	NA	+/-	NA	NA	+/-	NA	NA
16	Kumar *et al*, 2014	**Intervention 1:** Mamajjaka capsules	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+
		Intervention 2: Shilajatu capsules	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+

17	Ravindran *et al*, 2014	Varunadi Ghrita + Standard care	NA	NA	+	+	+	NA	NA	NA	NA	NA	NA
18	Somarathna *et al*, 2010	Ranahamsa Rasayana	+	NA	NA	NA	NA	NA	NA	NA	-	NA	NA
19	Gupta *et al*, 2010	Shilajatu Rasayana	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	SUMMARY	+	3	0	2	4	1	1	1	0	3	1	4
		+/-	3	3	2	6	3	1	0	2	1	0	0
		-	1	2	0	0	1	2	1	0	3	1	2

+: Evidence suggests significant immune enhancement; +/-: Ambiguous, confusing, or incomplete results; -: Evidence does not suggest significant immune enhancement.

[1]Sample size is 5 participants; significance values of any results not reported in the paper.

[2]paper reports only significance (*P* value) without reporting the value of NK cell activity.

[3]study I (parallel group study design) reports only significance (*P* value) without reporting the value of NK cell activity, and study II (crossover design) has less than optimal washout period, and there is a difference in numbers between text and table.

[4]paper reports only significance (*P* value) without reporting the value of T lymphocytes, B lymphocytes, NK cells, and cytokines.

[5]paper reports only significance (*P* value) without reporting the value of T lymphocytes and NK cells.

[6]paper reports only significance (*P* value) and mean difference without reporting the value of immunoglobulins and total or differential white blood cell counts.

NA: Not available; NK: Natural killer; WBC: White blood cell.

receiving *Vyagradhi Kashaya* developed loose stools and subsequently discontinued the treatment, and 3/40 patients receiving ginger-garlic developed burning sensation in the abdomen and were conservatively managed. After receiving *Ranahamsa Rasayana*, 2 patients were reported to have a mild burning sensation over the body, transient mouth ulceration, and mild drowsiness, which led to discontinuation of treatment[20].

Risk of bias assessment of included studies

As per RoB-2 tool, nine of the 20 RCTs described in the 19 included studies had high risk of bias, and the remaining 11 RCTs had some concerns of bias. Only three RCTs gave adequate information about randomization process; most of the RCTs used standard methods to measure the outcomes, leading to domain 4 having the highest number of studies with low risk of bias. Since none of the studies provided information about pre-specified statistical analysis plan, all 20 RCTs received a 'some concerns' rating for domain 5, leading to a similar rating for overall risk of bias. The risk of bias summary is presented in Figure 2 and Table 1, and the complete analysis of risk of bias assessment is available in Supplementary Table 4.

IRR

The IRR for study selection between the two reviewers was substantial, with Cohen's kappa value being 0.743 [95% confidence interval (CI): 0.714-0.772] and 0.681 (95%CI: 0.652-0.710) for title-abstract screening and full-text screening, respectively. For the methodological quality assessment, an agreement between the two reviewers with respect to the methodological quality was achieved with 16/20 RCTs,

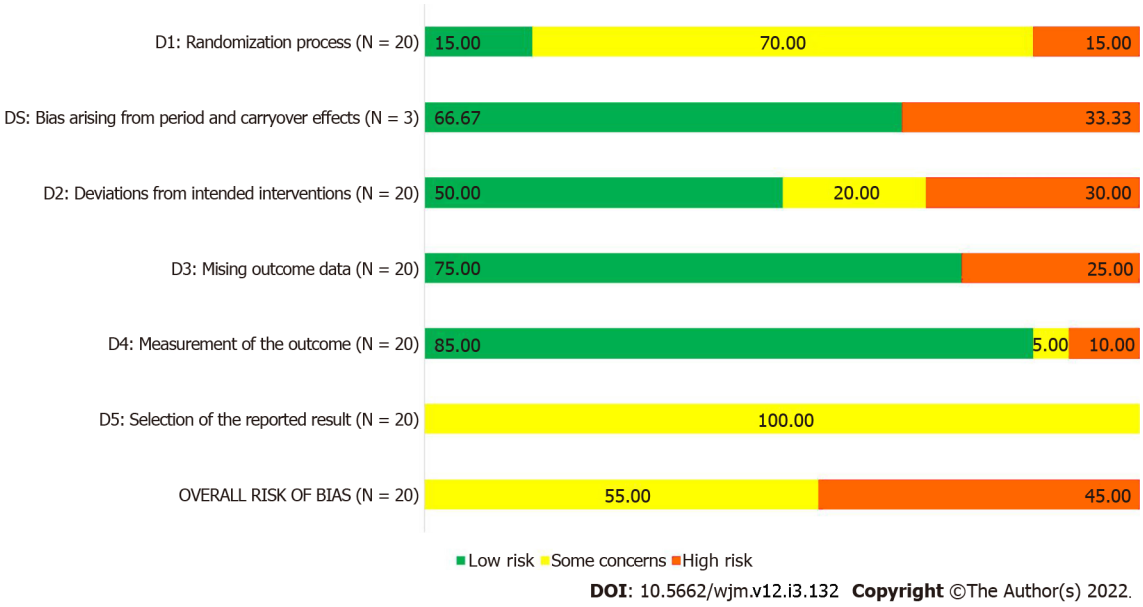

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Figure 2 Risk of bias summary of the included studies, as per RoB-2 tool.

with four papers needing mediation, leading to an inter-rater agreement of 84.21%. The Cohen's kappa value for risk of bias assessment was 0.636 (95%CI: 0.607-0.665), indicating substantial IRR.

DISCUSSION

The most prominent finding of our study is that while there is reasonably significant indirect evidence of immune system enhancement by different Ayurvedic interventions, especially *Cyavanaprasa*, the same cannot be said about direct evidence. In other words, we can say with some confidence that some Ayurvedic medicines have been shown to reduce the duration of illness and improve overall good health after long-term consumption, indirectly indicating that the immune system might have been boosted; however, the exact mechanism by which these effects occur is not yet clear. By looking at the summary of evidence in our review, it appears that enhancement of number and activity of NK cells and T helper cells might be responsible for such an enhancement, while the role of other components of immune system, including cytotoxic T cells, B lymphocytes, immunoglobulins, complement components, cytokines, and WBC counts is not clear. The high degree of inter-rater agreeability further points towards the accuracy of our observations.

We also observed some discomforting points about the included studies. Seven of the 20 interventions studied[19,20,25,28,32,34,36] were composite mixtures of a variety of Ayurvedic ingredients. By the nature of these individual studies, it is not possible to determine which component/s of these composite ingredients have prominent and contributory effect towards immune enhancement and which components have secondary roles such as excipients, flavoring and coloring agents or other non-essential functions. On the other hand, the exact mechanisms by which the remaining 13 individual Ayurvedic interventions impacted the immune system have been not adequately explored in the individual studies. Further contributing to the lack of clarity are the observations that there are some contradictory views wherein an apparent lack of effect in the results is claimed to have an effect (as in the case of cord blood IgG)[32] or contradictory correlations given for opposite directions of alterations in blood cytokine levels[24,26]. Next, as seen in Table 3, as many as six studies have confusing, ambiguous, or incomplete results for various reasons. The sample size of the included studies is not sufficiently large enough. None of the 20 RCTs described in the 19 studies included in the present review was able to score a 'low risk of bias' rating in the RoB-2 tool, had a mention of points indicative of well-conducted RCTs, such as protocol registration in a clinical trial registry, or had followed any reporting guidelines. All these factors generally tend to reduce confidence in the results presented in the studies, regardless of magnitude or statistical significance.

In the backdrop of these discouraging observations, it is apparent that, despite Ayurveda being an ancient science with an acclaimed and proven effect on immune system enhancement and despite the availability of modern medical research methodologies and technological advancements, we have not been able to know exactly how such an enhancement in immune function is brought about through the consumption of a variety of Ayurvedic medicines. The proponents of Ayurveda mostly seem to rely on the historical anecdotes, rather than using the benefits of scientific advancements to improve the general

confidence of the worldwide audience in this ancient science that has stood the test of time. While this lacuna on the part of Ayurvedic researchers has been observed and commented upon for quite some time now[37], solid steps to enhance systematic evidence generation in Ayurveda seem to be lacking. In fact, ancient Ayurvedic texts have already incorporated many of the basic tenets of modern scientific discovery, such as *Anumana* (logical questioning), *Yukti* (knowledge), *Tarka* (argument), and *Vyapti* (cause and effect relationship)[38]. It should be realized that before the advent of modern medicine, Ayurveda was the 'modern medicine' of those times. Perhaps it is time that proponents of Ayurveda and modern medicine come together with open minds to apply modern scientific methods to generate credible and reproducible evidence, so that there is rational and evidence-based integration of Ayurveda into mainstream medicine. Also, it is apt that this is done by Indian scientists, rather than waiting for the Western scientists to do it on our behalf, as in the case of turmeric, neem, *Basmati* rice[39, 40], *Pranayama*-meditation[41], and many others.

While our initial search resulted in a pool of over 12000 potentially relevant articles, we were able to identify only 19 RCTs that matched our eligibility criteria. During the process of literature screening, we observed that a large majority of research in this field is non-clinical and largely done *in vitro* or on animal models. It appears that a meaningful translation to clinical research is lacking for many Ayurvedic preparations, whose efficacy and safety were established in non-clinical models, for reasons unknown. It appears that Ayurvedic researchers are restricting themselves from entering clinical research, and the reasons for this hinderance should be sought out and resolved in order to boost clinical research in this field.

Our review should be interpreted in the backdrop of some limitations. We restricted to only papers published in English language, because of our familiarity with the language; we might have missed valid papers published in regional languages. We did not search other databases such as Embase or Scopus, since both these resources are paywalled, and our research was self-funded.

CONCLUSION

To conclude, various Ayurvedic preparations, both standalone and composite, appear to have an enhancing effect on the immune system, as evidenced indirectly through reduced illness variables, but the exact mechanism behind this enhancement is not fully established. There may be contributions from enhancement of NK cells and T helper cells, although the role of other immune system components is not clear. There were many inconsistencies and ambiguities with respect to the included studies. The numerous benefits of Ayurveda are being masked by a lack of proper research. The general need to improve the quality of research in Ayurveda is clearly visible, and the strong evidence thus generated, preferably by Indian researchers, will go a long way in fostering widespread acceptance of the immense knowledge of this ancient science by all stakeholders.

ARTICLE HIGHLIGHTS

Research background

Ayurveda is the Indian traditional system of medicine, and has a history since the 2nd century BC. Many Ayurvedic preparations have been anecdotally claimed to have immune-boosting properties and have been used for immune enhancement and general well-being. Pre-clinical research suggests that the immune enhancement is mediated through multiple immune system modulation and psychoneuroimmunological mechanisms.

Research motivation

We were interested to know the exact mechanisms of immune enhancement by Ayurvedic preparations when they are administered to healthy or sick humans.

Research objectives

The objectives of the present systematic literature review were to gather evidence towards the nature and mechanism of enhancement of human immune system by the administration of Ayurvedic preparations from published randomized clinical trials (RCTs).

Research methods

We prospectively registered the study protocol with PROSPERO. Based on predetermined eligibility criteria, search strategy was formulated and refined, and the same was used to search PubMed, DOAJ, Google Scholar, three dedicated Ayurveda research portals, two specialty Ayurveda journals, and reference lists for relevant records published until February 6, 2021. Baseline features and data pertaining to the nature and mechanism of immune system function were extracted from all eligible

records. Methodological quality was assessed using the Cochrane RoB-2 tool.

Research results

Our search strategy yielded a total of 12554 articles, and we found 19 studies reporting 20 RCTs (17 parallel group design, three crossover design) with 1661 unique patients to be eligible for inclusion. Healthy population was included in nine studies, of which one study included pregnant women and two included pediatric population; remaining studies included patients with different health conditions. A total of 21 Ayurvedic interventions were studied, out of which five were composite mixtures. Through indirect evidence, four interventions were seen to be associated with immune enhancement, and two interventions were associated with a lack of such an enhancement. The role of T helper cell and natural killer cell enhancement was reported to contribute to the enhancement of immune systems by three and four interventions, respectively. Evidence pointing to enhancement of other immune system components, including cytotoxic T cells, B lymphocytes, immunoglobulins, cytokines, complement components, leucocyte counts, and other components, was not found. Risk of bias was 'high' in 9/20 RCTs, and 'some concerns' of bias were found in the remaining 11/20 RCTs, according to RoB-2.

Research conclusions

Various Ayurvedic preparations, both standalone and composite, appear to have an enhancing effect on the immune system, as evidenced indirectly through reduced illness variables, but the exact mechanism behind this enhancement is not fully established. There may be contributions from enhancement of natural killer cells and T helper cells, although the role of other immune system components is not clear.

Research perspectives

There is a need to improve the quality of research in Ayurveda. Ayurvedic scholars should team up with experts of modern clinical research and generate credible and reproducible evidence towards immune system enhancement. This will enable widespread acceptance of the immense knowledge of Ayurveda, leading to its increased usage, and ultimately, a healthier society.

FOOTNOTES

Author contributions: Vallish BN contributed to acquisition of data, analysis and interpretation of data, drafting the article, making critical revisions related to important intellectual content of the manuscript, and final approval; Dang D contributed to acquisition of data, analysis and interpretation of data, making critical revisions related to important intellectual content of the manuscript, and final approval; Dang A contributed to conception and design of the study, acquisition of data, making critical revisions related to important intellectual content of the manuscript, and final approval.

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META-ANALYSIS

Assessment of diagnostic capacity and decision-making based on the 2015 American Thyroid Association ultrasound classification system

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Peer-review report's scientific quality classification

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Abstract

BACKGROUND

This study evaluates the American Thyroid Association (ATA) ultrasound (US) classification system for the initial assessment of thyroid nodules to determine if it indeed facilitates clinical decision-making.

AIM

To perform a systematic review and meta-analysis of the diagnostic value of the ATA US classification system for the initial assessment of thyroid nodules.

METHODS

In accordance with the PRISMA statement for diagnostic test accuracy, we selected articles that evaluated the 2015 ATA US pattern guidelines using a diagnostic gold standard. We analyzed these cases using traditional diagnostic parameters, as well as the threshold approach to clinical decision-making and decision curve analysis.

RESULTS

We reviewed 13 articles with 8445 thyroid nodules, which were classified according to 2015 ATA patterns. Of these, 46.62% were malignant. No cancer was found in any of the ATA benign pattern nodules. The Bayesian analysis post-test probability for cancer in each classification was: (1) Very-low suspicion, 0.85%; (2) Low, 2.6%; (3) Intermediate, 6.7%; and (4) High, 40.9%. The net benefit (NB), expressed as avoided interventions, indicated that the highest capacity to avoid unnecessary fine needle aspiration biopsy (FNAB) in the patterns that we studied

was 42, 31, 35, and 43 of every 100 FNABs. The NB calculation for a probability threshold of 11% for each of the ATA suspicion patterns studied is less than that of performing FNAB on all nodules.

CONCLUSION

These three types of analysis have shown that only the ATA high-suspicion diagnostic pattern is clinically useful, in which case, FNAB should be performed. However, the curve decision analysis has demonstrated that using the ATA US risk patterns to decide which patients need FNAB does not provide a greater benefit than performing FNAB on all thyroid nodules. Therefore, it is likely that a better way to approach the assessment of thyroid nodules would be to perform FNAB on all non-cystic nodules, as the present analysis has shown the ATA risk patterns do not provide an adequate clinical decision-making framework.

Key Words: Thyroid nodule; Thyroid cancer; Ultrasound; Bayesian analysis; Systematic review; Meta-analysis

Core Tip: There is no analysis that evaluates the real diagnostic value of the 2015 American Thyroid Association thyroid nodule risk patterns and their usefulness for clinical decision-making; thus, we undertook this study to quantify both values.

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INTRODUCTION

Many decisions in medicine involve trade-offs, such as weighing the balance between diagnosing patients with disease *vs* the cost of unnecessary additional testing for those who are healthy[1]. The traditional biostatistical approach to evaluating tests focuses on accuracy, evaluating calibration and discrimination, as well as using metrics such as sensitivity, specificity, or area under the curve (AUC). These methods have several advantages: They are mathematically simple, can be used for binary or continuous predictors, and are relatively easy to interpret. However, their clinical relevance is low, because there is no way to correctly discriminate between two or more diagnostic tests when there are differences in sensitivity or specificity among them. Furthermore, they do not take into consideration the consequences of the decisions made[2,3]. To address these issues, analytical methods for decision-making have been developed, which explicitly take into consideration the clinical consequences of decisions. They provide data about the clinical value of tests, including either the risks associated with an incorrect diagnosis, or the benefits of a correct diagnosis, and so can determine whether or not these tests should be used to guide decisions regarding patient care[4].

Ideally, the results of a diagnostic test should help physicians make a clear decision, meaning that, upon testing, we would either move from an epidemiological probability that a disease is present (testing threshold) to a lesser probability, and subsequent ruling out of the disease; or, on the contrary, the results could increase the probability to levels above the test-treatment threshold, and hence, point directly to treatment. However, sometimes the change in probability is higher than the testing threshold, but lower than the test-treatment threshold, in which case, the initial diagnostic test does not provide enough certainty to support decision-making regarding treatment, and additional diagnostic testing would therefore be required. This analytical process of diagnostic testing is known as "the threshold approach to clinical decision-making"[5,6] and it provides a clear, objective, and rational method to determine whether additional diagnostic testing is needed or not (Figure 1).

The sensitivity and specificity of a test cannot be used alone to estimate the probability of disease in a patient, but the two parameters when combined into one measure, called the likelihood ratio, may be used in conjunction with disease prevalence to estimate an individual patient's probability of having disease. This probability can then be transformed into a post-test probability through Bayesian analysis, and this post-test probability, when applied to the threshold approach to clinical decision-making, can then show us the true utility of a diagnostic test[7-9].

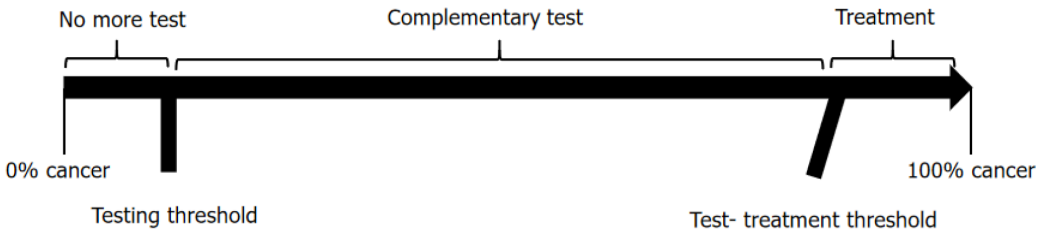

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Figure 1 The threshold approach to clinical decision-making.

Another method for clinical decision-making is decision curve analysis[2,10,11]. This method calculates a clinical "net benefit" for a diagnostic test *vs* treating all or no patients, across a range of threshold probabilities, defined as "the minimum probability of disease at which further intervention would be warranted". The net benefit, unlike accuracy metrics such as discrimination and calibration, incorporates the consequences of the decisions that were made based on the results of a diagnostic test. Therefore, if you look at the net benefit of a range of reasonable threshold probabilities (Pt) for any given intervention and, if your test has a high net benefit across the whole range, you can say that your test can help you to make an adequate decision regarding that intervention. It is clear, then, that if we use analytical methods for clinical decision-making, in addition to traditional diagnostic testing, we will have a better understanding and clinical use of the diagnostic test results.

In the context of thyroid nodule assessment, the role of ultrasound (US) has historically been very important. It was initially used only to identify the thyroid nodule and guide fine needle aspiration biopsy (FNAB), and later, it was further developed to identify nodule characteristics that would help differentiate between benign and malignant lesions, such as internal calcifications, hypoechoicity, increased central blood flow, infiltrative margins, taller than wider shape, absence of halo, solid nodule, and nodule size[12,13]. However, various meta-analyses have shown that none of these characteristics alone can differentiate with certainty between benign and malignant lesions[14-16].

The 2015 American Thyroid Association (ATA) Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer[17] reengineered these images, and, taking advantage of the practical aspects of US (non-invasive and readily accessible), described five sonographic patterns to establish the risk of malignancy: (1) Benign US features consist of purely cystic nodules, with no solid component, with an estimated risk of malignancy < 1%; (2) Very-low suspicion US features consist of spongiform or partially cystic nodules without any of the sonographic features described in low, intermediate, or high suspicion patterns, with an estimated risk of malignancy < 3%; (3) Low suspicion US features consist of isoechoic or hyperechoic solid nodule, or partially cystic nodule with eccentric solid areas, without microcalcification, irregular margin or extra thyroidal extension (ETE), or taller than wide shape, with an estimated risk of malignancy of 5%-10%; (4) Intermediate suspicion US features consist of hypoechoic solid nodule with smooth margins without microcalcifications, ETE, or taller than wide shape, with an estimated risk of malignancy of 10-20%; and (5) High suspicion US features consist of solid hypoechoic nodule or solid hypoechoic component of a partially cystic nodule with one or more of the following features: Irregular margins (infiltrative, microlobulated), microcalcifications, taller than wide shape, rim calcifications with small extrusive soft tissue component, evidence of ETE, with an estimated risk of malignancy between 70%-90%.

Based on these US descriptions and the size of the thyroid nodule, the ATA's intention is to standardize diagnostic behavior, from performing FNAB to simply keeping the thyroid nodule under observation, and the ATA patterns provide a clear and simple guideline to follow.

However, in the development of these US patterns, the origins of the percentages of malignancy suspicion assigned to each pattern is not clear, nor is the diagnostic accuracy of each pattern.

Several papers which have been published to date, both retrospective and prospective, have attempted to validate the risk patterns indicated in the ATA guidelines. These papers have shown similar findings in terms of malignancy rates in the categories of very low, low, and intermediate suspicion, although not in high suspicion, which have generally been found to be a lower percentage [18-40]. However, these studies only calculated the risk as a simple percentage and did not consider the diagnostic value in a clinical setting, which must be clearly established prior to decision-making.

Therefore, the objective of this study was to determine the real diagnostic value of the ATA classification system and to determine whether clinical decision-making based on this classification leads to an optimal management of thyroid nodules.

MATERIALS AND METHODS

Study design and data sources

We made a systematic review of the published literature related to the American Thyroid Association US classification system for the initial assessment of thyroid nodules[17] from 2016 to date.

Data extraction was performed in accordance with the PRISMA statement for Diagnostic Test Accuracy Studies[41] and by searching PubMed-Medline for all articles published in the English language with the keywords: Thyroid nodule, thyroid, ultrasound, US, ultrasonography, and 2015 ATA. Related articles suggested by PubMed were also retrieved. Bibliographies of retrieved articles were searched independently and checked for additional studies.

Study selection

Our criteria for eligibility were articles that clearly reported data related to the US patterns described in the ATA 2015 guidelines[17] and where the diagnosis of malignant or benign had been established either by histology reports, or two benign FNAB results.

Review process

Two authors (LMHL and ACM) independently reviewed the articles and established the criteria for inclusion in the pooled data analysis, with disagreements resolved through discussion. Characteristics of the included and excluded articles are presented in Figure 2.

Methodologic quality assessment

Methodologic quality was assessed using the QUADAS-2 (Quality Assessment of Diagnostic Accuracy Studies 2) criteria[42]. Both reviewers (LMHL and ACM) scored the 7-item tool independently and disagreements were resolved by consensus (among LMHL, ACM, and FRZR) *via* a face-to-face discussion about each disagreement (Table 1).

Data analysis

We used the Meta-DiSc, version 1.4 software (Ramon y Cajal Hospital, Madrid, Spain) in our meta-analysis[43]. The Mantel-Haenszel method of the random-effect model was used to calculate pooled sensitivity and specificity with corresponding 95% confidence intervals.

We analyzed these cases first using traditional diagnostic parameters and then the threshold approach to clinical decision-making and decision curve analysis.

To perform the threshold approach to clinical decision-making, it is important to understand that the indifference point for the choice between withholding therapy and performing a diagnostic test is a probability of disease designated here as the "testing" threshold (Tt). The indifference point for the choice between performing the diagnostic test and administering treatment is a probability of disease designated here as the "test-treatment" threshold (Ttrx).

Because the thresholds define these two indifference points, the physician can be guided by the calculated thresholds and estimated probability of disease in a given patient. As illustrated in Figure 1, the best choices are to withhold both treatment and the test if the probability of disease is smaller than the testing threshold, to administer treatment without testing if the probability of disease is greater than the test-treatment threshold, and to perform the test only if the probability of disease falls between the two thresholds.

The threshold levels were developed as follows[5]: The Tt consisted of the frequency of thyroid cancer, as reported in the 1015 ATA guidelines[17], estimated to be between 7% and 15%, making an average frequency of 11%.

For the Ttrx, we used the formula described by Pauker *et al*[5] shown in Figure 3. Using this formula, we estimated the Brx at 20% considering that the survival rate of a patient with papillary thyroid cancer is 98% with an early diagnosis, and 78% when there has been distant metastases[17,44]; Rrx at 4.2% when calculating an overall average rate of morbidity in total thyroidectomies, including permanent injury of the recurrent laryngeal nerve[45-47], injury to the external branch of the upper laryngeal nerve [48,49], hypoparathyroidism[50-53], and hematoma[54,55]; Rt at zero, as performing an US does not expose the patient to any risk; Pneg/nd at 0.98, representing true negatives, calculated based on US patterns[17] for benign and very low suspicion; and Pneg/d at 0.10 representing false negatives, as high-suspicion patterns detect 90% of cancers[17]. The resulting Ttrx was 67.2%.

To perform the decision curve analysis, the value for probability threshold (Pt), defined as the minimum probability of disease at which further intervention would be warranted[2,10], is a clinical judgement, and was calculated under five conditions: The first was set by simply identifying the general probability of cancer in a thyroid nodule, which is 11%, according to the ATA. The second was set to 28.1%, by taking an intermediate point between the Tt (11%) and the Ttrx (67.2%). The third condition was set to 3%, which is the probability of cancer in the very low pattern. The fourth condition was set to 7%, which is the lower range of probability of cancer according to the 2015 ATA guidelines. The fifth condition was set to 15%, which is the highest range of probability of cancer according to the 2015 ATA guidelines. We used these five Pt to calculate the net benefit of each ATA US risk pattern.

Table 1 Risk of bias and applicability judgments

Ref.	Risk of bias			Flow and timing	Applicability		
	Patient selection	Index test	Reference standard		Patient selection	Index test	Reference standard
Tang *et al*[25]	Low	Low	Low	Low	Low	Low	Low
Trimboli *et al*[28]	Low	Low	Low	Unclear	Low	Low	Low
Xu *et al*[29]	Low	Low	Low	Low	Low	Low	Low
Persichetti *et al*[30]	Low	Low	Low	Low	Low	Low	Low
Macedo *et al*[31]	Low	Low	Low	Low	Low	Low	Low
Chng *et al*[32]	Unclear	Unclear	Low	Low	High	Low	Low
Huang *et al*[34]	Low	Low	Low	Unclear	Low	Low	Low
Barbosa *et al*[35]	Unclear	Low	Low	Low	High	Low	Low
Hong *et al*[36]	Low	Low	Low	Low	Low	Low	Low
Valderrabano *et al*[37]	Low	Low	Low	Low	Low	Low	Low
Xiang *et al*[38]	Low	Low	Low	Low	Low	Low	Low
Gao *et al*[39]	Low	Low	Low	Low	Low	Low	Low
Shen *et al*[40]	Low	Low	Low	Low	Low	Low	Low

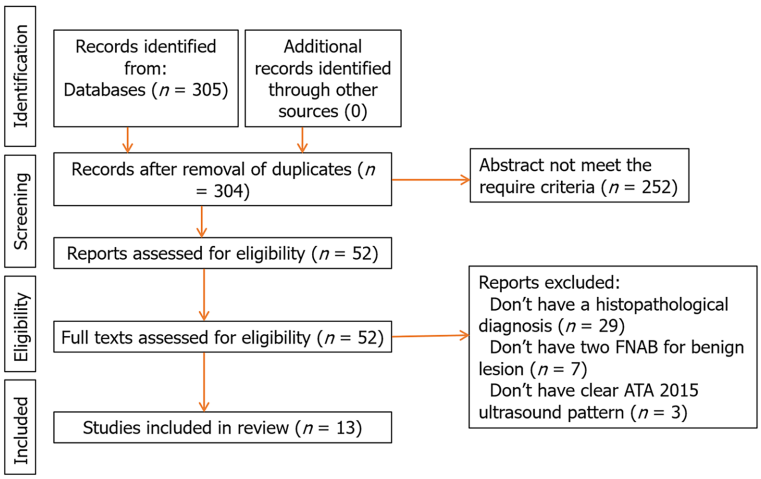

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Figure 2 Flowchart of study selection process.

$$Ttrx = \frac{(Pneg/nd)\ X\ (Rrx) - Rt}{(Pneg/nd)\ X\ (Rrx) + (Pneg/d)\ X\ (Brx)}$$

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Figure 3 Test-treatment threshold formula. Ttrx: Test-treatment threshold; Pneg/nd: Probability of a negative result in patients without disease; Rrx: Risk of treatment in patients without disease; Rt: Risk of diagnostic test; Pneg/d: Probability of a negative result in patients with disease; Brx: Benefit of treatment in patients with disease.

We calculated the net benefit, expressed as the number of unnecessary interventions avoided, in this case the number of FNABs avoided, using true negatives rather than true positives, and using the following formula: Net benefit for unnecessary interventions = (true negative count/total number of patients) – (false positive count/total number of patients) × (Pt /(1- Pt) in order to determine the number of unnecessary biopsies that had been performed, without missing any patients with cancer, in each of the ATA US patterns. We calculated these in all five possible Pt[10,11].

Once we were able to determine the best Pt by calculating the highest number of unnecessary FNABs avoided, we then calculated the net benefit for this Pt using curve decision analysis for each US pattern, using the following formula: Net benefit = (true positive count/total number of patients) – (false-positive count/total number of patients) × (Pt/1-Pt). This result would then be compared with the net benefit of performing FNAB on all thyroid nodules (Figure 4).

The resulting net benefit for each US pattern would have to be of greater value than the net benefit of performing FNAB on all thyroid nodules for it to be considered a diagnostic model that provides the correct identification of those thyroid nodules which can be safely excluded from FNAB.

We extracted the information, grouped it by author, and added all the cases together, obtaining sensitivity, specificity, and positive and negative predictive values for each of the categories. We determined the Youden's index using the following formula: Sensitivity + (specificity -1); and the positive likelihood ratio (LR) using the following formula: Sensitivity/(1-specificity). We then calculated post-test odds with Bayesian analysis using the following formula: Post-test odds = pretest odds × LR, and, in the final step, we converted the post-test odds into post-test probability.

Due to the design of this study, approval by an institutional review board was not required.

RESULTS

Our initial search retrieved 305 articles, and the summaries were reviewed by two authors (LMHL and ACM) to select those that met the required criteria. This resulted in 52 articles that we reviewed in their full text, before finally selecting 13 articles[25,28,29-32,34-40] which contained the required information in accordance with the design of the study. Nine were retrospective studies[28,29,32-37,39,40] and four were prospective studies[25,30,31,38] (Table 2 and Figure 3).

The data from 8445 thyroid nodules was obtained, of which 3937 (46.62%) were malignant and 4508 (53.38%) were benign. The average size of the tumors was 18.5 mm (5 mm to 71 mm).

When grouping the nodules into risk patterns, we found that the benign pattern was reported in only 6 of the 13 articles[30,32,34,36,39,40], for a total of 62 nodules in the category, and all of these corresponded to histopathologically benign nodules, therefore we decided to exclude this pattern from our analysis.

For the very-low suspicion pattern, there were a total of 848 cases. Of these, 832 were benign and 16 were malignant, meaning that in this pattern, the simple percentage of malignancy was 1.8%. The Youden's index was -0.18. The diagnostic value can be found in Table 3 and Figure 5.

There were 1800 nodules in the low-suspicion pattern. Of these, 1621 were benign and 179 were malignant, meaning that in this pattern, the simple percentage of malignancy was 9.4%. The Youden's index was -0.31. The diagnostic value can be found in Table 3 and Figure 6.

There were 1673 nodules in the intermediate-suspicion pattern. Of these, 1340 were benign and 333 were malignant, meaning that in this pattern, the simple percentage of malignancy was 19.9%. The Youden's index was -0.22. The diagnostic value can be found in Table 3 and Figure 7.

There were 4124 nodules in the high-suspicion pattern. Of these, 715 were benign and 3404 were malignant, meaning that in this pattern, the simple percentage of malignancy was 82.5%. The Youden's index was 0.71. The diagnostic value can be found in Table 3 and Figure 8.

After using Bayesian analysis to determine the post-test probability of cancer, our results were as follows: Very low, 0.85%; low, 2.6%; intermediate, 6.7%; and high, 40.9% (Figure 9).

The net benefit, expressed as the number of interventions (FNAB) avoided, with a Pt calculated for all five possibilities, can be seen in Figure 10.

The NB calculation for a Pt of 11% was: Very low, -0.01028; low, -0.002526; intermediate, 0.019821; and high, 0.393207. This means that the intermediate pattern was only able to identify 1.9 of every 100 patients with cancer, and the high pattern was only able to identify 39 of every 100 patients with cancer. In the very low and low pattern cases, there was no obvious interpretation for negative net benefit using this type of framework.

The true- and false-positive count for performing FNAB in all thyroid nodules is simply the number of patients with and without thyroid cancer, respectively. Calculating the net benefit for this strategy gave: (3937/8445) − (4508/8445) × (0.11/0.89) = 0.40022. The net benefit for each of the ATA suspicion patterns studied was less than that of performing FNAB on all nodules.

There was heterogeneity in the initial results, which could create a bias risk, so we made an analysis of the subgroups (Figure 10). The homogenous group was reduced to a population of 5151 thyroid nodules: 443 from the very low risk category with a 2.9% frequency of cancer, 316 cases from the low risk category with a 16.1% frequency of cancer, 946 from the intermediate risk category with a 20.7% frequency of cancer, and finally in the high risk category, 3446 nodules with an 81.5% frequency of cancer (Figure 11). After using Bayesian analysis to determine the post-test probability of cancer in the subgroup analysis, our results were: Very low, 0.7%; low, 1.8%; intermediate, 0.2%; and high, 37.4% (Figure 12).

Table 2 Studies and number of patients included

Ref.	Very low		Low		Intermediate		High	
	Cancer no	Cancer yes	Cancer no	Cancer yes	Cancer no	Cancer yes	Cancer no	Cancer yes
Tang *et al*[25]	7	1	20	5	17	7	0	8
Trimboli *et al*[28]	15	2	43	8	68	15	6	18
Xu *et al*[29]	36	2	190	21	212	59	109	277
Persichetti *et al*[30]	134	3	255	8	295	18	104	127
Macedo *et al*[31]	11	0	10	0	13	5	1	5
Chng *et al*[32]	16	1	60	10	18	12	13	27
Huang *et al*[34]	14	0	109	18	57	32	4	15
Barbosa *et al*[35]	4	1	33	10	27	9	10	46
Hong *et al*[36]	35	0	174	6	109	55	37	263
Valderrabano *et al*[37]	25	0	127	32	61	13	16	20
Xiang *et al*[38]	170	0	112	24	8	11	32	289
Gao *et al*[39]	178	0	339	20	107	55	233	1606
Shen *et al*[40]	187	6	149	17	348	42	150	708
Total	832	16	1621	179	1340	333	715	3409

Table 3 Pooled diagnostic value of ultrasound patterns studied

	Very Low	95%CI	Low	95%CI	Intermediate	95%CI	High	95%CI
Sensitivity	1%	0-1	5%	4-5	8%	8-9	87%	85-88
Specificity	82%	80-83	64%	63-65	70%	68-72	84%	83-85
Likelihood ratio (+)	0.07	0.03- 0.17	0.22	0.11-0.43	0.59	0.38-0.94	5.63	4.52-7.01

DISCUSSION

This study demonstrates how complicated it can be to interpret diagnostic tests and use them for clinical decision-making.

It is important to recognize that when we analyze decision-making based on diagnostic tests, there is no one "perfect test" that can either rule out or diagnose a disease with a 100% accuracy (in this discussion, thyroid cancer). Therefore, for every patient who undergoes a diagnostic test to help guide decision-making, there will always be three possible paths; the first is to keep the nodule under observation, the second is to do additional diagnostic testing, and the third is to proceed with treatment.

The results found in this study show an overall frequency of cancer of 46.6%. This frequency is higher than the 7% to 15% reported by the ATA because the data was obtained from reference center hospitals with a high volume of thyroid cancer cases. Although this could be considered as selection bias, it is important to emphasize that the diagnostic parameters which we used in this analysis, in particular sensitivity and specificity, and the decision-making based on these diagnostic tests, are not affected by the frequency of this disease.

We must also keep in mind that while indeed cancer was most frequent in the high-suspicion pattern, 15.5% of the cases were found among the very low, low, and intermediate-suspicion patterns.

It is also important to note that we did not include the benign pattern, as it was only reported in six of the studies analyzed, and of the 62 nodules reported with this pattern, all were confirmed to be benign. Therefore, it is reasonable to assume that any thyroid nodule that is purely cystic, regardless of size, is benign, and as such, we did not consider it necessary to include them in this study.

Another potential bias is that we had to exclude the size of the thyroid nodules, as detailed information for each nodule was not available in the articles that we reviewed. However, we do not consider this as selection bias, because the average of sizes reported in the articles that we reviewed was 18.5 mm (ranging from 5 mm to 71 mm), which is within the typical range found in most nodules in the day-to-day medical practice. We therefore consider that this variable is not of particular importance in deciding which patients will need FNAB. Nodule size has always generated controversy in terms of how it might affect the diagnosis of malignancy[56-59].

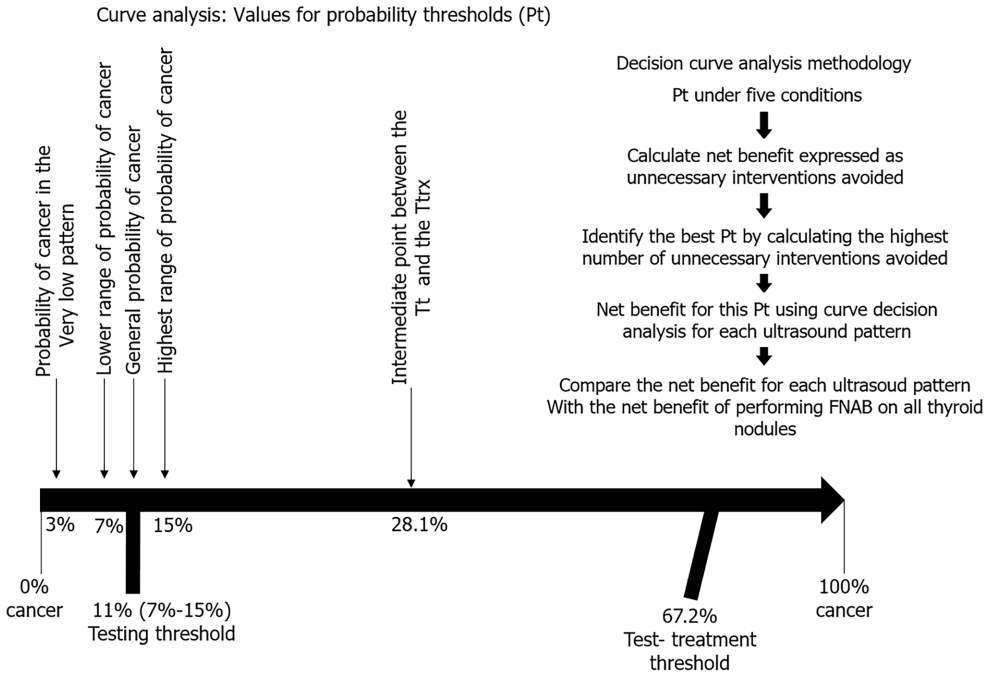

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Figure 4 Decision curve analysis methodology.

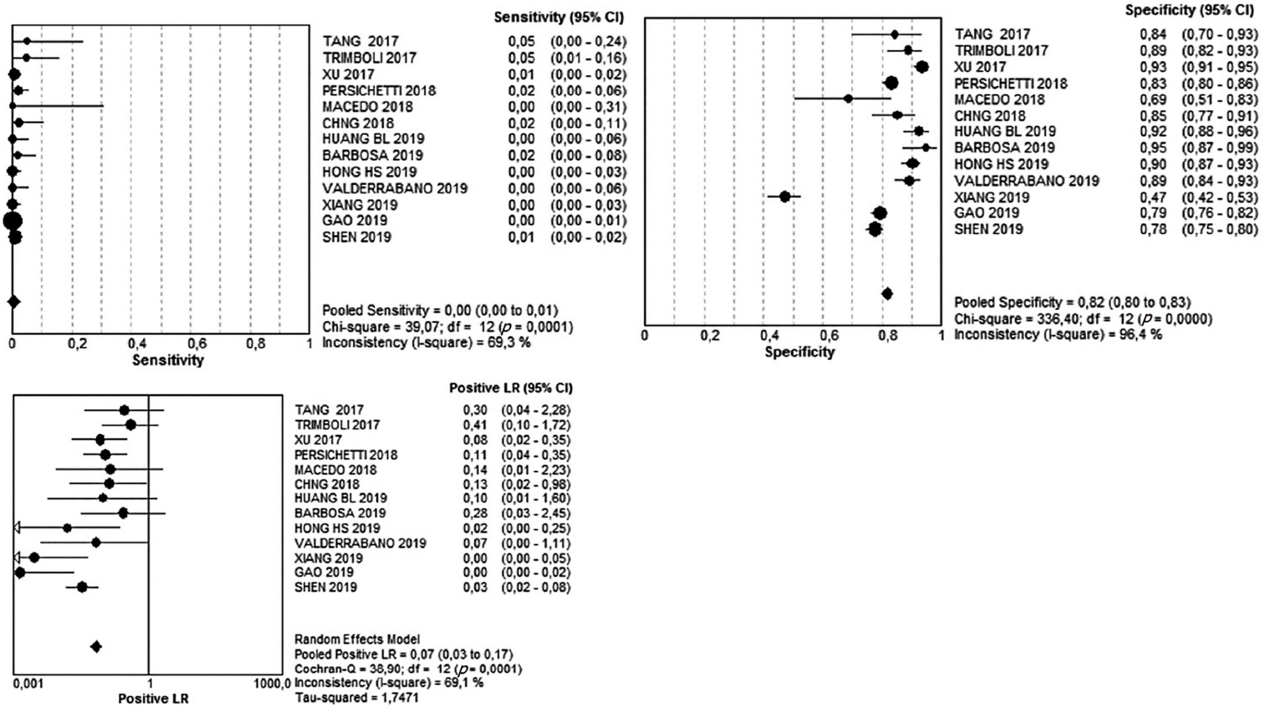

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Figure 5 Pooled diagnostic value of very low risk pattern.

The final limitation in our study is that most of the US images, and consequently their ATA US classifications, were performed retrospectively. However, this is unlikely to be significant, as the cases came from high-volume thyroid disease reference centers, and were interpreted by highly qualified US experts[60,61].

Our results show that, if we were to assume that the simple percentage for the presence of cancer in each US pattern was a reliable diagnostic tool, they would coincide perfectly with the ranges published by the 2015 ATA guidelines[17], in that the very low pattern had a 1.8% malignancy rate and the ATA reports < 3%; in the low pattern, a 9.4% malignancy rate and the ATA reports 5% to 10%; in the

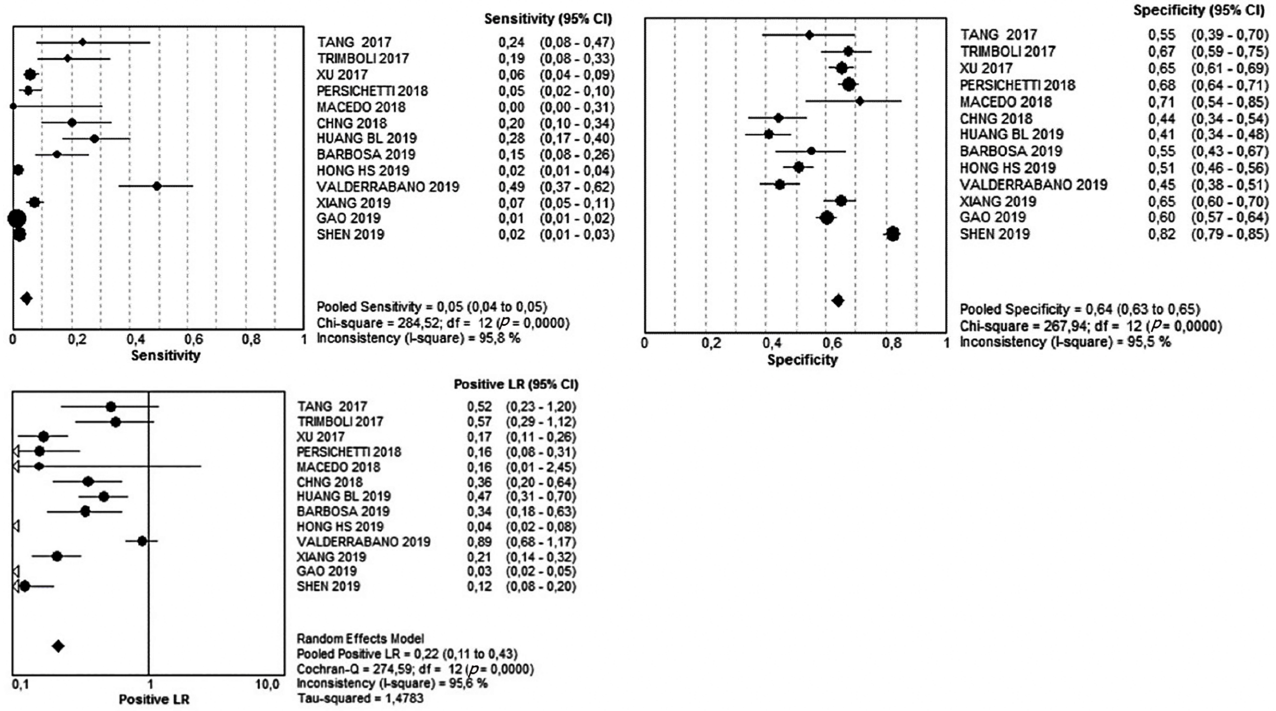

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Figure 6 Pooled diagnostic value of low risk pattern.

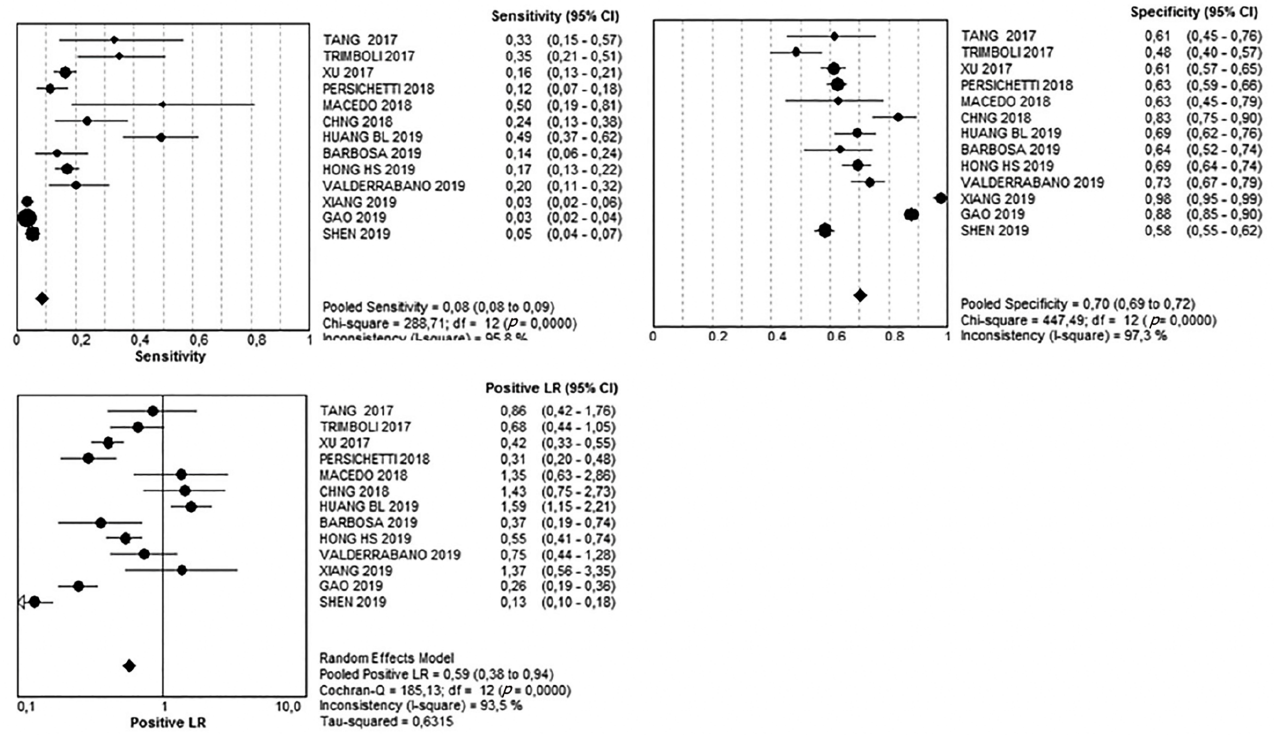

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Figure 7 Pooled diagnostic value of intermediate risk pattern.

intermediate pattern, a 19.9% malignancy rate and the ATA reports 10% to 20%; and finally, in the high pattern, an 82.5% malignancy rate and the ATA reports between 70% and 90%.

However, when we analyzed the results of the traditional biostatistical approach to evaluating tests and Youden's index, together with the threshold approach to clinical decision-making and curve decision analysis, we can see that these US patterns were no longer as clear, or as practical.

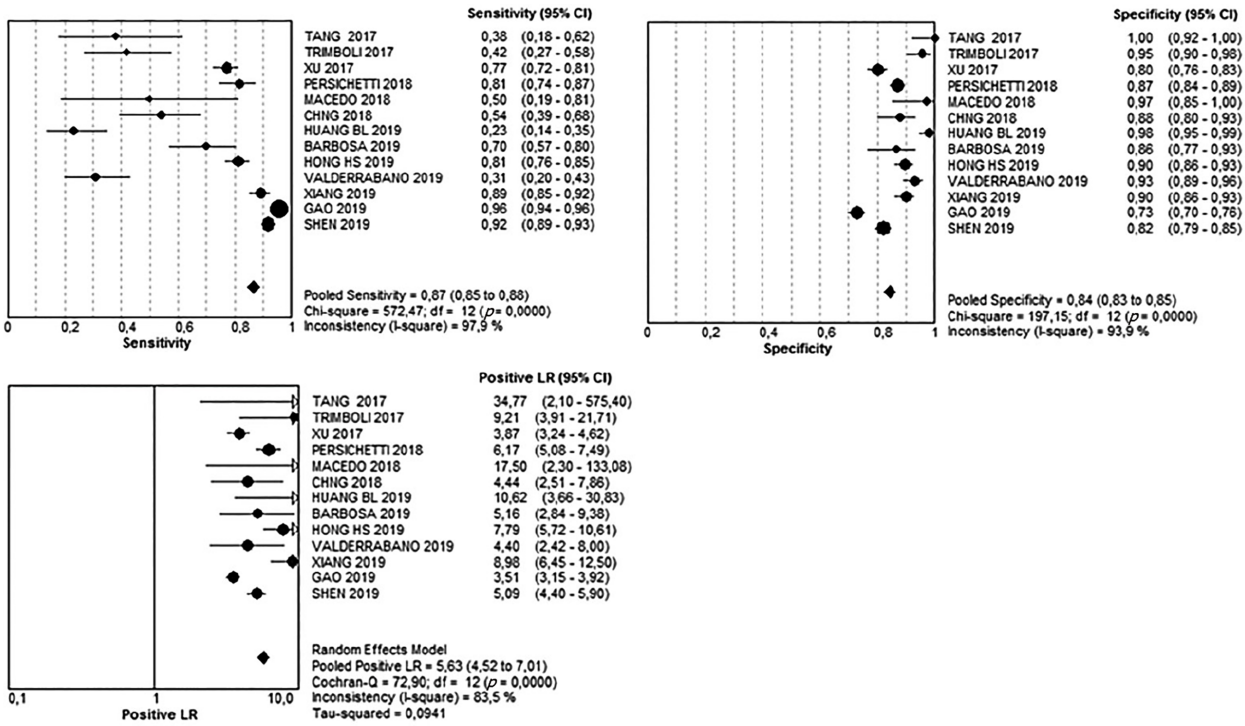

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Figure 8 Pooled diagnostic value of high risk pattern.

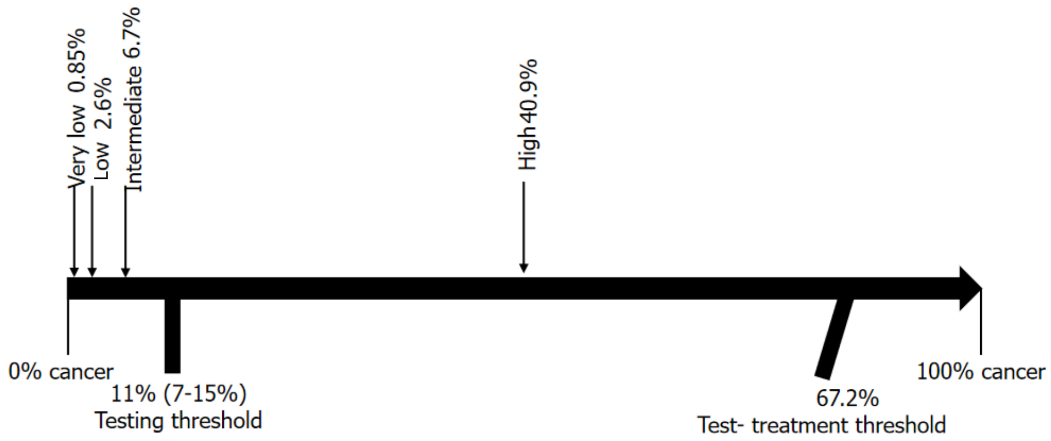

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Figure 9 Results by Bayesian analysis and the threshold approach to clinical decision-making.

When we analyzed a traditional diagnostic parameter, we can see that the very low, low, and intermediate patterns had a regular to good specificity, but a very low sensitivity, therefore they could, in theory, be used to correctly identify and rule out patients who do not have the disease, with very few false-positive results. In effect, this is the reason that the ATA has grouped thyroid nodules into patterns to guide decisions regarding whether to do further FNAB testing or not. However, our study showed that relying strictly on these patterns would have resulted in false positives: For very low, 18.1%; low, 36%; and intermediate, 29.7%, meaning that there would have been patients with cancer that went undetected using the US patterns, and, because the Youden's index for these three patterns was below 0, they would have been identified as "without diagnostic value".

On the other hand, the high pattern gave a more accurate diagnostic value with a sensitivity of 86.6%, specificity of 84.1%, and Youden´s J index of 0.7, and so, this pattern is better able to discriminate between malignant and benign cases, and therefore, can be used reliably.

When analyzing decisions based on the threshold approach to clinical decision-making, we were able to determine that the testing threshold was 11% and the test-treatment threshold was 67.2%. Once we determined the values of the US patterns using Bayesian analysis, we concluded that the very low

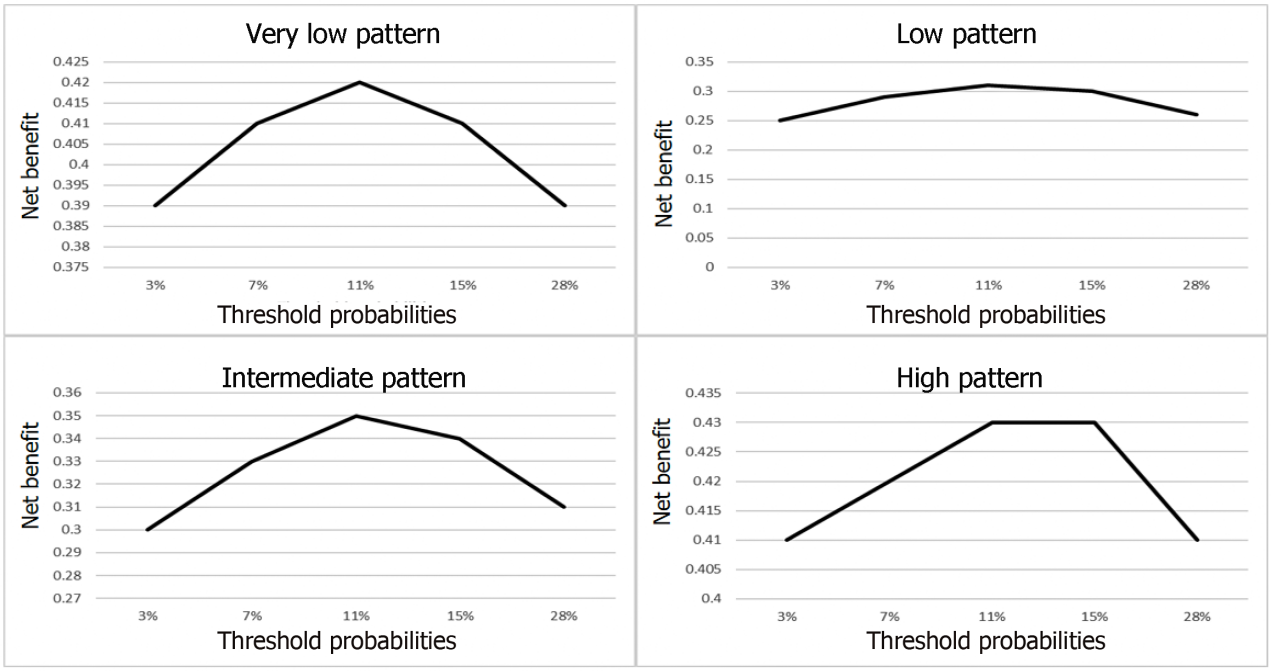

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Figure 10 Net benefit, number of interventions avoided, in study patterns.

pattern had a cancer risk of 0.3%, low of 1.5%, and intermediate of 3.4%, meaning that these three patterns would be below the testing threshold and, as a logical consequence of this method of analysis, thyroid nodules classified within these three patterns could simply be left under observation, since by definition, cancer had been ruled out, rendering further testing unnecessary. However, decisions using this type of analysis would have resulted in 15.5% of the cancers in this study going undiagnosed, troubling if we keep in mind that intermediate-risk nodules are mostly isoechoic nodules, which could be follicular carcinoma, a potentially high-risk thyroid cancer with a poor prognosis. If physicians do not perform FNAB on Bethesda IV intermediate-risk nodules, this group of potentially high-risk cancers can be missed.

The high-risk pattern, on the other hand, analyzed in the same way, raises the probability of thyroid nodule cancer to 40.3%, which is between the testing threshold (11%) and the test-treatment threshold (67.2%), therefore indicating the need to do additional FNAB testing.

With regard to heterogeneity, we made an analysis by subgroups, and it is interesting to note that the frequency of malignancy was quite similar to the initial analysis. Even more importantly, after using Bayesian analysis with the threshold approach to clinical decision-making, the results were still the same, where, in the groups of very low, low, and intermediate risk, cancer was ruled out, and the high-risk group fell in the area where additional testing would be necessary.

When analyzing decision-making based on the net benefit expressed as the number of unnecessary interventions avoided, we made our calculations using several different Pt scenarios, with a Pt of 11% being the one that most frequently matched with the test threshold defined in the analysis discussed above. We made this analysis, because the classification of US patterns was intended to determine who should undergo FNAB and who could be kept under observation, in order to reduce the number of unnecessary FNABs. When evaluating the capacity to avoid unnecessary FNABs, we found that the highest capacity for avoidance was: 42 of 100 FNABs in the very low pattern with a Pt of 11%, 31 of 100 FNAB in the low pattern with a Pt of 11%, 35 of 100 FNAB in the intermediate pattern with a Pt of 11%, and 43 of 100 FNABs in the high-risk pattern with a Pt of 11%. This means that attempting to avoid unnecessary FNABs is unadvisable, since over half of them were indeed necessary, and a cancer diagnosis would have been missed if FNAB had not been performed.

When calculating NB with a Pt of 11%, the very low and low patterns had a net benefit of less than zero, so there was no obvious interpretation for negative net benefit using this type of framework. In the case of intermediate patterns, NB could only detect slightly fewer than 2 out of every 100 patients with cancer, and in high-risk patterns, it could only detect 39 out of every 100 patients with cancer.

Further still, when comparing the net benefit of each US risk pattern studied *vs* that of systematically performing FNAB on all thyroid nodules, we found that none were higher than that of performing FNAB on all thyroid nodules. This clearly demonstrates that using these US categories to guide the decision regarding who should undergo FNAB is inferior to performing FNAB on all thyroid nodules.

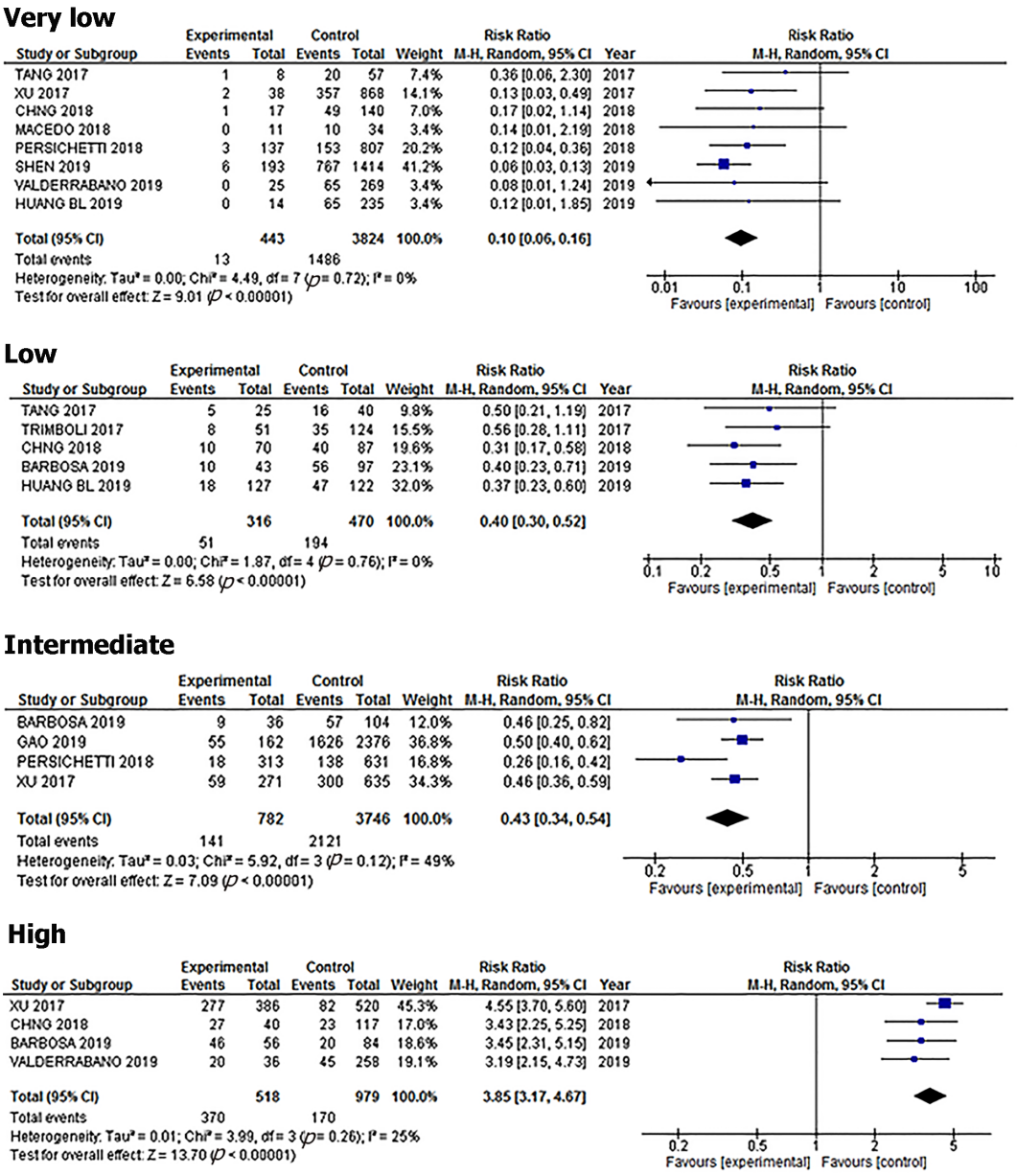

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Figure 11 Pooled subgroups analysis.

It is also important to understand that clinical decisions should not be made based on simple percentages. Instead, it is preferable to apply a rigorous study based on clinical decision models, which, although they may seem complicated to calculate, are, in reality, the only way to make an accurate professional diagnosis for the thyroid nodule patient. It is also clear that the clinically low aggressiveness of malignant thyroid cancer has allowed for a margin of error since it can be detected in a formerly undiagnosed patient at a later date. However, this diagnostic behavior would be unprofessional, as any patient who consults a physician for a thyroid nodule expects an accurate diagnosis.

From a practical standpoint, the results of this study indicate to the physician that, when evaluating thyroid nodules by US, only the high-risk and benign categories are clinically useful, indicating FNAB for high-risk cases and observation for the benign pattern. However, if the US shows a pattern of very low, low, or intermediate risk, the physician should recommend FNAB, as opposed to the current recommendations of observation only, as there is a risk of cancer of up to 15% that could go undiagnosed.

CONCLUSION

It is clear from our three types of analysis, that the only ATA diagnostic pattern that is clinically useful

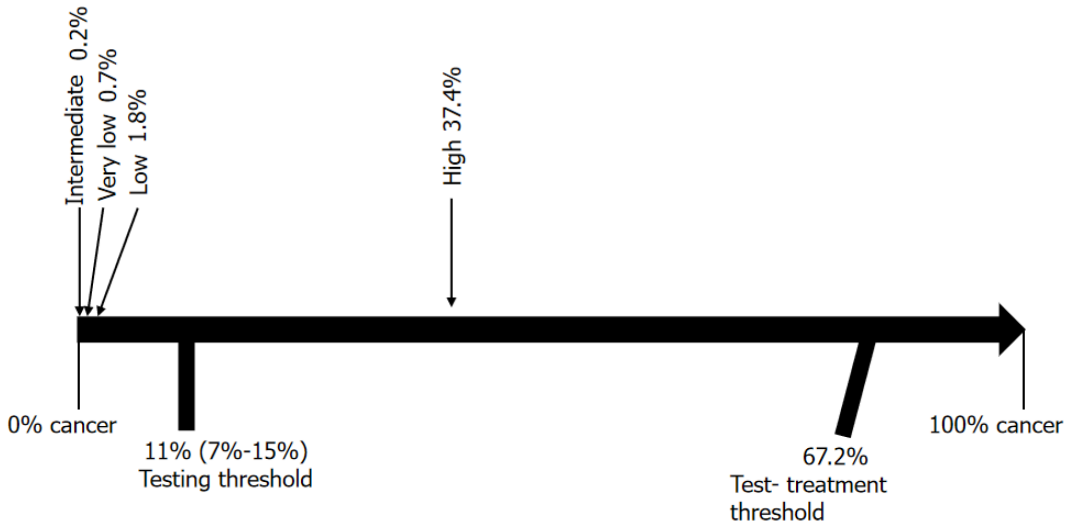

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Figure 12 Bayesian subgroups analysis and the threshold approach to clinical decision-making.

is the high-suspicion pattern, in which case, without a doubt, FNAB should be performed. However, and even more importantly, curve decision analysis has demonstrated that using these US risk patterns to decide which patients need FNAB does not provide a greater benefit than performing FNAB on all thyroid nodule patients. Therefore, we conclude that a better way to approach the assessment of thyroid nodules would be to perform FNAB on all non-cystic nodules, as the present study has shown that the ATA risk patterns do not provide an adequate clinical decision-making framework.

ARTICLE HIGHLIGHTS

Research background

It is important to make clinical decisions with the best evidence available, but the 2015 American Thyroid Association (ATA) Ultrasound (US) Guide does not yet have sufficient evidence. Therefore it should be studied and evaluated whether or not it is useful in making clinical decisions during the initial evaluation of thyroid nodules.

Research motivation

The real diagnostic value and its usefulness in clinical decision-making of the ATA 2015 US guide should be known.

Research objectives

To perform a systematic review and meta-analysis of the diagnostic value of the American Thyroid Association US system for the initial assessment of thyroid nodules.

Research methods

A meta-analysis study of the diagnostic value of the ATA 2015 ultrasonographic patterns was carried out and this diagnostic value was used to evaluate, through threshold and decision curve analysis, whether it is useful in decision-making during the initial evaluation of thyroid nodules.

Research results

The results showed that the US guided studies had no diagnostic value for decision-making in selecting which nodule should undergo or not FNAB.

Research conclusions

Physicians should continue doing FNAB to all solid or mixed thyroid nodules.

Research perspectives

An alternative diagnostic method must continue to be sought, which resolves the question of which nodule should undergo and which not FNAB.

FOOTNOTES

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META-ANALYSIS

Participant attrition and perinatal outcomes in prenatal vitamin D-supplemented gestational diabetes mellitus patients in Asia: A meta-analysis

Sumanta Saha, Sujata Saha

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Abstract

BACKGROUND

The role of vitamin D supplementation in gestational diabetes mellitus (GDM) patients is unclear.

AIM

To determine the burden and risk of post-randomization GDM patient attrition from vitamin D-supplemented arms of randomized controlled trials (RCTs). The auxiliary aim was to compare the effects of nutritional supplements on their fasting blood glucose (FPG) levels and perinatal outcomes.

METHODS

RCTs were searched in the PubMed, Embase, and Scopus databases. Random-effect prevalence and pairwise meta-analysis were performed for the primary objective. The auxiliary aim was to compare the effects of nutritional supplements on their fasting blood glucose (FPG) levels and perinatal outcomes. Fixed-effect network meta-analyses were undertaken for the secondary goals. All analyses were performed using Stata software, and statistical significance was determined at $P < 0.05$.

RESULTS

Thirteen RCTs from Iran and China were reviewed. The participant attrition burden in vitamin D recipients was 6% [95% confidence interval (CI): 0.03, 0.10], and its risk did not vary from non-recipients. Vitamin D and calcium co-supplementation reduced the cesarean section incidence in GDM patients [risk ratio (RR): 0.37; 95%CI: 0.18, 0.74]. The hyperbilirubinemia or hospitalization risk in their newborns decreased with vitamin D supplementation (RR: 0.47; 95%CI: 0.27, 0.83) and co-supplementation with calcium (RR: 0.35; 95%CI: 0.16, 0.77) or omega-

3 fatty acids (RR: 0.25; 95%CI: 0.08, 0.77). Vitamin D and probiotics co-supplementation decreased newborn hyperbilirubinemia risk (RR: 0.28; 95%CI: 0.09, 0.91). FPG levels and macrosomia risk did not vary across interventions.

CONCLUSION
In RCTs, vitamin D supplementation or co-supplementation in GDM patients showed a low participant attrition burden and low risk of cesarean section, newborn hyperbilirubinemia, and newborn hospitalization.

Key Words: Diabetes; Gestational; Vitamin D; Prenatal care; Nutrition therapy

Core Tip: This meta-analysis was conducted on efficacy trials testing the effect of vitamin D in gestational diabetes mellitus (GDM) patients and/or their neonates. The post-randomization attrition burden of GDM patients from vitamin D-supplemented trial arms was low. The risk of hyperbilirubinemia and hospitalization in newborns was low with vitamin D and its omega-3 fatty acids and calcium co-supplemented forms. Vitamin D co-supplementation with calcium and probiotics reduced the risk of cesarean section and newborn hyperbilirubinemia, respectively. Compared to omega-3 fatty acids, the risk of hyperbilirubinemia and hospitalization among neonates was low when it was co-supplemented with vitamin D.

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INTRODUCTION

Gestational diabetes mellitus (GDM) is a condition of glucose intolerance that is detected or diagnosed for the first time during pregnancy. The prevalence of GDM in pregnancy is between 4% and 18%, depending on the diagnostic criteria used[1]. The treatment of GDM is crucial as it can cause perinatal complications such as cesarean section (CS) in the mother and macrosomia in her newborn[2]. The benefits of standard GDM care with medical nutrition, lifestyle modification, and self-blood glucose monitoring are inconsistent across different treatment outcomes. For example, it decreases macrosomia risk but not CS occurrence compared to non-GDM care recipients[3]. Therefore, researchers have investigated the role of standard GDM care adjuncts for better perinatal outcomes. In this regard, vitamin D has drawn substantial attention due to the plausible association of its deficiency and GDM[4]. Although several randomized controlled trials (RCTs)[5] have assessed vitamin D efficacy in GDM patients, the burden and risk of post-randomization participant attrition from vitamin D-supplemented arms of these trials remain unclear. Notably, participant attrition happens even in adequately conducted RCTs[6]. Besides, the efficacy of vitamin D, its co-supplements, and other supplements included in these trials, remain unclear. Existing meta-analyses have compared how vitamin D affects the occurrence of perinatal outcomes and maternal fasting blood glucose (FPG) levels[7,8]. However, these did not distinguish how the effects of vitamin D can be differentiated from its co-supplemented forms (like with calcium) and other non-vitamin D supplements (*e.g.*, omega-3 fatty acids) included in these trials. This meta-analysis article attempted to address these underexplored areas of perinatal medicine.

Intervention description

The fat-soluble vitamin D hormone is available from the diet and nutritional supplements in the inactive D2 (ergocalciferol) and D3 (cholecalciferol) forms[9,10]. Cholecalciferol is further synthesized in the skin from sunlight. The pre-vitamin D undergoes hydroxylation in the liver and forms the albumin-bound circulatory 25-hydroxyvitamin D[9,11,12]. This active form of vitamin D causes calcium absorption by its action on the intestine and kidneys[10]. The physiologic role of vitamin D in pregnancy occurs *via* its binding to its receptors in the uteroplacental tissue[9,12]. The dietary allowance and the tolerable upper limit of vitamin D in pregnancy are 600 and 4000 IU, respectively[9].

The vitamin D supplementation effects on GDM mothers and their neonates have been assessed in several RCTs. Commonly tested oral dosages of vitamin D are 200-500 IU daily[13,14] or 50000 IU 2-3 weekly[15-18]. While some RCTs supplemented vitamin D as a mono-supplement, others co-supplemented it with zinc, calcium, and magnesium[14,16].

Objective

This review aimed to determine the burden and risk of post-randomization GDM patient attrition from vitamin D-supplemented arms of RCTs. Additionally, it determined the changes in FPG levels and risk of different perinatal outcomes (neonatal hyperbilirubinemia, newborn hospitalization, microsomia, and CS) across nutritional supplements tested in these RCTs.

MATERIALS AND METHODS

Registration and reporting

A pre-published protocol exists for this review, and it is registered in the PROSPERO (CRD42020180634) [19,20]. The preliminary findings of this review were presented at a conference[21]. This report adheres to The Preferred Reporting Items for Systematic Review and Meta-Analysis 2020 statement (Supplementary Table 1)[22].

Inclusion criteria

Trial design: Parallel arm RCTs of any duration.

Trial population: GDM patients of any age irrespective of their gestational age and previous GDM history.

Intervention arm/s: Prenatal vitamin D or its co-supplemented form with other nutrients orally.

Comparator arm: No nutritional supplements or placebo and/or prenatal nutritional supplement/s that does not contain vitamin D.

Primary outcome: GDM patients leaving the trial post-randomization during the intervention period. The participants excluded from analysis by trialists were not the outcome of interest.

Secondary outcomes (post-nutrient supplementation outcomes): Mean FPG levels and its standard deviation and CS frequency. Other outcomes of interest included macrosomia, hyperbilirubinemia, and hospitalization of newborns.

The diagnosis and management of GDM and the dosages and regimen of the nutritional supplements were accepted as per the trialists.

Exclusion criteria

Study designs other than that stated above (*e.g.*, crossover study, observational study). Non-GDM type of diabetes including type 1 and type 2 diabetes.

Data source

The title and abstract of the articles published in the English language were searched in the PubMed, Embase, and Scopus databases irrespective of the date of publication and geographic boundary. Additionally, the bibliographies of articles included in this review were searched. The search string used to search in the PubMed was composed of the following words and phrases: "vitamin D" OR calciferol OR "vitamin D2" OR ergocalciferol OR "vitamin D3" OR cholecalciferol AND gdm OR "gestational diabetes." Identical search strings were used in the remaining databases. The complete search string with their electronic links, when available, are presented in Supplementary Table 2.

Study selection and data abstraction

After uploading the retrieved citations to a reference handling software, the title and abstract of the articles were skimmed against the above eligibility criteria. Full-text reading transpired when articles appeared eligible or dubious for inclusion in this review. Figure 1 depicts the reasons for the elimination of articles read in full text. Salient detail abstraction about the trials (including its registration number and country of conduct), participants, interventions tested in respective treatment arms, and the outcomes of interest transpired.

Risk of bias evaluation

Using the Cochrane risk of bias (RoB) tool for RCTs, the following RoB components of the reviewed trials were evaluated[23]. The randomization method and successive allocation concealment method of interventions to different treatment arm participants were used to judge the selection bias. Utilizing the blinding mechanism used for trial personnel and participants and that of outcome assessors, performance and detection bias evaluation occurred, respectively. The attrition bias risk evaluation was assessed by comparing the frequency and reason of missing outcome data across intervention arms. By comparing trial findings with the pre-stated intentions of trialists, the risk of reporting bias was assessed. Any other bias besides those mentioned above was classified as miscellaneous bias.

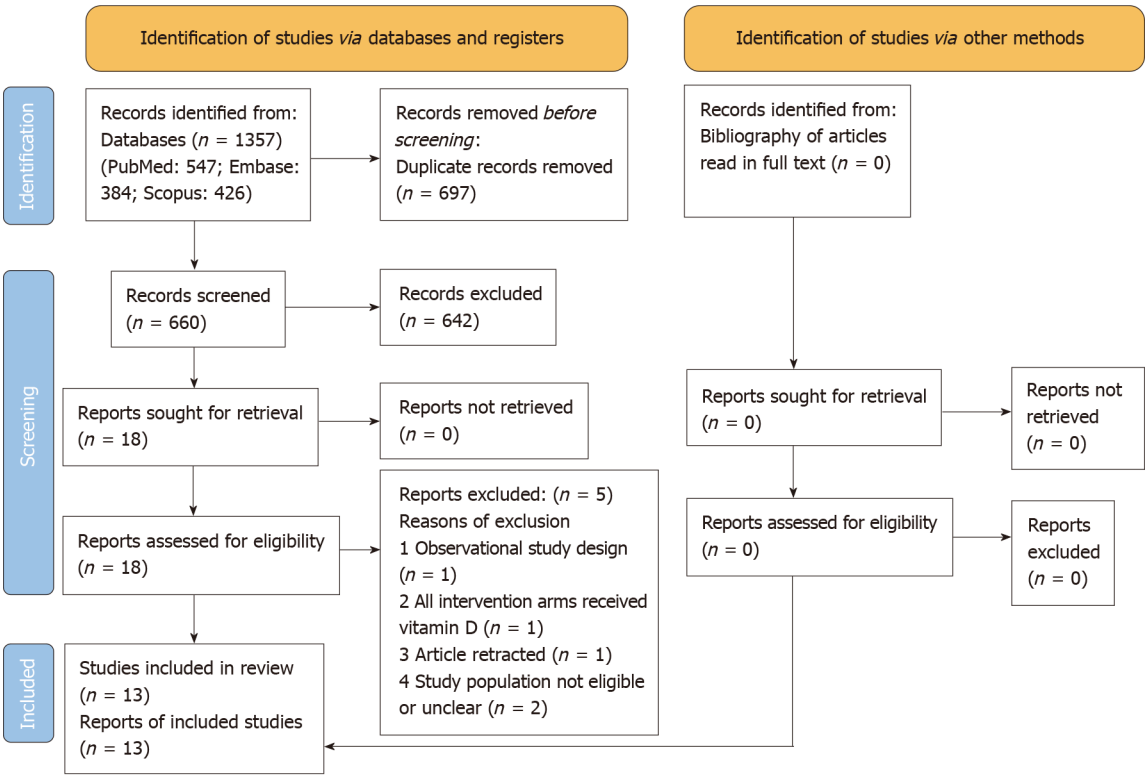

Figure 1 Preferred Reporting Items for Systematic Review and Meta-Analysis flow chart. Citation: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, Shamseer L, Tetzlaff JM, Akl EA, Brennan SE, Chou R, Glanville J, Grimshaw JM, Hróbjartsson A, Lalu MM, Li T, Loder EW, Mayo-Wilson E, McDonald S, McGuinness LA, Stewart LA, Thomas J, Tricco AC, Welch VA, Whiting P, Moher D. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021; 372: n71. For more information, visit: http://www.prisma-statement.org/.

Review authors' role

The review authors performed the database search, study selection, data abstraction, and RoB assessment independently and resolved any conflict in an opinion by discourse. A third-party opinion or contact with the trialists was not required.

Analysis prevalence meta-analysis

The overall prevalence of post-randomization participant attrition from the vitamin D-supplemented arms was estimated using random-effect (DerSimonian and Laird) prevalence meta-analysis (exact binomial method). Trials with zero numerators, when all participants followed up until the end of the trial period, did not get included in the analysis.

Pairwise meta-analysis

A random effect pairwise meta-analysis model (DerSimonian and Laird) contrasted the participant attrition risk between vitamin D recipients and non-recipients and determined the summary effect in the risk ratio (RR). When any cell of the 2 × 2 table had no event, 0.5 got added to all cells. Forest plots were used to present the results of prevalence and pairwise meta-analysis.

Statistical heterogeneity evaluation

Heterogeneity was determined using χ^2 statistics (statistical significance determined at $P < 0.1$) and was successively quantified using I^2 statistics (at values 25%, 50%, and 75% heterogeneity were classified as low, moderate, and high, respectively)[24].

Supplementary analysis (network meta-analysis)

A frequentist method network meta-analysis (NMA) ensued for each outcome to determine the relative efficacy across various supplements tested in the reviewed trials. For FPG, the weighted mean difference was estimated, and its values were included in mg/dL (FPG values in mmol/L got converted into mg/dL). A fixed-effect NMA ensued for categorical outcomes (effect size estimated in RR) due to the absence of freedom for heterogeneity in respective models. An augmentation method was used when these binomial outcomes had zero events.

Transitivity
The NMA models did not include open-label trials to minimize the intransitivity risk. Local and global inconsistency models were used to assess inconsistency.

Network map
Utilizing network maps, a visual conceptualization of the relationship across various nutritional supplements tested in the trials transpired for each outcome. The nodes represent the intervention types received, and it enlarges with the increase in sample size receiving these. The node connectors represent the trials testing the interventions represented by the nodes, and it thickens as the no of trials increases.

League tables and intervention ranking
The effect sizes and their corresponding confidence interval (CI) are presented in league tables. The diagonal cells of these tables represented the interventions compared. The surface under the cumulative ranking curve values got utilized to predict the best supplement for outcomes with statistically significant effect sizes.

Subgroup analysis
Subgroup analysis and meta-regression were not applicable, as the heterogeneity was not high in the prevalence and pairwise meta-analysis.

Publication bias
Small study effect assessment for the pairwise meta-analysis ensued using funnel plot and Egger's test. The RoB across studies included in the NMA models occurred by identifying any selective reporting that deviates from the pre-stated notions[25].

Sensitivity analysis
The prevalence and pairwise meta-analysis iteration happened by dropping a study (every time the analysis was repeated) and by a fixed-effect model, respectively.

Certainty assessment
For statistically significant meta-analysis results, the Grading of Recommendations Assessment, Development, and Evaluation approach[26] was used to determine the evidence quality.

Analytic tools
The metaprop, meta, and network packages of Stata statistical software (version 16) were used for the prevalence, pairwise, and network meta-analysis, respectively. The statistical significance was determined at $P < 0.05$ and 95%CI.

RESULTS

Scope of this review
The database search retrieved 1357 citations (PubMed: 547; Embase: 384; Scopus: 426) (Figure 1). The last date of the search was July 4, 2021. Five articles read in full text were excluded[18,27-30]. Additional searches did not produce new articles. The review included 13 publications with 1109 GDM patients' data from Iran[14-17,31-37] and China[13,38]. The salient features of these trials are presented in Table 1.

RoB evaluation
The trials were primarily at low RoB except one at high RoB (due to lack of blinding of study personnel and participants) (Table 2)[34].

Meta-analysis
Prevalence and pairwise meta-analysis: The pooled prevalence of participant attrition among vitamin D recipients was 6% (95%CI: 0.03, 0.10, I^2: 38.04%) (Figure 2), and its risk did not vary from non-vitamin D recipients (Figure 3). Although the funnel plot (Figure 4) appeared somewhat asymmetrical, Egger's test did not suggest any small study effect ($P = 0.6602$).

***NMA*:** Figure 5 depicts the network maps. The maps revealed a lack of direct comparison between any supplement and following nutrients co-supplemented with vitamin D- calcium or magnesium-zinc-calcium combination or evening prime rose oil. The global and local inconsistency tests for any of the outcomes were not suggestive of any inconsistency. The league tables are shown in Tables 3 and 4. Vitamin D (RR: 0.47; 95%CI: 0.27, 0.83) and its co-supplementation with probiotic (RR: 0.28; 95%CI: 0.09, 0.91), omega-3 fatty acids (RR: 0.25; 95%CI: 0.08, 0.77), and calcium (RR: 0.35; 95%CI: 0.16, 0.77)

Table 1 Salient features of the reviewed trials

Ref.	Design	Participants	Interventions	Outcomes
Jamilian *et al*[37], 2016	Randomized, double-blind, placebo-controlled clinical trial; Intervention arms: Two; Single-centered trialTrial duration: 6 wk. Trial conducted in: Iran; Obtained ethical clearance and participant consent. Funding information provided. Clinical trial registration number: IRCT201509115623N52	Participants diagnosed with GDM (used ADA criteria); 60 participants randomized into different treatment arms (vitamin D3 and evening primrose oil: n = 30, placebo: n = 30); Mean age of participants: -Vitamin D3 and evening primrose oil receiving group: 28.4 ± 6.2 yr; -Placebo receiving group: 29.6 ± 4.3 yr	Two intervention arms: (1) 1000 IU of vitamin D and 1000 mg of evening primrose oil daily for 6 wk; and (2) Placebo	Attrition from vitamin D supplemented arm: n = 3; Other outcomes reported: Fasting plasma glucose
Jamilian *et al*[17], 2017	Randomized, double blinded, placebo-controlled clinical trial; Intervention arms: four; Single centered trial; Trial duration: 6 wk. Trial conducted in: Iran; Obtained ethical clearance and participant consent. Funding information provided. Clinical trial registration number: IRCT201605135623N78	Participants diagnosed with GDM (used ADA criteria); 140 participants randomized into different treatment arms (vitamin D and omega-3 fatty acid receiving group: n = 35, vitamin D receiving arm: n = 35, omeag-3 fatty acid receiving arm: n = 35, placebo receiving arm: n = 35); Mean age of participants: -Vitamin D and omega-3 fatty acid receiving group: 31.2 ± 4.3 yr; -Vitamin D receiving group: 31.5 ± 7.0 yr; -Omega-3 receiving group: 30.7 ± 3.5 yr; -Placebo receiving group: 30.7 ± 4.1 yr	Four intervention arms: (1) Vitamin D and omega-3 fatty acid: 50000 IU of vitamin D two weekly and 1000 mg omega-3 fatty acid twice daily; (2) Vitamin D: 50000 IU vitamin D every 2 wk; (3) Omega-3 fatty acid: 1000 mg omega-3 fatty acids two times a day; and (4) Placebo	No attrition from vitamin D supplemented arm; Other outcomes reported: Fasting plasma glucose
Jamilian *et al*[33], 2019a	Randomized, double-blind, placebo-controlled; Intervention arms: 3; Trial conducted in: Iran; Single centered trial; Trial duration: 6 wk; Obtained ethical clearance and participant consent. Funding information provided. Trial ID: IRCT201706075623N119	Participants diagnosed with GDM (used ADA criteria); 90 participants randomized into different treatment arms (probiotic arm: n = 30, vitamin D and probiotic arm: n = 30, placebo arm: n = 30); Mean age of participants: -Probiotic arm: 31.2 ± 5.9 yr; -Vitamin D and probiotic arm: 28.9 ± 6.1 yr; -Placebo arm: 29.9 ± 3.7 yr	Three intervention arms: (1) Probiotic: 8 × 109 CFU/g; (2) Vitamin D3 (50,000 IU) every 2 wk plus 8 × 109 CFU/g probiotic; Placebo	No attrition from vitamin D supplemented arm; Other outcomes reported: (1) Newborn hyperbiliru-binemia; (2) Newborn hospitalization; (3) Macrosomia; and (4) Cesarean section. Fasting plasma glucose
Jamilian *et al*[36], 2019b	Randomized, double-blind, placebo-controlled. Intervention arms: 2; Trial conducted in: IranSingle centered trialTrial duration: 6 wkObtained ethical clearance and participant consent. Funding information provided.Trial ID: IRCT201704225623N109	Participants diagnosed with GDM (used ADA criteria); 60 participants randomized into different treatment arms (vitamin D-magnesium-zinc-calcium arm: n = 30, placebo arm: n = 30). Mean age of participants: -Vitamin D-magnesium-zinc-calcium arm: 27.7 ± 4.0 yr; -Placebo arm: 29.1 ± 4.1 yr	Two intervention arms: (1) Vitamin D (200 IU) along with 100 mg magnesium, 4 mg zinc, 400 mg calcium twice daily; and (2) Placebo	No attrition from vitamin D supplemented armOther outcomes reported: (1) Newborn hyperbiliru-binemia; (2) Newborn hospitalization; (3) Macrosomia; and (4) Cesarean section. Fasting plasma glucose
Asemi *et al* [31], 2014a	Randomized, double-blind, placebo-controlled trial. Intervention arms: 2; Trial conducted in: Iran; Single centered trial; Trial duration: 6 wk; Obtained ethical clearance and participant consent. Funding information provided.Trial ID: IRCT201305115623N7	Participants diagnosed with GDM (used ADA criteria); 50 participants randomized into different treatment arms (vitamin D arm: n = 25, placebo arm: n = 25). Mean age of participants: -Vitamin D arm: 31.1 ± 5.5 yr; -Placebo arm: 30.8 ± 6.2 yr	Two intervention arms: (1) Vitamin D: 50,000 IU vitamin D3 pearl two times during the trial period (at baseline and day 21); and (2) Placebo	Attrition from vitamin D supplemented arm: n = 3; Other outcomes reported: (1) Newborn hyperbiliru-binemia; (2) Newborn hospitalization; (3) Macrosomia; and (4) Cesarean section
Asemi *et al* [16], 2014b	Randomized, placebo-controlled clinical trial. Intervention arms: TwoMulti-centric trial. Trial duration: 6 wk. Trial conducted in: IranOb-tained ethical clearance and participant consent. Funding information provided. Clinical trial registration number: IRCT201311205623N11	Participants diagnosed with GDM (used ADA criteria); 56 participants randomized into different treatment arms (vitamin D and calcium: n = 28, placebo receiving group: n = 28). Mean age of participants: -Vitamin D and calcium receiving arm: 28.7 ± 6.0 yr; -Placebo receiving arm: 30.8 ± 6.6 yr	Two intervention arms: (1) 1000 mg calcium carbonate daily and 50000 U vitamin D3 at the baseline and day 21 of the study; and (2) Placebo	Attrition from vitamin D supplemented arm: n = 3. Other outcomes reported: Fasting plasma glucose
Karamali *et al*[32], 2016	Randomized, double-blind, placebo-controlled trial; Intervention arms: 2; Trial conducted in: Iran; Multicentric trialTrial duration: 6 wk; Obtained ethical clearance and participant consent. Funding information provided. Trial ID: IRCT201407115623N23	Participants diagnosed with GDM (used ADA criteria); 60 participants randomized into different treatment arms (vitamin D and calcium arm: n = 30; placebo arm: n = 30). Mean age of participants: -Vitamin D and calcium arm: 28·7 ± 6·1 yr; -Placebo arm: 31·6 ± 6·3 yr	Two intervention arms: (1) Vitamin D3 (50000 IU) at baseline and day 21 along with 1000 mg calcium carbonate daily; and (2) Placebo	No attrition from vitamin D supplemented arm; Other outcomes reported: (1) Newborn hyperbiliru-binemia; (2) Newborn hospitalization; (3) Macrosomia; and (4) Cesarean section
Karamali *et al*[14], 2018	Randomized, double-blind, placebo-controlled trial; Intervention arms: 2; Single centered trial. Trial duration: 6 wk; Trial conducted in: Iran; Obtained ethical clearance	Participants diagnosed with GDM (used ADA criteria); 60 participants randomized into different treatment arms; (Magnesium, zinc, calcium and vitamin D supplements arm: n = 30;	Two intervention arms: (1) 100 mg magnesium, 4 mg zinc, 400 mg calcium and 200 IU vitamin D two times a day for 6 wk; and (2) Placebo	No attrition from vitamin D supplemented arm; Other outcomes reported: Fasting plasma glucose

	(participant consent information unclear). Funding information provided. Trial registration details: Unclear	Placebo arm: *n* = 30); Mean age of participants: -Magnesium, zinc, calcium and vitamin D: 30.0 ± 4.5 yr; -Placebo arm: 31.1 ± 4.2 yr		
Razavi *et al*[35], 2017	Randomized, double-blind, placebo-controlled, Intervention arms: 4; Trial conducted in: Iran. Single centered trial. Trial duration: 6 wk; Obtained ethical clearance and participant consent. Funding information provided. Trial ID: IRCT201701305623N106	Participants diagnosed with GDM (used ADA criteria); 120 participants randomized into different treatment arms (vitamin D and omega-3 arm: *n* = 30; omega-3 arm: *n* = 30; vitamin D arm: *n* = 30; placebo: *n* = 30); Mean age of participants: -Vitamin D and omega-3 arm: 29.9 ± 4.0 yr; -Omega-3 arm: 29.7 ± 3.6 yr; -Vitamin D arm: 29.9 ± 5.0 yr; -Placebo: 29.2 ± 3.4 yr	Four intervention arms: (1) Vitamin D (50000 IU): Two weekly two times a day; (2) Vitamin D (50000 IU) two weekly plus 1000 mg omega-3 fatty acids two times a day; (3) 1000 mg omega-3 fatty acids two times a day; and (4) Placebo	No attrition from vitamin D supplemented arm; Other outcomes reported: (1) Newborn hyperbilirubinemia; (2) Newborn hospitalization; (3) Macrosomia; and (4) Cesarean section
Valizadeh *et al*[34], 2016	Randomized controlled trial. Investigators and patients were not blinded. Intervention arms: 2; Single centered trial; Trial conducted in: Iran; Trial duration: Until delivery; Obtained ethical clearance and participant consent. Funding information provided. Trial ID: IRCT2012101611144N1	Participants diagnosed with GDM (used ADA criteria); 96 participants randomized into different treatment arms (vitamin D arm: *n* = 48; no supplement arm: *n* = 48); Mean age of participants: -Vitamin D arm: 32.0 ± 5.5 yr; -No supplement arm: 32.4 ± 4.7 yr	Two intervention arms: (1) 700000 IU vitamin D3 in total (regimen differed by gestational age of GDM patients); and (2) Comparison group did not receive any supplementation	Attrition from vitamin D supplemented arm: *n* = 4; Other outcomes reported: (1) Newborn hyperbilirubinemia; (2) Macrosomia; (3) Cesarean section; and (4) Fasting plasma glucose
Yazdchi *et al*[15], 2016	Randomized, double-blinded placebo-controlled clinical trial; Intervention arms: 2; Multi-center trial; Trial duration: 8 wk. Trial conducted in: Iran; Obtained ethical clearance and participant consent. Funding information provided. Clinical trial registration number: IRCT201306253140N11	Participants diagnosed with GDM (used International Association of Diabetes and Pregnancy Study Groups criteria); 76 participants randomized into different treatment arms: Vitamin D arm: *n* = 38; placebo arm: *n* = 38; Mean age of participants: -Vitamin D arm: 31.64 ± 4.40 yr; -Placebo arm: 32.11 ± 3.61 yr	Two intervention arms:(1) 50000 IU vitamin D3 oral capsules two weekly for 8 wk; and (2) Placebo	Attrition from vitamin D supplemented arm: *n* = 4; Other outcomes reported: Fasting plasma glucose
Zhang *et al*[38], 2016	Randomized, double-blind, placebo-controlled trial. Intervention arms: 4; Single centered trial. Trial duration: 24-28 wk of pregnancy to delivery; Trial conducted in: China; Obtained ethical clearance and participant consent. Funding information provided. Clinical trial registration details: Unclear	Participants diagnosed with GDM (criteria unclear). 133 participants randomized into different treatment arms (low dose vitamin D: *n* = 38; medium dose vitamin D: *n* = 38; high dose vitamin D: *n* = 37; placebo: *n* = 23); Mean age of participants: -Placebo arm: 29.8 ± 4.7; -Low dose vitamin D arm: 30.3 ± 5.1; -Medium dose vitamin D arm: 29.4 ± 4.9; -High dose vitamin D arm: 30.1 ± 4.5	Four intervention arms: (1) Low dose vitamin D: 200 IU daily; (2) Medium dose vitamin D: 2000 IU monthly; and (3) High dose vitamin D: 50000 IU every 2 wk. Placebo	Attrition from vitamin D supplemented arm: *n* = 4
Li and Xing[13], 2016	Randomized, double-blinded clinical trial. Intervention arms: 2. Multi-centric trial. Trial duration: 16 wk. Trial conducted in: China; Obtained ethical clearance and participant consent. Funding information provided. Clinical trial registration details: Unclear	Participants diagnosed with GDM (used ADA criteria)103 participants randomized into different treatment arms (yoghurt with vitamin D: *n* = 52, plain yoghurt: *n* = 51); Mean age of participants: -Yoghurt supplemented with vitamin D receiving arm: 29.0 ± 5.3 yr; -Plain yoghurt arm: 28.3 ± 4.1 yr	Two intervention arms: (1) Yoghurt was supplemented with 500 IU of vitamin D3 twice daily for 16 wk; and (2) plain yoghurt: Twice daily for 16 wk	Attrition from vitamin D supplemented arm: *n* = 4. Other outcomes reported: Fasting plasma glucose

ADA: American diabetes association.

decreased the risk of newborn hyperbilirubinemia. Vitamin D (RR: 0.47; 95%CI: 0.27, 0.83) and its co-supplementation with omega-3 fatty acids (RR: 0.25; 95%CI: 0.08, 0.77) and calcium (RR: 0.35; 95%CI: 0.16, 0.77) reduced the risk of newborn hospitalization. The incidence of CS in GDM patients was lower with vitamin D and calcium co-supplementation (RR: 0.37; 95%CI: 0.18, 0.74). Vitamin D and omega-3 fatty acid co-supplementation in GDM patients decreased the risk of hyperbilirubinemia (RR: 0.30; 95%CI: 0.09, 0.98) and hospitalization (RR: 0.30; 95%CI: 0.09, 0.98) in their newborns compared to omega-3 supplementation alone.

The surface under the cumulative ranking curve values suggested vitamin D and calcium co-supplementation in GDM patients as the best supplement for reducing the CS requirement, and vitamin D and omega-3 fatty acid co-supplementation as the best supplement for reducing the risk of hospitalization and hyperbilirubinemia in their newborns (Table 5). The macrosomia risk and FPG levels (league table not shown) did not vary among the interventions.

***RoB* across studies:** Evaluation of *RoB* across studies suggests that the trials primarily adhered to their pre-stated analytic notions.

Table 2 Risk of bias assessment of respective trial included in the review[23]

Ref.	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias); All outcomes	Blinding of outcome assessment (detection bias); All outcomes	Incomplete outcome data (attrition bias); All outcomes	Selective reporting (reporting bias)	Other bias
Jamilian *et al* [37], 2016	Low	Unclear; Comment: Precise mechanism unclear	Unclear; Comment: Precise mechanism unclear	Low	Low	Low	Low
Jamilian *et al* [17], 2017	Low	Unclear; Comment: Precise mechanism unclear	Unclear; Comment: Precise mechanism unclear	Unclear	Low	Low	Low
Jamilian *et al* [33], 2019a	Low risk	Unclear risk; Comment: Precise mechanism unclear	Low risk	Low risk	Low risk	Low risk	Low risk
Jamilian *et al* [36], 2019b	Low risk	Unclear risk; Comment: Precise mechanism unclear	Low risk	Low risk	Low risk	Low risk	Low risk
Asemi *et al* [31], 2014a	Low risk	Unclear risk; Comment: Precise mechanism unclear	Low risk	Low risk	Low risk	Low risk	Low risk
Asemi *et al* [16], 2014b	Low	Low	Low	Low	Low	Low	Low
Karamali *et al* [32], 2016	Low risk	Unclear risk; Comment: Precise mechanism unclear	Low risk	Low risk	Low risk	Low risk	Low risk
Karamali *et al* [14], 2018	Low	Unclear; Comment: Precise mechanism unclear	Unclear; Comment: Precise mechanism unclear	Low	Low	Low	Low
Razavi *et al* [35], 2017	Low risk	Unclear risk; Comment: It's unclear if the bottles were sequentially numbered and identical in appearance	Low risk	Low risk	Low risk	Low risk	Low risk
Valizadeh *et al* [34], 2016	Low risk	Unclear risk	High risk; Comment: Both investigators and participants were not blinded		Low risk	Low risk	Low risk
Yazdchi *et al* [15], 2016	Low	Unclear	Unclear	Low	Low	Low	Low
Zhang *et al* [38], 2016	Low	Unclear; Comment: Precise mechanism unclear	Unclear; Comment: Precise mechanism unclear	Unclear; Comment: Precise mechanism unclear	Low	Low	Low
Li and Xing [13], 2016	Low	Unclear; Comment: Precise mechanism unclear	Unclear; Comment: Precise mechanism unclear	Low	Low	Low	Low

Sensitivity analysis: On repeating the prevalence meta-analysis by dropping one study each time, the prevalence ranged between 5% and 8%. The pairwise meta-analysis findings were identical to the preliminary model when a fixed-effect model-based iteration occurred.

Table 3 League table. Outcomes: cesarean section (left lower triangle) and newborn hyperbilirubinemia (right upper triangle). Interventions of interest: represented in diagonal cells

Interventions and effect sizes							
Vitamin D and probiotic	1.14 (0.22, 5.92)[1]	0.79 (0.19, 3.27)	0.59 (0.16, 2.20)	0.73 (0.18, 2.96)	**0.28 (0.09, 0.91)**[2]	0.34 (0.09, 1.32)	0.42 (0.10, 1.69)
0.76 (0.27, 2.19)	Vitamin D and omega-3 fatty acid	0.70 (0.17, 2.79)	0.52 (0.16, 1.75)	0.64 (0.14, 2.99)	**0.25 (0.08, 0.77)**	**0.30 (0.09, 0.98)**	0.37 (0.09, 1.44)
1.48 (0.52, 4.21)	1.94 (0.71, 5.28)	Vitamin D and calcium	0.75 (0.29, 1.96)	0.91 (0.25, 3.35)	**0.35 (0.16, 0.77)**	0.43 (0.16, 1.19)	0.53 (0.18, 1.55)
0.69 (0.29, 1.68)	0.91 (0.44, 1.90)	0.47 (0.21, 1.07)	Vitamin D	1.22 (0.37, 3.96)	**0.47 (0.27, 0.83)**	0.57 (0.27, 1.22)	0.71 (0.28, 1.78)
0.68 (0.30, 1.54)	0.89 (0.34, 2.35)	0.46 (0.17, 1.20)	0.97 (0.45, 2.13)	Probiotic	0.39 (0.14, 1.09)	0.47 (0.14, 1.60)	0.58 (0.16, 2.07)
0.54 (0.25, 1.18)	0.71 (0.35, 1.46)	**0.37 (0.18, 0.74)**	0.78 (0.52, 1.19)	0.80 (0.42, 1.56)	Placebo	1.22 (0.64, 2.33)	1.50 (0.72, 3.14)
0.68 (0.24, 1.90)	0.89 (0.40, 1.99)	0.46 (0.17, 1.22)	0.98 (0.49, 1.96)	1.00 (0.39, 2.58)	1.24 (0.63, 2.45)	Omega-3 fatty acid	1.23 (0.46, 3.28)
1.23 (0.33, 4.57)	1.61 (0.45, 5.79)	0.83 (0.23, 2.97)	1.76 (0.56, 5.53)	1.81 (0.52, 6.33)	2.25 (0.78, 6.52)	1.81 (0.51, 6.37)	Magnesium, zinc, calcium, and vitamin D

[1]Effect sizes in risk ratio with its 95% confidence interval in parenthesis.
[2]Cells with bold-faced values depict a statistically significant decrease in effect size.
In the right upper and the left lower triangle, the columns and rows depict the reference treatment, respectively.

Table 4 League table: Outcomes: Macrosomia (left lower triangle) and newborn hospitalization (right upper triangle). Interventions of interest: Represented in diagonal cells

Interventions and effect sizes							
Vitamin D and probiotic	1.27 (0.24, 6.66)[1]	0.88 (0.21, 3.69)	0.66 (0.18, 2.49)	0.97 (0.21, 4.41)	0.31 (0.09, 1.03)	0.38 (0.10, 1.49)	0.47 (0.11, 1.91)
0.94 (0.11, 8.45)	Vitamin D and omega-3 fatty acid	0.70 (0.17, 2.79)	0.52 (0.16, 1.75)	0.76 (0.15, 4.02)	**0.25 (0.08, 0.77)**[2]	**0.30 (0.09, 0.98)**	0.37 (0.09, 1.44)
3.36 (0.13, 88.67)	3.56 (0.14, 93.17)	Vitamin D and calcium	0.75 (0.29, 1.96)	1.10 (0.26, 4.59)	**0.35 (0.16, 0.77)**	0.43 (0.16, 1.19)	0.53 (0.18, 1.55)
0.98 (0.13, 7.29)	1.03 (0.18, 6.09)	0.29 (0.01, 6.77)	Vitamin D	1.46 (0.39, 5.50)	**0.47 (0.27, 0.83)**	0.57 (0.27, 1.22)	0.71 (0.28, 1.78)
1.93 (0.19, 20.18)	2.05 (0.15, 27.32)	0.58 (0.02, 20.11)	1.98 (0.17, 22.68)	Probiotic	0.32 (0.10, 1.07)	0.39 (0.10, 1.53)	0.48 (0.12, 1.97)
0.37 (0.08, 1.77)	0.40 (0.08, 1.85)	0.11 (0.01, 1.98)	0.38 (0.11, 1.36)	0.19 (0.02, 1.55)	Placebo	1.22 (0.64, 2.33)	1.50 (0.72, 3.14)
0.63 (0.08, 4.84)	0.67 (0.12, 3.71)	0.19 (0.01, 4.45)	0.64 (0.13, 3.13)	0.33 (0.03, 3.83)	1.69 (0.45, 6.30)	Omega-3 fatty acid	1.23 (0.46, 3.28)
1.87 (0.14, 25.22)	1.98 (0.15, 26.45)	0.56 (0.02, 19.45)	1.91 (0.17, 21.96)	0.97 (0.05, 18.42)	5.00 (0.62, 40.28)	2.96 (0.25, 34.96)	Magnesium, zinc, calcium, and vitamin D

[1]Effect sizes in risk ratio with its 95% confidence interval in parenthesis.
[2]Cells with bold-faced values depict a statistically significant decrease in effect size.
In the right upper and the left lower triangle, the columns and rows depict the reference treatment, respectively.

DISCUSSION

Overall, this review included 13 publications sourcing data from 1109 GDM patients from Iran and China. The RoB across the trials was primarily low except for one with a high RoB component. The burden of attrition of GDM patients from the vitamin D supplemented arms post-randomization was 6%, and this risk did not vary from GDM patients who did not receive the supplement. Vitamin D and calcium co-supplementation benefited the GDM patients (decreased the CS incidence) and their neonates (decreased hyperbilirubinemia and hospitalization risk). Vitamin D alone and its omega-3 fatty

Table 5 The surface under the cumulative ranking curve values. Outcomes: Newborn hyperbilirubinemia, newborn hospitalization, and cesarean section

Intervention	Outcomes					
	Newborn hyperbilirubinemia		Newborn hospitalization		Cesarean section	
	SUCRA	Mean rank	SUCRA	Mean rank	SUCRA	Mean Rank
Vitamin D and omega-3 fatty acid	81.8	2.3[1]	81.1	2.3[1]	46.4	4.8
Vitamin D and probiotic	76.2	2.7	70.7	3.0	66.3	3.4
Probiotic	62.2	3.6	69.5	3.1	36.6	5.4
Vitamin D and calcium	67.9	3.3	67.2	3.3	87.6	1.9[1]
Vitamin D	52.8	4.3	52.4	4.3	39.0	5.3
Magnesium, zinc, calcium, and vitamin D	32.4	5.7	32.2	5.7	73.8	2.8

[1]The best rank corresponding to the highest SUCRA value. SUCRA: Surface under the cumulative ranking curve.

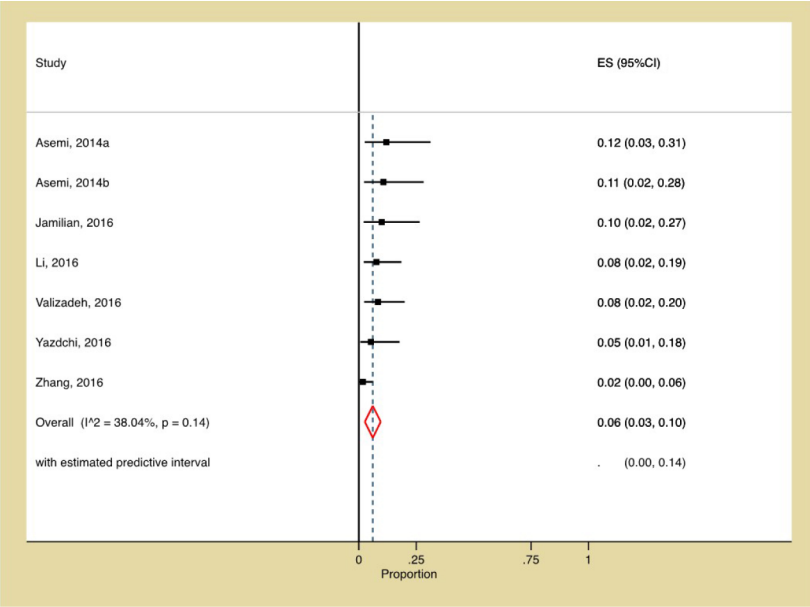

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Figure 2 Forest plot showing the overall weighted prevalence of post-randomization participant attrition from vitamin D supplementation trials in gestational diabetes mellitus patients. The diamond centers on the summary of the prevalence estimate, and the width indicates the corresponding 95% confidence interval. Articles with identical author names and years are suffixed with alphabets: Asemi *et al*[31], 2014, Asemi *et al*[16], 2014. CI: Confidence interval; ES: Effect size.

acid added form both reduced the newborn's risk of hyperbilirubinemia and hospitalization. For these outcomes, co-supplementation of vitamin D and omega-3 fatty acids was superior to omega-3 fatty acids alone. Combining vitamin D with probiotics was effective in reducing the risk of newborn hyperbilirubinemia.

Quality of evidence

Using the Grading of Recommendations Assessment, Development, and Evaluation approach[26], the NMA-generated evidence was double downgraded to low quality. This decision stood on the fact that the statistically significant findings were unlikely to be generalizable as study participants were mostly from Iran; thus, a fixed-effect model NMA was used for the categorical outcomes, and the trials had few unclear RoB components.

Comparison with existing literature

Regarding the prevalence of participant attrition, to the best of our knowledge, no literature is available to contrast with the findings of this review, perhaps due to its conceptual novelty. Concerning the perinatal outcomes, existing reviews suggested that vitamin D supplementation decreases the risk of

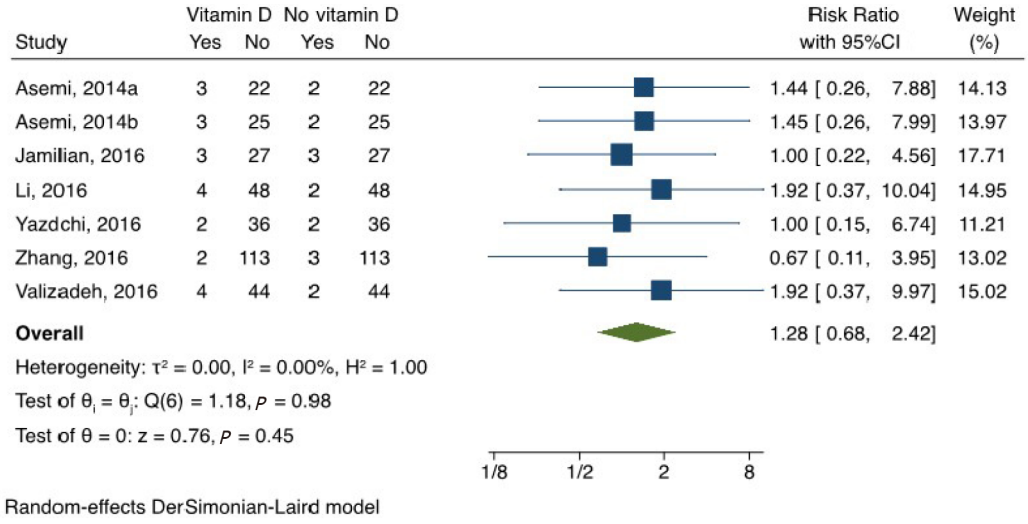

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Figure 3 Forest plot (pairwise meta-analysis; random-effect model) comparing missing outcome data between vitamin D recipients and non-recipients. Articles with identical author names and years are suffixed with alphabets: Asemi, 2014a[31], Asemi, 2014b[16].

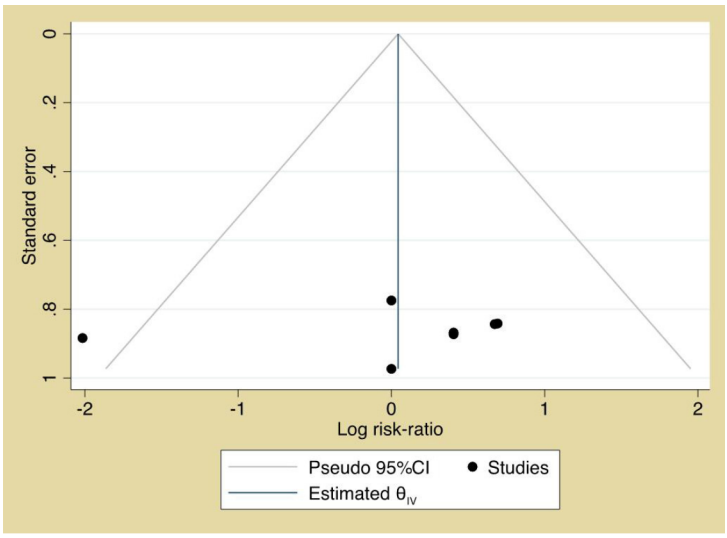

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Figure 4 Funnel plot for pairwise meta-analysis. Outcome: Post-randomization participant attrition from vitamin D-supplemented treatment arm/s.

CS, macrosomia, neonatal hyperbilirubinemia, and newborn hospitalization[8,39]. However, unlike this paper's findings, these reviews[8,39] did not sort out how perinatal outcomes vary across vitamin D, its co-supplemented forms, and other (non-vitamin D) supplements tested in these trials.

Strengths and weaknesses

The key strength of this review is its incorporation of RCTs only, the highest level of epidemiological evidence. The intransitivity risk in the NMA models is perhaps low due to the exclusion of the trial at a high RoB component. Furthermore, beyond reviewing post-randomization GDM patients' attrition burden from vitamin D-supplemented trial arm/s and its risk, this is plausibly the first study that attempted to distinguish the efficacy between vitamin D and its co-supplemented forms in GDM patients.

Despite these strengths, this study also had a few limitations. This review could not incorporate non-English language publications (if any) as the review authors are competent in handling publications in the English language only. The anticipated generalizability of the evidence generated in this study was low due to the homogenous nature of the study population. Although the prevalence meta-analysis estimate appeared weak due to its inclusion of a trial with a high RoB component, the sensitivity analysis did not observe any fluctuation upon excluding the trial from the model.

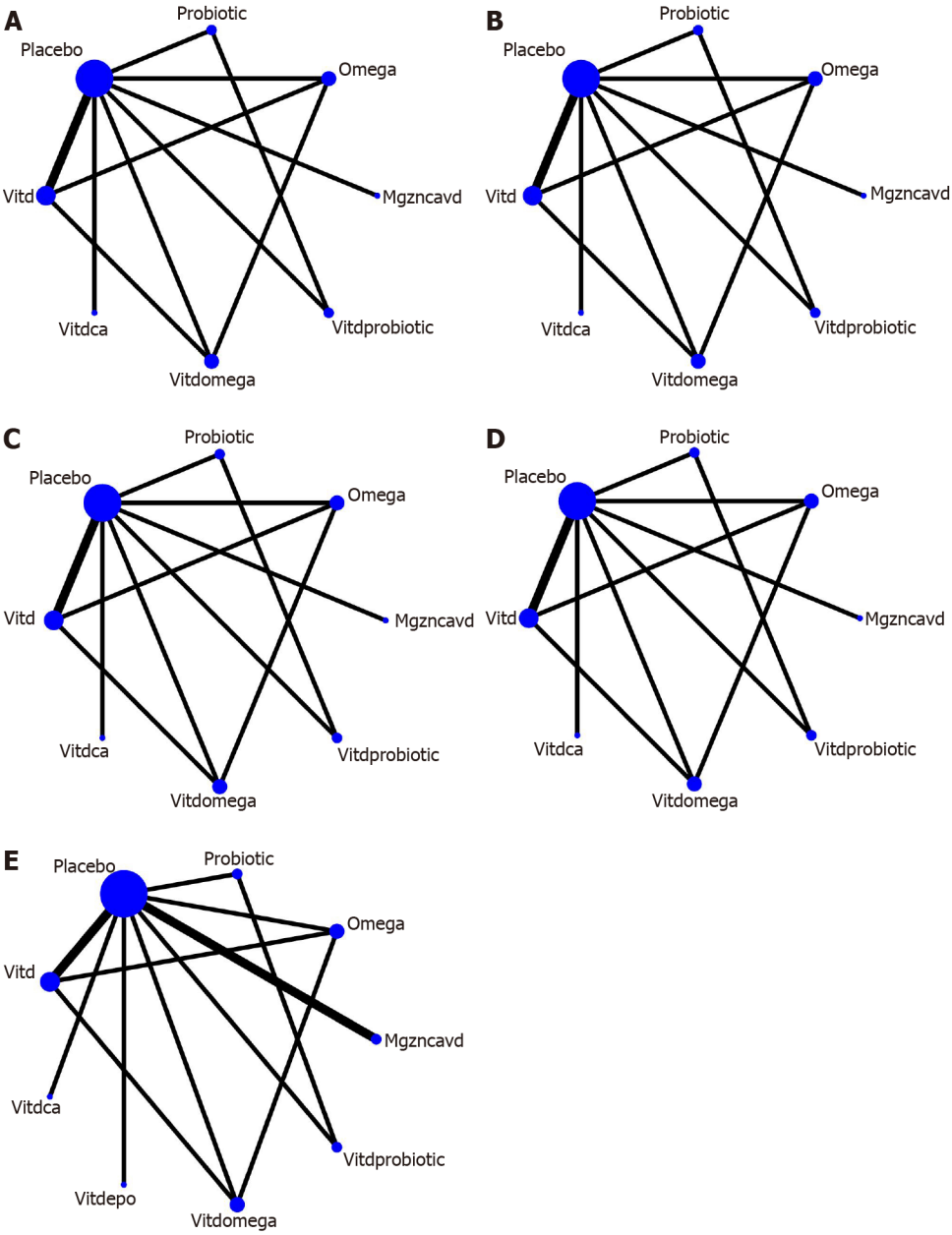

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Figure 5 Network map. A: Outcome: Newborn hyperbilirubinemia; B: Outcome: Newborn hospitalization; C: Outcome: Macrosomia; D: Outcome: Cesarean section; E: Outcome: Fasting plasma glucose. Interventions in the model: Placebo, probiotic, omega-3 fatty acids (omega), magnesium-zinc-calcium and vitamin D (mgzncavd), vitamin D and probiotic (vitdprobiotic), vitamin D and omega-3 fatty acids (vitdomega), vitamin D and calcium (vitdca), and vitamin D (vitd).

Implications

The low prevalence of post-randomization attrition of GDM patients from the vitamin D-supplemented intervention arms in RCTs suggests good adherence to the supplement and might encourage trialists across the globe to conduct identical efficacy trials. Given the substantial burden of vitamin D deficiency and insufficiency in Iranian pregnant females[40] (and most trials included in this review were from Iran), from a public health point of view, this study's findings might help the local health authority in reviewing the scope of routine prenatal supplementation of vitamin D and its co-supplemented forms with calcium, omega-3 fatty acids, and probiotics in GDM patients.

CONCLUSION

In RCTs testing the efficacy of vitamin D supplementation, the post-randomization attrition burden in vitamin D-supplemented GDM patients was low. Prenatal vitamin D and its co-supplemented form with calcium, omega-3 fatty acids, and probiotics each can curb certain perinatal complications' risks in

GDM patients and their neonates.

ARTICLE HIGHLIGHTS

Research background

The role of vitamin D in gestational diabetes mellitus (GDM) is not established. Several randomized controlled trials (RCT) have tested it.

Research motivation

The burden and risk of participant attrition from vitamin D receiving treatment arm/s of these trials are unclear. Also, the effect of vitamin D and its co-supplemented forms and other supplements on the mother's glycemic control and perinatal outcomes remains unclear.

Research objectives

This study aimed to address these issues.

Research methods

Eligible clinical trials were retrieved by searching the PubMed, Embase, and Scopus databases. The burden and risk of participant attrition got determined by random-effect prevalence and pairwise meta-analysis, respectively. The effect of different nutritional supplements on the perinatal outcomes got estimated by fixed-effect network meta-analysis. All analysis ensued in Stata statistical software (v16).

Research results

The database search produced 13 RCTs conducted in Iran and China. The participant attrition from vitamin D treated arms was 6% (95% confidence interval [CI]: 0.03, 0.10), and this risk did not vary from its non-recipient arms. The cesarean section risk decreased with the combined supplementation of vitamin D and calcium [risk ratio (RR): 0.37; 95%CI: 0.18, 0.74]. The vitamin D alone and its co-supplemented forms with calcium and omega-3 fatty acids decreased the risk of newborn- hyperbilirubinemia or hospitalization. The probiotics co-supplemented form of vitamin D decreased newborn hyperbilirubinemia risk (RR: 0.28; 95%CI: 0.09, 0.91). The fasting plasma glucose levels didn't vary across the compared interventions.

Research conclusions

This study suggests that vitamin D supplementation is a relatively well-tolerated intervention in GDM patients resulting in relatively low participant attrition from RCTs testing it. Also, this study suggests that some nutritional supplements can be beneficial in reducing perinatal outcomes.

Research perspectives

Given the low burden of participant attrition from the vitamin-supplemented arms of RCTs, future trialists may find the conduct of RCTs with a larger sample size reasonable to produce rigorous results.

FOOTNOTES

Author contributions: Sumanta Saha conceptualized, designed, analyzed, and drafted the first and final versions of the manuscript; all authors contributed to the study selection, data abstraction, and risk of bias assessment of this manuscript; all authors have read and approved the final manuscript.

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META-ANALYSIS

Global prevalence of occult hepatitis C virus: A systematic review and meta-analysis

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Abstract

BACKGROUND

Occult hepatitis C infection (OCI) is characterized by the presence of hepatitis C virus (HCV) RNA in the liver, peripheral blood mononuclear cells (PBMC) and/or ultracentrifuged serum in the absence of detectable HCV-RNA in serum. OCI has been described in several categories of populations including hemodialysis patients, patients with a sustained virological response, immunocompromised individuals, patients with abnormal hepatic function, and apparently healthy subjects.

AIM

To highlight the global prevalence of OCI.

METHODS

We performed a systematic and comprehensive literature search in the following 4 electronic databases PubMed, EMBASE, Global Index Medicus, and Web of Science up to 6th May 2021 to retrieve relevant studies published in the field. Included studies were unrestricted population categories with known RNA status in serum, PBMC, liver tissue and/or ultracentrifuged serum. Data were extracted independently by each author and the Hoy *et al* tool was used to assess the quality of the included studies. We used the random-effect meta-analysis model to estimate the proportions of OCI and their 95% confidence intervals (95%CI). The Cochran's *Q*-test and the I^2 test statistics were used to assess heterogeneity between studies. Funnel plot and Egger test were used to examine publication bias. R software version 4.1.0 was used for all analyses.

RESULTS

The electronic search resulted in 3950 articles. We obtained 102 prevalence data from 85 included studies. The pooled prevalence of seronegative OCI was estimated to be 9.61% (95%CI: 6.84-12.73) with substantial heterogeneity [I^2 = 94.7% (95%CI: 93.8%-95.4%), P < 0.0001]. Seropositive OCI prevalence was estimated to be 13.39% (95%CI: 7.85-19.99) with substantial heterogeneity [I^2 = 93.0% (90.8%-94.7%)]. Higher seronegative OCI prevalence was found in Southern Europe and Northern Africa, and in patients with abnormal liver function, hematological disorders, and kidney diseases. Higher seropositive OCI prevalence was found in Southern Europe, Northern America, and Northern Africa.

CONCLUSION

In conclusion, in the present study, it appears that the burden of OCI is high and variable across the different regions and population categories. Further studies on OCI are needed to assess the transmissibility, clinical significance, long-term outcome, and need for treatment.

Key Words: Occult hepatitis C virus infection; Prevalence; Worldwide; Peripheral blood mononuclear cells; Hepatitis C virus

Core Tip: This study showed that the burden of seropositive and seronegative occult hepatitis C infections (OCIs) is high and variable in different regions and population categories. Patients with hematological disorders, kidney diseases, and abnormal liver function showed the highest OCI prevalence.

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INTRODUCTION

In 2019, the World Health Organization (WHO) estimated that 58 million people are living with hepatitis C virus (HCV)[1], making HCV infection a major global public health problem[2,3]. Each year, more than 1.5 million people around the world are newly infected with HCV[4] and more than 290000 people die from it[5]. HCV infection is increasingly affecting healthcare particularly in highly endemic areas[3]. The prevalence of HCV varies greatly between regions and ranges from 0.2% to 20% in the general population[6]. HCV infection can lead to liver cirrhosis (10%-20% of cases) and hepatocellular carcinoma (HCC) (1%-5% of cases)[7].

The principal multiplication site for HCV is hepatocytes, but evidence of HCV replication has been reported in peripheral blood mononuclear cells (PBMC) and other extrahepatic organs[8,9].

Occult hepatitis C infection (OCI) was first described by Castillo *et al*[10] in 2004. This new form of hepatitis is defined as the absence of RNA in serum and its presence in hepatocytes, PBMC or ultracentrifuged serum[3,11-13]. OCI is further classified as seronegative OCI in subjects who are anti-HCV negative and seropositive OCI in those who are anti-HCV positive[14]. Seropositive OCI individuals represent those chronically infected with HCV who have recovered (absence of RNA in serum) either spontaneously or following treatment. There are asymptomatic carriers of OCI with normal liver enzyme levels and some with abnormal liver function[10,15-23]. OCI can also lead to hepatic attacks including cases of liver cirrhosis and even HCC in high-risk groups[24]. The first syntheses performed at the global level and in the Middle East and the Eastern Mediterranean showed highly variable OCI prevalence (ranging from 0%-89%) according to the population groups including apparently healthy individuals, patients with hematological disorders, chronic liver disease, HIV, patients who have achieved a sustained virological response (SVR), and transplant recipients[20,25,26]. The review conducted in the Middle East and Eastern Mediterranean revealed that high frequencies of OCI were recorded in patients with chronic liver disease, HIV, and injecting drug users[20]. In the review by Hedayati-Moghaddam *et al*[20], no statistically significant difference was observed in the variability of OCI prevalence across countries, patient anti-HCV status, and HCV detection method. OCI highlights multiple concerns including the potential for transmission of this form of infection through blood transfusion or hemodialysis[27]. To date, there is no global data synthesis on the prevalence of OCI in different population categories. To eradicate HCV infection by 2030 as recommended by WHO, making data available on the burden of OCI is crucial[28,29]. The objective of this systematic review and meta-analysis is to determine the global prevalence of OCI and evaluate the potential factors resulting in heterogeneity between the population groups and regions. Findings from this review may help prioritize population groups and regions most at risk for OCI screening and managing programs.

MATERIALS AND METHODS

Study design

We used the preferred reporting items for systematic reviews and meta–analyses (PRISMA) checklist to design this systematic review (Supplementary Table 1)[30]. The systematic review was declared in the PROSPERO international database under the number CRD42021252763.

Inclusion criteria

We included all studies without time restriction, published in peer-reviewed journals in English or in French and which fulfilled the following criteria: having a cross-sectional or case-control study design and for cohorts and clinical trials, only the baseline data were considered. We considered studies with patients of all ages tested for seropositive OCI (anti-HCV positive) and for seronegative OCI (anti-HCV negative). One study could contribute to several prevalence data that we called effect ratings. We included studies that detected HCV RNA by molecular methods in PBMCs, hepatocytes or ultracentrifuged serum[11,15,20,31]. To strengthen the robustness of our estimates, we considered only studies with at least 10 participants.

Exclusion criteria

We excluded all studies that did not provide an opportunity to extract data on OCI prevalence, and studies with no baseline data for longitudinal study. Case reports, studies selecting participants with an already known OCI result, comments on an article, reviews, editorials, duplicates and studies for which the full article or abstract could not be found were also excluded.

Search strategy

We performed a systematic and comprehensive literature search in 4 electronic databases: PubMed, EMBASE, Global Index Medicus, and Web of Science from inception until 6th May 2021 to retrieve relevant studies published in the field. The electronic search strategy conducted in PubMed covered the key words of OCI (Occult Hepatitis C OR Occult Viral hepatitis C OR Occult Hepatitis C Virus OR Occult HCV) and was adapted to other databases. We also manually searched all included studies and previous systematic reviews on the topic to identify additional references. The references cited in this article were checked in the Reference Citation Analysis website (https://www.referencecitation-analysis.com/).

Study selection

The duplicate articles found in the databases were removed using EndNote software. Two investigators (JTEB and SK) independently selected articles on the basis of title and abstract using Rayyan review platform. The full texts of selected articles were then read by 22 authors on the basis of the eligibility criteria. Disagreements were resolved through discussion and consensus.

Data extraction

Data were extracted independently by each author *via* the Google Forms for articles that met the inclusion criteria. The data extracted were as follows; the name of the first author, the date of publication, the period of recruitment of the participants, the design of the study, the sampling method, the number of study sites, the time of collection of the data, country, United Nations Statistics Division (UNSD) region, type of population studied, patient demographic details such as gender, age, and location of recruitment, OCI type (seronegative or seropositive), risk of bias assessment, detection test, target detected, type of sample used, number of samples tested, and number of samples positive for OCI. All disagreements regarding eligibility and data collected were resolved by discussion and consensus.

Appraisal of the methodological quality of the included studies and risk of bias

We used the Hoy *et al*[32] tool to assess the quality of the included studies (Supplementary Table 2). This tool takes into account 10 elements to assess the internal and external validity of prevalence studies. For each item, a score of 1 is assigned to a "yes" response and a score of 0 is assigned to the other responses ("no", "not clear", "not applicable"). Basically, a study was considered to be low risk, moderate risk, or high risk of bias if the total score was 0-3, 4-6, and 7-10, respectively.

Data synthesis and analysis

To estimate proportions of OCI and their 95% confidence intervals (95%CI), we chose the random-effect meta-analysis model due to the heterogeneity expected for observational studies. The I^2 statistics and Cochran's Q-test were used to assess heterogeneity between studies[33]. The I^2 cut-offs > 50% indicate substantial heterogeneity. Potential sources of heterogeneity were explored by subgroup analyses and meta-regression including covariates: study design, sampling, setting, timing of samples collection, countries, WHO region, UNSD region, country income level, age range, population categories, OCI diagnostic method, and sample types. We used Funnel plot and Egger test to examine publication bias [34]. R software version 4.1.0 was used for all analyses[35,36].

RESULTS

Study selection and characteristics

The electronic search identified 3950 articles (EMBASE (2025), Web of Science (1183), PubMed (706), and Global Index Medicus (36) (Figure 1). The eligibility review of 179 articles resulted in the exclusion of 94 and the inclusion of 85. The excluded articles and the individual reasons for exclusion are presented in Supplementary Table 3, while the included articles are indicated in Supplementary Text 1.

Characteristics of the included studies

Overall, we obtained 102 prevalence data from the 85 included studies (75 seronegative OCI, 24 seropositive OCI, and 3 seropositive OCI and/or seronegative OCI (Supplementary Tables 4 and 5). The prevalence data were published from 1995 to 2021 and for studies with data reported, the participants were recruited from 2002 to 2019. The majority of the prevalence data were cross-sectional design (94

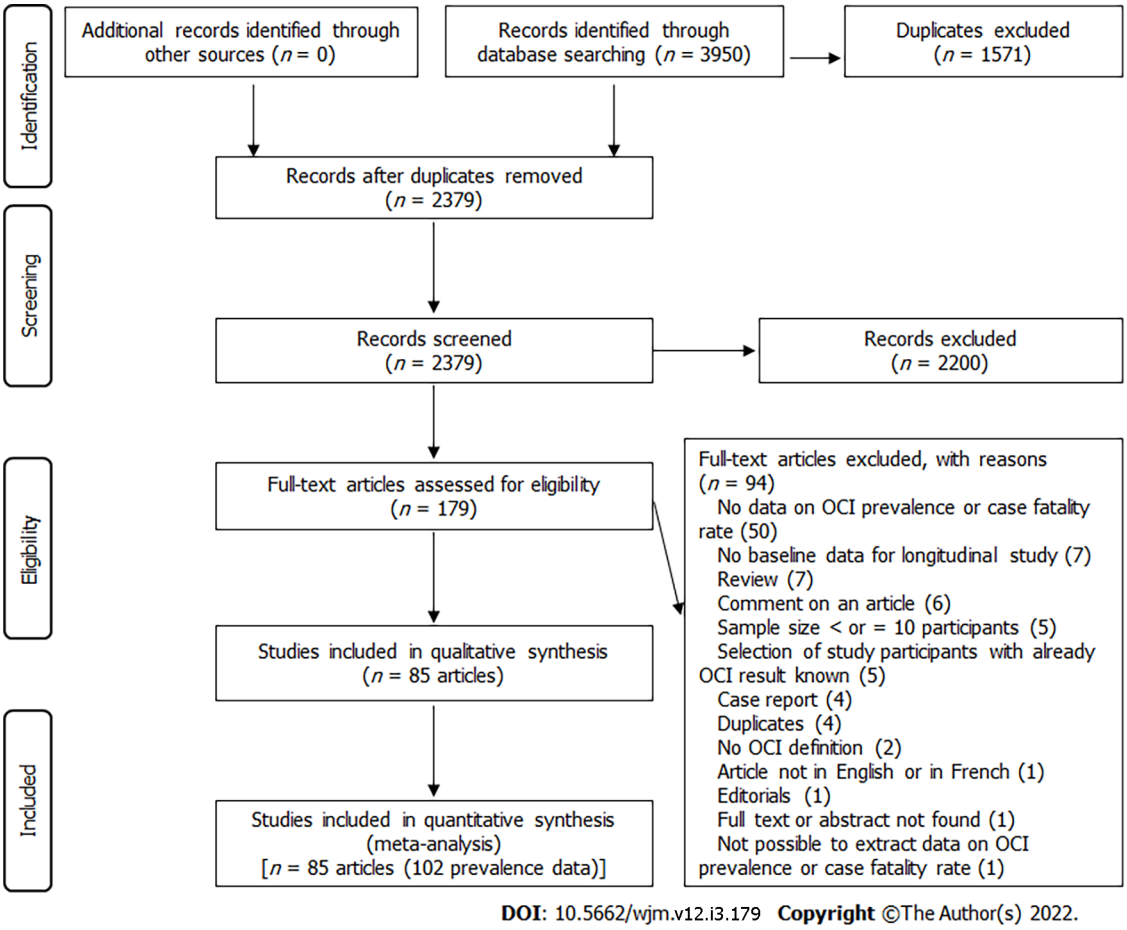

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Figure 1 PRISMA flow-chart of studies selected for the meta-analysis.

out of 102) with non-random sampling (97 out of 102) and consecutive sampling methods (95 out of 102). The setting of the study was hospital-based (98 out of 102) and monocentric (83 out of 102). Prevalence data were reported predominantly in the Eastern Mediterranean (51 out of 102) and in European (41 out of 102) WHO regions. The highest numbers of prevalence data were from high-income countries (40 out of 102) and upper-middle-income countries (35 out of 102). Prevalence data predominantly involved adults (33 out of 102), patients on hemodialysis (25 out of 102), and patients who achieved a SVR (15 out of 102). The most used sample type was PBMC (86 out of 102). The OCI diagnosis was performed using classical RT-PCR (49 out of 102) or real-time RT-PCR (44 out of 102). In most prevalence data, the risk of bias was moderate (64 out of 102) (Supplementary Table 6).

Prevalence of seronegative occult C infection

A total of 75 prevalence data reporting seronegative OCI were conducted across 4 WHO regions: America, Eastern Mediterranean, Europe, and Western Pacific (Figure 2). The pooled OCI prevalence was estimated to be 9.6% (95%CI: 6.8-12.7) in samples of 8535 participants with high heterogeneity [I^2 = 94.7% (95%CI: 93.8%-95.4%), P < 0.0001] (Figure 3A, Table 1, and Supplementary Figure 1). There was significant publication bias (Supplementary Figure 2, P = 0.006). Trim-and-fill adjusted analysis indicated a lower prevalence of 5.3% (95%CI: 2.9-8.2) with an addition of 10 studies.

Prevalence of seropositive occult C infection

Prevalence data reporting seropositive OCI were conducted in 4 WHO regions including America, Eastern Mediterranean, Europe, and Western Pacific (Figure 2). Overall, seropositive OCI prevalence was estimated to be 13.3% (95%CI: 7.8-19.9) with a total of 2642 participants from 24 prevalence data (Figure 3B). High heterogeneity was observed in the overall estimate of the prevalence of seropositive OCI [I^2 = 93.0% (90.8%-94.7%), P < 0.0001]. There was a significant publication bias (Supplementary Figure 3, P = 0.017). Trim-and-fill adjusted analysis indicated a lower prevalence of 5.3% (95%CI: 1.4-10.7) with an addition of 8 studies.

Seronegative and/or seropositive occult C infection

Prevalence data reporting seronegative and/or seropositive OCI were conducted in 2 WHO regions

Table 1 Summary of meta-analysis results for the global prevalence of occult hepatitis C virus infection

	Prevalence % (95%CI)	95% prediction interval	Studies (*n*)	Participants (*n*)	[1]H (95%CI)	[2]I^2 (95%CI)	*P* heterogeneity
Seronegative OCI							
Overall	9.6 (6.8-12.7)	(0-44.1)	75	8535	4.3 (4-4.6)	94.7 (93.8-95.4)	< 0.001
Trim-and-fill adjusted analysis	5.3 (2.9-8.2)	(0.0-45.1)	85	NA	5.1 (4.8-5.4)	96.2 (95.7-96.6)	< 0.001
Cross-sectional	9.3 (6.5-12.6)	(0-43.6)	68	8250	4.4 (4.1-4.8)	94.9 (94.1-95.6)	< 0.001
Low risk of bias	10.2 (5.9-15.5)	(0-45.9)	28	3372	4.3 (3.8-4.8)	94.6 (93.1-95.7)	< 0.001
Seropositive OCI							
Overall	13.4 (7.9-20)	(0-52)	24	2642	3.8 (3.3-4.3)	93 (90.8-94.7)	< 0.001
Trim-and-fill adjusted analysis	5.3 (1.4-10.7)	(0.0-49.9)	32	NA	4.5 (4.0-5.0)	95.1 (93.9-96.0)	< 0.001
Cross-sectional	12.5 (7.2-18.7)	(0-48.5)	23	2530	3.5 (3.1-4.1)	92 (89.3-94)	< 0.001
Low risk of bias	12.8 (4.6-23.6)	(0-57.6)	9	1659	3.6 (2.8-4.6)	92.3 (87.5-95.2)	< 0.001

[1]H is a measure of the extent of heterogeneity, a value of H =1 indicates homogeneity of effects and a value of H >1 indicates potential heterogeneity of effects.
[2]I^2 describes the proportion of total variation in study estimates due to heterogeneity, a value > 50% indicates the presence of heterogeneity.
OCI: Occult hepatitis C virus infection; *n*: Number; 95%CI: 95% confidence interval; NA: Not applicable.

(Eastern Mediterranean and Europe). Overall, seronegative and/or seropositive OCI prevalence was estimated to be 12.6% (95%CI: 1.2-32.2) with a total of 285 participants from 3 prevalence data. High heterogeneity was observed in the overall estimate of the prevalence of seronegative and/or seropositive OCI [I^2 = 93.0% (83.0%-97.1%), P < 0.0001].

Subgroup analyses and meta-regression

Seronegative OCI: Higher proportions of seronegative OCI were estimated for studies which selected participants by non-probabilistic sampling (P = 0.001), conducted in Spain and Egypt (P < 0.001), in Southern Europe and Northern Africa (P < 0.001), or in countries with lower-middle income economies (P = 0.045), investigated children (P = 0.01) or patients with abnormal liver function, hematological disorders, and kidney diseases (P < 0.001), and detected OCI cases by real-time RT-PCR (P < 0.001) or by examining liver tissue (P < 0.001) (Supplementary Table 7). The heterogeneity of the prevalence of seronegative OCI was explained at 84.0% (R^2 = 84.0%) (Supplementary Table 8).

Seropositive OCI: Higher proportions of seropositive OCI were estimated for studies performed as case controls (P < 0.001), conducted in Italy, United States of America, and Egypt (P < 0.001), in Southern Europe, Northern America, and Northern Africa (P = 0.001), or by examining liver tissue and PBMC (P = 0.023). The heterogeneity of the prevalence of seropositive OCI was explained at 46.2% (R^2 = 46.2%).

DISCUSSION

This systematic review summarized the prevalence of seronegative and seropositive OCI in relevant articles published between 1995 and 2021 in 17 countries across 4 WHO regions: America, Europe, Eastern Mediterranean, and Western Pacific. Overall, we found a high prevalence of seronegative OCI (9.61%) and seropositive OCI (13.39%), respectively. Higher seronegative OCI prevalence was found in Southern Europe and Northern Africa and in patients with abnormal liver function, hematological disorders, and kidney diseases. Higher seropositive OCI prevalence was found in Southern Europe, Northern America, and Northern Africa.

Many studies have previously shown that multiple transfused subjects are at high risk of HCV infection[20,25,37-39]. Seronegative OCIs aligned well with classical HCVs and were very predominant in subjects with hematological disorders and renal diseases in this study. It is therefore important to implement screening measures for OCI in blood transfusion banks, dialysis and/or transplant units[40].

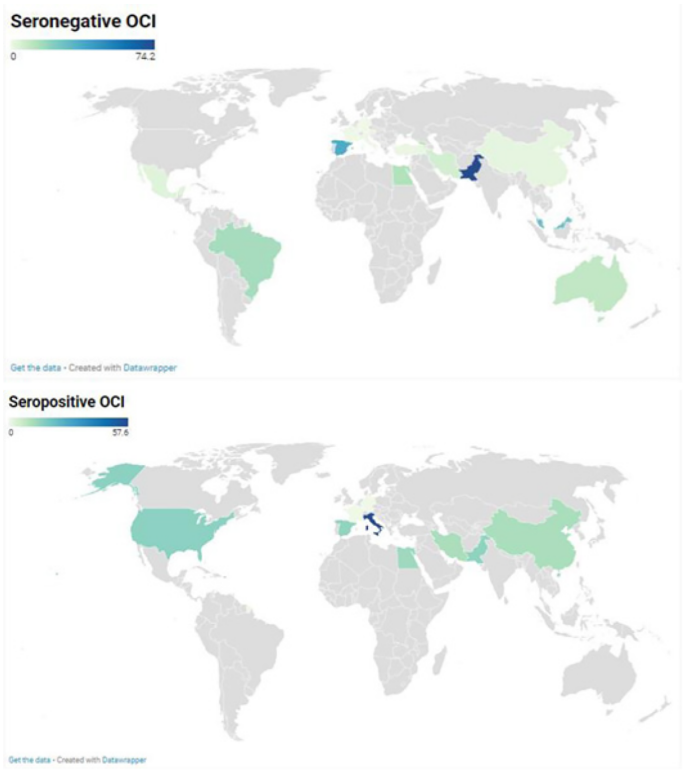

Figure 2 Global prevalence of seronegative and seropositive occult hepatitis C virus infection.

As in the present review, it has also been shown previously that patients with abnormal liver functions are at high risk of OCI[26]. There is, however, a significant residual heterogeneity in our estimates that could be related to the different types of chronic liver disease that we did not take into account. North Africa and particularly Egypt is the country with the highest prevalence of HCV in the world[41,42]. The findings of the present study corroborate this fact and show higher seronegative and seropositive OCI prevalence in North Africa (Egypt). However, it should be noted that Southern Europe and North America also showed high prevalence of OCI in this study, while most other regions were absent or poorly represented in the estimates. HIV patients, people who inject drugs and men who have sex with men are groups known to be at high risk of HCV infection and were poorly represented in this review. Additional studies characterizing the epidemiology of HCV in these groups are awaited to fully explain the global epidemiology of OCI and more specifically in the WHO regions of Africa and South-East Asia. In a first global review without meta-analysis conducted in 2017, Dolatimehr *et al*[25] reported OCI prevalence of 0%-45% and 0%-2% in hemodialysis patients (10 studies) and kidney transplant recipients (2 studies), respectively. In a conference abstract, Fu *et al*[26] reported a highly variable prevalence of OCI in different population groups ranging from 0% in patients with autoimmune hepatitis, 9% in patients with cryptogenic liver disease, 22% in patients with chronic liver disease who achieved a SVR, 33% in patients with long-standing abnormal liver-enzyme levels to 89% in patients with abnormal levels of serum aminotransferases. More recently, Hedayati-Moghaddam *et al*[20] reported the prevalence of OCI in the Middle East and Eastern Mediterranean in several population categories. This last systematic review also revealed a significant variability in OCI prevalence according to the category of the population with 19% for patients with hematological disorders, 12% for HIV-infected patients, 12% for patients with chronic liver diseases, 9% for hemodialysis patients, 8% for multitransfused patients, and 4% for apparently healthy populations. Similar to the reviews mentioned above, our study also noted a statistically significant difference in the prevalence of seronegative OCI according to population categories. However, it should be mentioned that the above reviews only included participants tested for PBMC unlike our work which also considered liver biopsies and ultracentrifuged serum. The strong heterogeneity recorded in our work could also be explained by the differences in HCV prevalence according to the regions with the areas of high HCV endemicity which should also be the areas of high prevalence of OCI[11]. As the different risk factors and the different approaches for controlling HCV infection also vary widely between studies, regions and populations, this could also potentially represent a considerable source of the variability observed in our work. We should also cite the examples of a history of accidental exposure to infected needle sticks, history of blood transfusion, history of surgery, history of endoscopy, history of unsafe sexual intercourse, history of liver disease, the length and frequency of dialysis sessions, immunodepression, injecting drugs, tattoos or imprisonment. Other potential sources of heterogeneity in our estimates may also include gender of

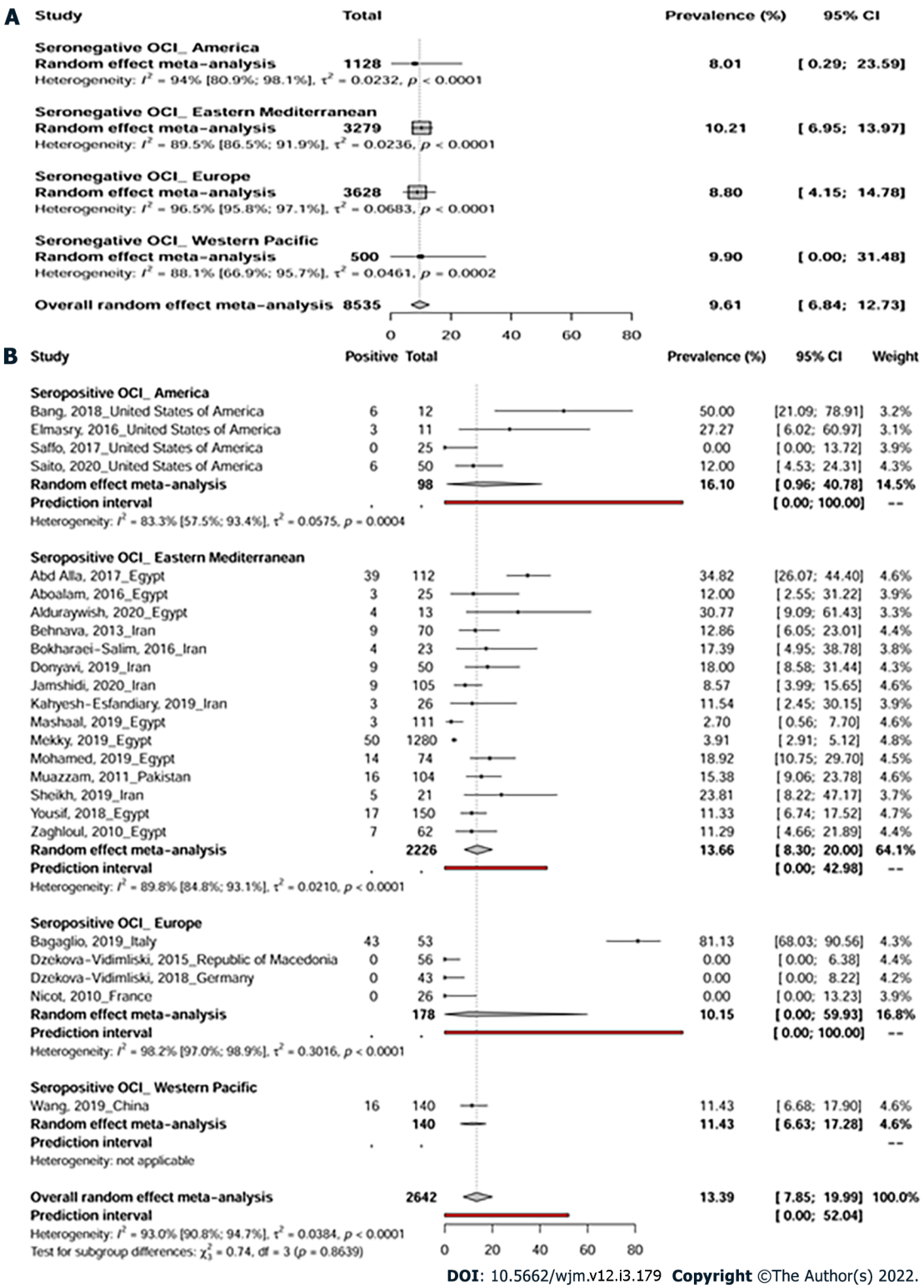

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Figure 3 The pooled global prevalence of seronegative (A) and seropositive (B) occult hepatitis C virus infection.

participants, sample size, and year of participant recruitment. Our study revealed that although PBMC are an excellent non-invasive sampling approach for diagnosing OCI, liver tissue exhibits superior sensitivity for seronegative OCI. It was also found that the ultracentrifuged serum obtained was not an insignificant fraction in patients positive for OCI. These results suggest that it is potentially insufficient to test for OCI in one type of sample. We also observed that real-time RT-PCR was significantly more sensitive for the detection of seronegative OCI. This suggests a further improvement in the sensitivity of molecular techniques for OCI detection.

Our study is limited due to the included studies where the WHO Africa and South-East Asia regions are not represented. Our OCI prevalence could therefore be over- or underestimated. Substantial

residual statistical heterogeneity in prevalence measures was identified in all aggregate and subgroup meta-analyses. Despite these limitations, the main strength of our study is that we identified a very large number of studies, which covered multiple categories of symptomatic, apparently healthy populations and those at high risk of HCV infection. We also accounted for the variability of the prevalence according to the anti-HCV serostatus.

Our results suggest that the implementation of screening programs for OCI in high-risk populations, especially patients with hematologic complications, hemodialysis patients, and patients with chronic liver disease should be initiated. More studies are needed to assess the transmissibility, clinical significance, long-term outcome, and need for OCI treatment.

CONCLUSION

In conclusion, it appears that the burden of seronegative and seropositive OCI is high and very variable according to regions and categories of populations.

ARTICLE HIGHLIGHTS

Research background

In 2004, Castillo *et al* first described an unknown form of hepatitis C in humans that was different from the common chronic hepatitis C and was called occult hepatitis C Infection (OCI).

Research motivation

To eradicate hepatitis C virus (HCV) by 2030, as recommended by the WHO, it is crucial to determine the burden of OCI across the different regions of the world and in different population categories.

Research objectives

Highlight the global prevalence of seronegative and seropositive OCI according to population categories and regions of the world.

Research methods

The authors searched PubMed, EMBASE, Global Index Medicus, and Web of Science databases from inception to May 6, 2021. Data were extracted independently by each author and the Hoy *et al* tool was used to assess the quality of included studies. Prevalence and 95% confidence intervals were determined using random-effect meta-analysis.

Research results

The authors included 85 articles out of the 3950 identified by the electronic search. The combined prevalence of seronegative OCI was 9.61% (95%CI: 6.84-12.73) and the prevalence of seropositive OCI was 13.39% (95%CI: 7.85-19.99). For variations by region, seropositive OCI prevalence was higher in Southern Europe, Northern America, and Northern Africa, and seronegative OCI prevalence was higher in Southern Europe and Northern Africa. For variations by population categories, seronegative OCI prevalence was higher in patients with abnormal liver function, hematological disorders, and kidney diseases.

Research conclusions

The burden of OCI is high and greatly variable according to world regions and population categories.

Research perspectives

Consideration should be given to the implementation of screening programs for OCI in high-risk populations such as patients with hematologic disorders, kidney disease, and those with abnormal liver function.

FOOTNOTES

Author contributions: Kenmoe S, Mbaga DS, and Riwom Essama SH were responsible for conception and design of the study as well as project administration; Mbaga DS, Kenmoe S, Njiki Bikoï J, Takuissu GR, Amougou Atsama M, Atenguena Okobalemba E, Ebogo-Belobo JT, Bowo-Ngandji A, Oyono MG, Magoudjou-Pekam JN, Kame-Ngasse GI, Nka AD, Feudjio AF, Zemnou-Tepap C, Velhima EA, Ndzie Ondigui JL, Nayang Mundo RA, Touangnou-Chamda SA, Kamtchueng Takeu Y, Taya-Fokou JB, Mbongue Mikangue CA, Kenfack-Momo R, and Kengne-Nde C were responsible for the data curation and interpretation of results; Kengne-Nde C and Kenmoe S were responsible for

statistical analysis; Kenmoe S, Mbaga DS, and Riwom Essama SH were responsible for the project supervision; Kenmoe S and Mbaga DS wrote the original draft; All authors critically reviewed the first draft and approved the final version of the paper for submission, and have read and approved the final manuscript.

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LETTER TO THE EDITOR

Severe acute respiratory syndrome coronavirus 2 pandemic and surgical diseases: Correspondence

Pathum Sookaromdee, Viroj Wiwanitkit

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Abstract

This letter to editor discussing on the publication on severe acute respiratory syndrome coronavirus 2 pandemic and surgical diseases. Concerns on procedures are raised and discussed.

Key Words: Pediatric; Surgery; COVID-19

Core Tip: This letter to editor discussing on the publication on severe acute respiratory syndrome coronavirus 2 pandemic and surgical diseases: Concerns on study techniques and clinical implication are raised and discussed.

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TO THE EDITOR

We read with interest a case report on "Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic related morbidity and mortality in patients with pediatric surgical diseases: A concerning challenge" by Vaos and Zavras[1]. We would like to share ideas on this report. Basically, the adaptation of medicine to the coronavirus disease 2019 (COVID-19) is necessary. For surgery, to save lives while maintaining excellent surgical standards, dynamic prioritizing of SARS-CoV-2 infected and surgical patient groups is critical[2]. In emergency departments, non-intensive care wards, and

operating rooms, strict segregation of patient groups inhibits virus spread, while appropriately training and carefully selecting hospital staff allows them to confidently and successfully perform their respective clinical roles[2]. How to find a solution in surgery need a good systematic study.

In this report, a literature retrospective review is done. However, there is no clear information on searching technique and extracting of data. There is no interrelationship network analysis of recruited literatures and it does not follow standard meta-analysis technique, bioinformatics interrelationship analysis and bibliometric analysis. The summarization is based on crude summary on surgical cases, without adjustment to the background condition of the cases (age, underlying disease, surgical intervention, *etc.*). Also, there is no study on the correlation with the stages of COVID-19 background in different recruited publication. It should not possible to recommend the new guidelines for management of pediatric surgical cases. For pediatric surgery, a meta-analysis on each specific condition with specific aim or target for study, such as comparison of surgical approach, should be the best method to find out the solution during the current COVID-19 crisis. Good example of the studies in this kind are reports by Chan *et al*[3,4].

FOOTNOTES

Author contributions: Sookaomdee P and Wiwanitkit gave ideas, analyzed the data and wrote the manuscript; all authors have read and approve the final manuscript.

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