ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2021 January 20; 11(1): 1-14

Published by Baishideng Publishing Group Inc

World Journal of Methodology

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Bimonthly Volume 11 Number 1 January 20, 2021

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World Journal of Methodology

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The *WJM* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Yun-Xiaojian Wu*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Ji-Hong Liu*.

NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Gerhard Litscher

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2222-0682/editorialboard.htm

PUBLICATION DATE
January 20, 2021

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

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ONLINE SUBMISSION
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World Journal of Methodology

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World J Methodol 2021 January 20; 11(1): 1-14

DOI: 10.5662/wjm.v11.i1.1

ISSN 2222-0682 (online)

META-ANALYSIS

Epidemiological burden of parents being the index cases of COVID-19 infected children

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Author contributions: Sumanta Saha prepared the concept and design, performed the data analysis, and drafted the manuscript; Sujata Saha hard edited the manuscript; both authors contributed to the study selection and risk of bias assessment and agree with the content of the final version of the manuscript.

Conflict-of-interest statement: The authors do not have any conflict of interest to declare.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Abstract

BACKGROUND

In the ongoing coronavirus disease 2019 (COVID-19) pandemic, when children remain home-confined secondary to the closure of schools, little is known of the burden of the parents being their index case.

AIM

To determine the prevalence of parents being the index case of COVID-19 infected children.

METHODS

A database search in PubMed and Scopus ensued to recruit studies reporting the index case information of COVID-19 infected individuals aged ≤ 18. The reviewed articles' quality evaluation included the use of the National Heart, Lung, and Blood Institute's tool. A random-effect meta-analysis ensued to determine the prevalence of the parent being and not-being the index case. Heterogeneity was assessed by I^2 and Chi^2 statistics. The publication bias was evaluated by funnel plots and Egger's test.

RESULTS

Overall, this review included 13 eligible studies sourcing data from 622 children of 33 nations. Study designs were heterogeneous and primarily included descriptive reports (38.4%). The prevalence of parent being the index case was 54% (95%CI: 0.29-0.79; I^2: 62.3%, Chi^2 $P < 0.001$). In > 70% of children, their index-case parent was symptomatic due to COVID-19 at the time of infection transmitting. Studies for which a risk of bias assessment was possible were of fair quality.

CONCLUSION

There is a substantial global burden of parents being the index case of COVID-19 infected children, and frequently these parents are symptomatic. Therefore, from

Manuscript source: Invited manuscript

Specialty type: Medical laboratory technology

Country/Territory of origin: India

Peer-review report's scientific quality classification
Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: December 3, 2020
Peer-review started: December 3, 2020
First decision: December 11, 2020
Revised: December 17, 2020
Accepted: December 27, 2020
Article in press: December 27, 2020
Published online: January 20, 2021

P-Reviewer: Carnevale S, Konakci G, Pan SL
S-Editor: Fan JR
L-Editor: A
P-Editor: Yuan YY

a public health perspective, early detection of these parents is crucial.

Key Words: COVID-19 pandemic; COVID-19; Index case epidemiology; Patient zero epidemiology; Pediatrics

Core Tip: During the ongoing coronavirus disease 2019 (COVID-19) pandemic situation, while schools remain closed and children primarily stay at home, the prevalence of parent to child COVID-19 transmissibility remains unknown. Therefore, this meta-analysis chiefly quantifies this epidemiological burden. Globally, this burden was substantial (about 54%) and was highest in Asia. The majority of these parents (> 70%) were symptomatic. This study highlights the public health importance of early detection of COVID-19 infected parent index cases to decrease transmission to their wards.

Citation: Saha S, Saha S. Epidemiological burden of parents being the index cases of COVID-19 infected children. *World J Methodol* 2021; 11(1): 1-14
URL: https://www.wjgnet.com/2222-0682/full/v11/i1/1.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i1.1

INTRODUCTION

In March 2020, the World Health Organization declared the coronavirus disease 2019 (COVID-19) epidemic that originated in China as a pandemic[1]. By August 2020, the toll of COVID-19 cases crossed 22 million globally[2]. Our knowledge of COVID-19 has increased at a remarkably fast pace as new research findings became available. Nevertheless, COVID-19 has been less well studied in children, as their reported number of cases, disease severity, and fatality have been less than the adults[3,4]. However, as the testing of the disease increased in different nations and because young children often fail to use face masks effectively[5], the COVID-19 cases in children are rising and it can be severe in children with comorbidities like congenital heart disease and malnutrition[6]. The inability of the children to report their symptoms or contact history is a major challenge in identifying pediatric COVID-19 infection[7]. In the ongoing pandemic scenario, while children often stay at home with parents as schools remain closed in various nations[8], the household transmission risk of the infection from their parents remain high. Moreover, contemporary research[4] has primarily focused on the possibility of children being the index case and not on the other way around when parents can be the index case. Consequently, it's imperative to investigate the vulnerability of children's infectivity from their parents.

Therefore, in this study, we primarily quantified the epidemiologic burden of parents being the index cases of COVID-19 transmission in children.

MATERIALS AND METHODS

This review report adheres to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guideline[9] and is registered in the PROSPERO (CRD 42020209006)[10].

Inclusion criteria

Population: COVID-19 diagnosed children aged ≤ 18.

Study design: Articles reporting the index cases of the above study population was incorporated. Publications of all types including experimental and observational studies, case reports, case series, letters, descriptive reports, and editorials were eligible.

Exclusion criteria

Studies were excluded if its study population included pregnant females.

Search strategy

The search for title and abstract of articles published in the English language took place in the PubMed and Scopus databases irrespective of the publication date and geographical boundary. The last date of the search was Sep 12, 2020. We also searched the Google search engine and the bibliography of the reviewed articles. The search terms used to search the PubMed were: "index case*" OR "index patient*" OR "patient zero" AND child* OR pediatric* OR infant OR toddler OR Adolescent and "Syndrome Coronavirus 2" OR "coronavirus" OR "COVID-19" OR "COVID 19."

Study selection, data abstraction, and risk of bias assessment

Following the uploading of the database search results to the Rayyan systematic review software[11] and successive duplicate elimination, we skimmed the remaining excerpts while matching these against the eligibility criteria. Articles likely to meet these criteria or when decision-making was not possible by reading the title and abstracts only, a full-text reading ensued. Salient features like the study design, inclusion criteria, COVID-19 diagnostic method of children and their index case, relation to the index case, the symptom and death information of the index case, and the number of COVID-19 positive children were abstracted using a pre-piloted form.

The risk of bias assessment (RoB) evaluation categorized each study as good, fair, or poor utilizing the National Heart, Lung, and Blood Institute's tool[12]. For case reports, descriptive reports (not categorizable to any study design), letters, and editorials, we did not perform RoB assessment as validated tools to appraise such articles don't exist. We independently selected studies, abstracted data, and assessed the RoB, and resolved all disagreements by discussion. For experimental studies, the RoB assessment was planned by the Cochrane tool[13]; however, it was not used as the reviewed studies did not include a clinical trial.

Meta-analysis

Using the random-effect (DerSimonian and Laird) meta-analysis (exact binomial method with Freeman-Tukey double arcsine transformation), we estimated the weighted overall and subgroup prevalence of parents being the index case of COVID-19 infected children. Subgrouping was done by continent, country, COVID-19 diagnostic method used in children and their index case, and the index case's symptom status (symptomatic versus asymptomatic) and death. Heterogeneity was assessed by I^2 (catego-rized as low, moderate, or high at values of 25%, 50%, and 75%)[14] and P value of Chi^2 statistics (statistically significant at $P < 0.1$). Exploring the reasons for heterogeneity were not necessary as it was moderate. The publication bias judgment included the use of funnel plots and Egger's test, and sensitivity analysis repeated the meta-analysis while dropping a study each time.

Analytic software Stata (version 16) was used to perform analysis. $P < 0.05$ and 95% confidence interval (CI) estimate the statistical significance.

RESULTS

Scope of the review

The database search retrieved 51 citations. After eliminating the duplicates, out of the 30 articles skimmed, we read 22 manuscripts in full-text and finally reviewed 13 studies[15-27] published in 2020 (Figure 1). These articles chiefly constituted of descriptive reports (38.4%) and case series (23.1%). Other article variants were cross-sectional study (15.4%), research letter (15.4%), and case report (7.7%). Cumulatively, the studies sourced data of 622 children of 33 nations from four continents (Asia, Europe, North America, and South America).

Primarily the COVID-19 infection in the children was diagnosed by reverse transcription polymerase chain reaction (RT-PCR) (99.7%). The index case was mainly an immediate family member or unknown person (96%) for children whose index case was not their parent (n = 276). Table 1 depicts the salient features of the reviewed articles.

Risk of bias assessment

The cross-sectional and case-series studies were of fair quality (Table 2).

Table 1 Salient features of the reviewed studies

Ref.	Country	Continent	Study design	Study period (2020)	Inclusion criteria	COVID-19 diagnosis confirmation in children	Age of COVID-19 infected children with index case	No. of COVID-19 infected children with index case		COVID-19 infected index case/s'			
								Parent	Not-parent[1]	Age	COVID-19 diagnosis Ascertainment	Symptoms around the time of contact with children	Death due to COVID-19[2]
Götzinger *et al*[15], 2020	21 European nations except France[3]	Europe	Case series	1 Apr-24 Apr	≤ 18 yr SARS-CoV-2 infected individuals	RT-PCR	Median age 5 yr (IQR 5–12)	324	24 (sibling); 234 (immediate family member or unknown)	Unclear	History	Unclear	Unclear
Kim *et al*[16], 2020	South Korea	Asia	Cross-sectional	20 Jan- 6 Apr	≤ 18 yr SARS-CoV-2 infected individuals	RT-PCR	15 yr	0	1 (sibling)	16 yr	RT-PCR	Symptomatic	No
								0	1 [unknown (not parent or sibling)]	Unclear	Unclear	Unclear	Unclear
Luo *et al*[20], 2020	China	Asia	Case report	NA	NA	RT-PCR	Average 7 yr	2	0	39 yr	RT-PCR	Symptomatic	No
Merza *et al*[21], 2020	Iraq	Asia	Case series	18 Mar-07 April	Hospitalized conformed COVID-19 cases	RT-PCR	Average 11 yr	3	0	45 yr	RT-PCR	Symptomatic	No
Silva *et al*[22], 2020	Brazil	South America	Descriptive report	NA	NA	Rapid test	Average 10.5 yr	2	0	2 index cases: Male: 51 yr; female: 42 yr	RT-PCR	Symptomatic (both)	No
Song *et al*[23], 2020	China	Asia	Descriptive report	NA	NA	RT-PCR	Average 3.94 yr	5	0	Parents (n = 3): Average age 40.33 yr	RT-PCR	Symptomatic	No
								0	2 (grandparent)	Grandparent (n = 1): 60 yr		Symptomatic	No
Torres *et al*[24], 2020	Chile	South America	Cross-sectional	NA	All school staff and randomly selected students	RT-PCR	Unclear	0	7 (school staff)	Unclear	RT-PCR	Unclear	Unclear
Yang *et al*[25], 2020	Taiwan	Asia	Descriptive report	NA	NA	RT-PCR	11 yr	0	1 (grandparent)	85 yr	RT-PCR	Symptomatic	Yes
Yung *et al*[26], 2020	Singapore	Asia	Case series	5 Mar–30 Apr	Paediatric household contacts of confirmed COVID-19 cases	RT-PCR	Unclear	7[4]	2 (grandparent or another adult except parent)[5]	Unclear	RT-PCR	Unclear	Unclear

Zhang *et al*[27], 2020	China	Asia	Research letter	28 Jan–15 Mar	Secondary COVID-19 cases	RT-PCR	Average 3 yr	2	0	Unclear	RT-PCR	One case: Mild symptoms. Other case: Moderate symptom	Unclear
Danis *et al*[17], 2020	France	Asia	Descriptive report	NA	NA	RT-PCR	9 yr	0	1 (a visitor/tourist)	Unclear	RT-PCR	Yes	No
James *et al*[18], 2020	United States	North America	Descriptive report	NA	NA	RT-PCR	≤ 18 yr	0	2 (a pastor and his wife)	Two index cases aged 56 and 57 yr	RT-PCR	During contact: Initially asymptomatic, then symptomatic	No
Jung *et al*[19], 2020	South Korea	Asia	Research letter	NA	NA	RT-PCR	Average 5.5 yr	1	0	The mother: 40 yr	RT-PCR	During contact asymptomatic (symptomatic after RT-PCR diagnosis)	No
								0	1 (another patient who stayed in the same room)	For 9 years old: Unclear	Unclear	Unclear	Unclear

[1]In parenthesis the COVID-19 infected child/children's relation to the index case.
[2]Upto the time period for which index case data reported or at the time of contact–whichever reported in the reviewed studies.
[3]Austria, Belgium, Bulgaria, Croatia, Denmark, Estonia, Germany, Greece, Hungary, Ireland, Italy, Lithuania, Norway, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey, and the United Kingdom.
[4]Either mother or father the index case; another non-parent may be an index case for some of the children.
[5]May also have a parent index case. RT-PCR: Reverse transcription polymerase chain reaction; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; NA: Not applicable; COVID-19: Coronavirus disease 2019.

Prevalence estimates

The overall weighted prevalence of parents being the index case of COVID-19 in children was 54% (95%CI: 0.29-0.79; I^2: 62.3%, Chi^2 $P < 0.001$) (Figure 2). Continent-wise this was highest in Asia (75%; 95%CI: 0.45-0.97), followed by Europe (58%; 95%CI: 0.52-0.63). The index cases' RT-PCR-based COVID-19 diagnosis (60%; 95%CI: 0.20-0.94) was four percentage point higher than a history-based diagnosis (56%; 95%CI: 0.52-0.60). The prevalence of parent-index-case being symptomatic or not dying due to COVID-19 was about 73% each at the time of disease transmission to their children (Table 3).

The crude prevalence of parents not being the index case was 46% (95%CI: 0.21-0.71; I^2: 62.3%; Chi^2 $P < 0.01$) (Figure 3). It was substantial in the North (100%) and South (89%) United States. Only 27.0% (95%CI: 0.00-0.67; I^2: 44.3%) of these cases were symptomatic (Table 4).

Heterogeneity and publication bias

Overall, the heterogeneity was moderate. The funnel plots (Figures 4 and 5) and the Egger's test (index case parent: $P = 0.198$; index case non-parent: $P = 0.488$) were not

Table 2 Risk of bias assessment using National Heart, Lung, and Blood Institute's tool[12]

Study design: Case series

Ref.	1. Was the study question or objective clearly stated?	2. Was the study population clearly and fully described, including a case definition?	3. Were the cases consecutive?	4. Were the subjects comparable?	5. Was the intervention clearly described?	6. Were the outcome measures clearly defined, valid, reliable, and implemented consistently across all study participants?	7. Was the length of follow-up adequate?	8. Were the statistical methods well-described?	9. Were the results well-described?	Quality rating (Good, fair, or poor)
Götzinger *et al*[15], 2020	Yes	Yes	CD	Yes	NA	Yes	NA	Yes	Yes	Fair
Merza *et al*[21], 2020	Yes	Yes	CD	Yes	NA	Yes	NA	Yes	Yes	Fair
Yung *et al*[26], 2020	Yes	Yes	Yes	Yes	NA	Yes	NA	Yes	Yes	Fair

Study design: Cross-sectional study

Ref.	1. Was the research question or objective in this paper clearly stated?	2. Was the study population clearly specified and defined?	3. Was the participation rate of eligible persons at least 50%?	4. Were all the subjects selected or recruited from the same or similar populations (including the same time period)? Were inclusion and exclusion criteria for being in the study prespecified and applied uniformly to all participants?	5. Was a sample size justification, power description, or variance and effect estimates provided?	6. For the analyses in this paper, were the exposure(s) of interest measured prior to the outcome(s) being measured?	7. Was the timeframe sufficient so that one could reasonably expect to see an association between exposure and outcome if it existed?	8. For exposures that can vary in amount or level, did the study examine different levels of the exposure as related to the outcome (*e.g.*, categories of exposure, or exposure measured as continuous variable)?	9. Were the exposure measures (independent variables) clearly defined, valid, reliable, and implemented consistently across all study participants?	10. Was the exposure(s) assessed more than once over time?	11. Were the outcome measures (dependent variables) clearly defined, valid, reliable, and implemented consistently across all study participants?	12. Were the outcome assessors blinded to the exposure status of participants?	13. Was loss to follow-up after baseline 20% or less?	14. Were key potential confounding variables measured and adjusted statistically for their impact on the relationship between exposure(s) and outcome(s)?	Quality rating (Good, fair, or poor)
Kim *et al*[16], 2020	Yes	Yes	NA	Yes	No	No	No	NA	Yes	NA	CD	NA	NA	No	Fair

Torres *et al* [24], 2020	Yes	Yes	NA	Yes	Yes	No	No	NA	Yes	NA	CD	NA	NA	No	Fair

CD: Can't determine; NA: Not applicable.

suggestive of any small study effect.

Sensitivity analysis

On dropping a study each time and repeating the meta-analysis, the prevalence estimates of the parent being the index case ranged between 49%-65%.

DISCUSSION

Altogether, we reviewed the data of 622 children from four different continents. The parents were the index cases in a substantial proportion of COVID-19 infected children and were highest in Asia. In seven out of ten COVID-19 infected children, the index parent case was symptomatic at the time of disease acquiring.

During this COVID-19 pandemic, schools remain closed in several nations, presuming that it would minimize the risk of transmission of the severe acute respiratory syndrome coronavirus 2 from children to adults[8]. Henceforth, children across the globe primarily remain at home, which increases their odds of getting COVID-19 from their parent/s since the latter are at risk of acquiring the disease from the community due to their required outdoor activities. As our findings suggest that a considerable proportion of the parents were the index cases of COVID-19 transmission to their children, their early detection and isolation is crucial to ensure early intervention. However, as we observed the highest global burden of parents being the index cases of COVID-19 in children in Asian nations, isolation of infected parents may not be unchallenging in these countries since many households lack an adequate number of rooms. Notably, the cities are more population-dense in Asia than those in the rest of the globe[28].

Strengths and limitations

The chief rigor of this study is its novelty to review an unexplored area of COVID-19 literature. Besides, the evidence synthesized in this paper is likely to be comprehensive as the database search criteria did not restrict to any geographic boundary or date range. Additionally, as we did not exclude from meta-analysis the studies with zero numerators, our estimates plausibly did not compromise with the sample size and power of the analysis. However, there are a few weaknesses in our paper. Mostly the

Table 3 Subgroup wise weighted prevalence of parent being the index case in coronavirus disease 2019 infected children

Prevalence of COVID-19 infected children with parents being the index case									
Subgroup	Category	Number of studies	Number of COVID-19 positive children	Number of COVID-19 positive children with parent being the index case	Weighted prevalence of COVID-19 positive children with parent being the index case		95% prediction interval	Heterogeneity measures	
					%	95%CI		I^2 (%)	Q (P value)
Continent	Asia	8	28	20	75.0	0.45-0.97	0.1-1.0	31.8	0.17
	Europe	2	583	324	58.0	0.52-0.63	Inestimable	-	-
	North America	1	2	0	0.0	0.00-0.84	Inestimable	-	-
	South America	2	9	2	11.0	0.00- 0.47	Inestimable	-	-
Country	21 European nations except France[1]	1	582	324	56.0	0.52-0.60	Inestimable	-	-
	France	1	1	0	0.0	0.00-0.98	Inestimable	-	-
	Brazil	1	2	2	100.0	0.16-1.00	Inestimable	-	-
	Chile	1	7	0	0.0	0.00-0.41	Inestimable	-	-
	China	3	11	9	87.0	0.54-1.00	Inestimable	-	-
	Iraq	1	3	3	100.0	0.29-1.00	Inestimable	-	-
	Singapore	1	9	7	78.0	0.40-0.97	Inestimable	-	-
	South Korea	2	4	1	18.0	0.00-0.77	Inestimable	-	-
	Taiwan	1	1	0	0.0	0.00-0.98	Inestimable	-	-
	United States	1	2	0	0.0	0.00-0.84	Inestimable	-	-
COVID-19 diagnosis in children	RT-PCR	12	620	344	50.0	0.24-0.76	0.0-1.0	63.4	0
	Rapid Method	1	2	2	100.0	0.16-1.00	Inestimable	-	-
COVID-19 diagnosis in the index case	RT-PCR	10	36	21	60.0	0.20-0.94	0.0-1.0	69.0	0.00
	History	1	582	324	56.0	0.52-0.60	Inestimable	-	-
	Unclear	2	4	1	18.0	0.00-0.77	Inestimable	-	-
COVID-19 index patient clinical presentation	Symptomatic	8	20	14	73.0	0.33-1.00	0.0-1.0	44.2	0.08
	Unclear	5	602	332	36.0	0.06-0.72	0.0-1.0	77.2	0.00
COVID-19 index patient mortality	Died	1	1	0	0.0	0.00-0.98	Inestimable	-	-
	Not died	6	17	12	74.0	0.29-1.00	0.0-1.0	48.5	0.08
	Unclear	6	604	334	44.0	0.13-0.78	0.0-1.0	74.1	0.00
Overall	NA	13	622	346	54.0	0.29-0.79	0.0-1.0	62.3	0.00

[1]Austria, Belgium, Bulgaria, Croatia, Denmark, Estonia, Germany, Greece, Hungary, Ireland, Italy, Lithuania, Norway, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey, and the United Kingdom. RT-PCR: Reverse transcription polymerase chain reaction; NA: Not applicable; COVID-19: Coronavirus disease 2019.

reviewed articles were not of any particular study design to allow a proper risk of bias assessment. Furthermore, distinguishing index-parents by gender or biological origin (*i.e.*, blood relation or stepparent) was impossible due to the lack of reporting of this information in the reviewed articles. Finally, for deaths among index cases, as we made estimates depending on the period for which the articles reported their data, we could not account for COVID-19-related deaths in them that might have happened beyond this period.

Table 4 Subgroup wise weighted prevalence of parent not being the index case in coronavirus disease 2019 infected children

Prevalence of COVID-19 infected children with parents not being the index case									
Subgroup	Category	Number of studies	Number of COVID-19 positive children	Number of COVID-19 positive children with parent not being the index case	Weighted prevalence of COVID-19 positive children with parent not being the index case		95% prediction interval	Heterogeneity measures	
					%	95% CI		I^2 (%)	Q (P value)
Continent	Asia	8	28	8	25.0	0.03-0.55	0.0-0.9	31.8	0.17
	Europe	2	583	259	42.0	0.37-0.48	Inestimable	-	-
	North America	1	2	2	100.0	0.16-1.00	Inestimable	-	-
	South America	2	9	7	89.0	0.53-1.00	Inestimable	-	-
Country	21 European nations except France[1]	1	582	258	44.0	0.40-0.48	Inestimable	-	-
	France	1	1	1	100.0	0.03-1.00	Inestimable	-	-
	Brazil	1	2	0	0.0	0.00-0.84	Inestimable	-	-
	Chile	1	7	7	100.0	0.59-1.00	Inestimable	-	-
	China	3	11	2	13.0	0.00-0.46	Inestimable	-	-
	Iraq	1	3	0	0.0	0.00-0.71	Inestimable	-	-
	Singapore	1	9	2	22.0	0.03-0.60	Inestimable	-	-
	South Korea	2	4	3	82.0	0.23-1.00	Inestimable	-	-
	Taiwan	1	1	1	100.0	0.03-1.00	Inestimable	-	-
	United States	1	2	2	100.0	0.16-1.00	Inestimable	-	-
COVID-19 diagnosis in children	RT-PCR	12	620	276	50.0	0.24-0.76	0..0-1.0	63.4	0.00
	Rapid method	1	2	0	0.0	0.00-0.84	Inestimable	-	-
COVID-19 diagnosis in the index case	RT-PCR	10	36	15	40.0	0.06-0.80	0.0-1.0	68.9	0.00
	History	1	582	258	44.0	0.40-0.48	Inestimable	-	-
	Unclear	2	4	3	82.0	0.23-1.00	Inestimable	-	-
COVID-19 index patient clinical presentation	Symptomatic	8	20	6	27.0	0.00-0.67	0.0-1.0	44.3	0.08
	Unclear	5	602	270	64.0	0.28-0.94	0.0-1.0	77.2	0.00
Overall	NA	13	622	276	46.0	0.21-0.71	0.0-1.0	62.3	0.00

[1]Austria, Belgium, Bulgaria, Croatia, Denmark, Estonia, Germany, Greece, Hungary, Ireland, Italy, Lithuania, Norway, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey, and the United Kingdom. RT-PCR: Reverse transcription polymerase chain reaction; NA: Not applicable; COVID-19: Coronavirus disease 2019.

Implications

At present, while we are still learning about the transmission dynamics of COVID-19 in children, this study provides a preliminary estimate of the epidemiological burden of the parents being their index case. Our findings emphasize the importance of isolating COVID-19 positive parents when they are living with their children in the same household to break the chain of transmission. Moreover, as most index case parents were symptomatic, early COVID-19 testing in adults, particularly in those residing with their wards is mandated, to ensure early diagnosis and isolation.

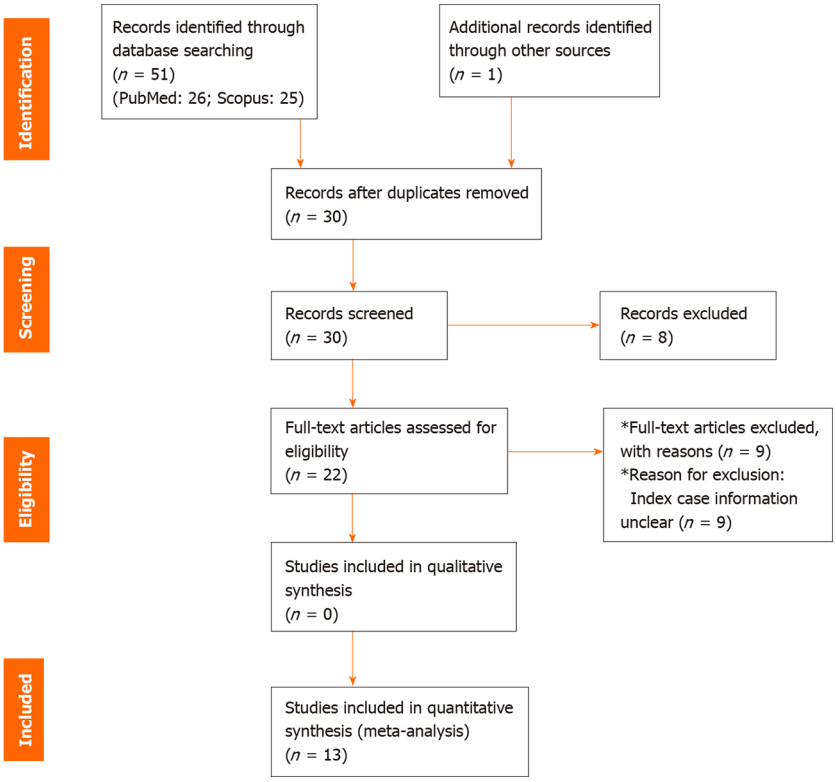

Figure 1 PRISMA diagram[9].

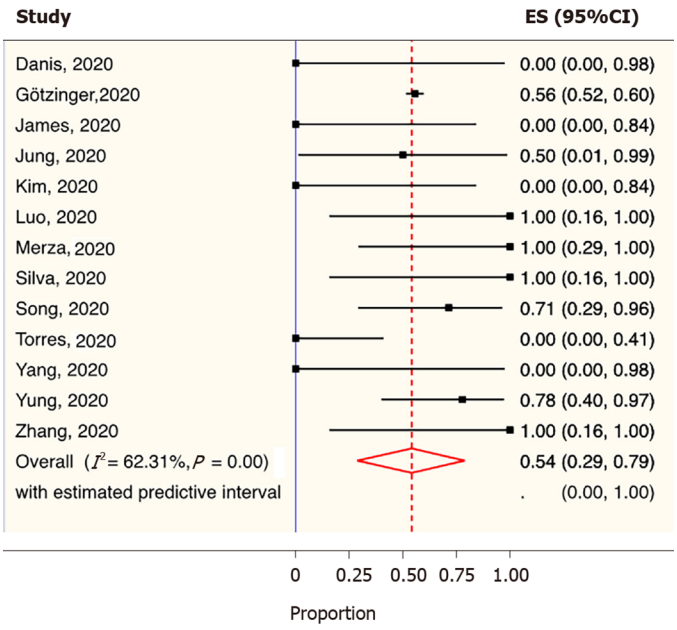

Figure 2 Forest plot depicting the overall weighted prevalence of parent being the index case in coronavirus disease 2019 infected children. The diamond is centred on the summary of the prevalence estimate, and the width indicates the corresponding 95% confidence interval. CI: Confidence interval.

CONCLUSION

In COVID-19 infected children, parents are frequently the index cases than any other individual. A considerable proportion of these parents are expected to be symptomatic when they transmit the disease to their wards. In this context, early detection of COVID-19 infected parents is likely to be a chief public health initiative.

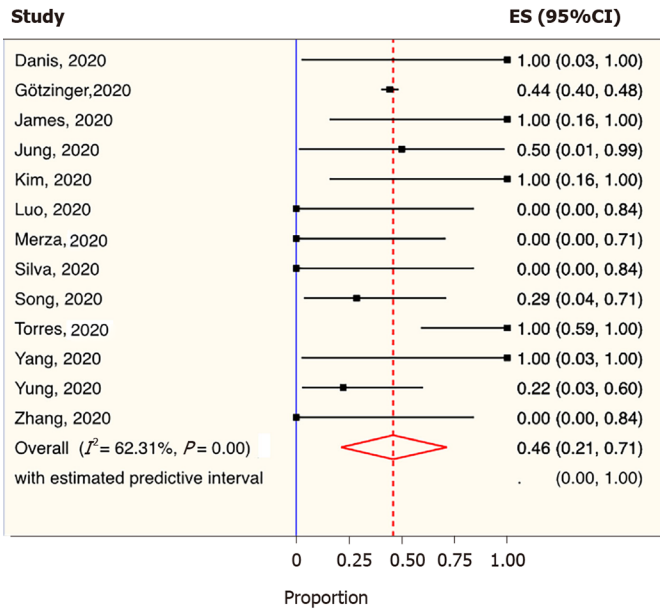

Figure 3 Forest plot depicting the overall weighted prevalence of parent not being the index case in coronavirus disease 2019 infected children. The diamond is centred on the summary of the prevalence estimate, and the width indicates the corresponding 95% confidence interval. CI: Confidence interval.

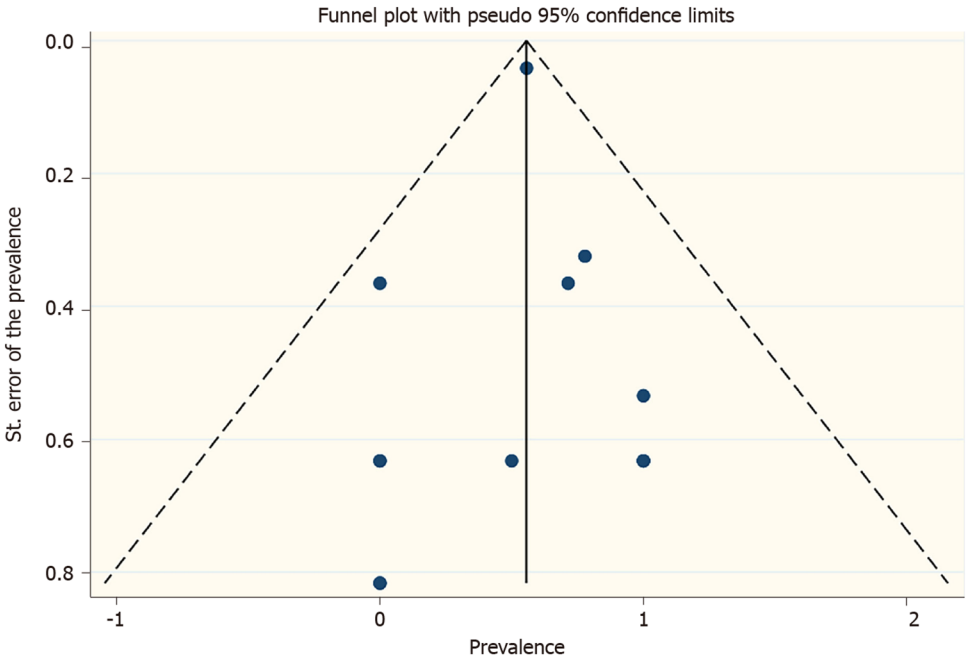

Figure 4 Funnel plot. Outcome: Parent being the index case of COVID-19 infected children.

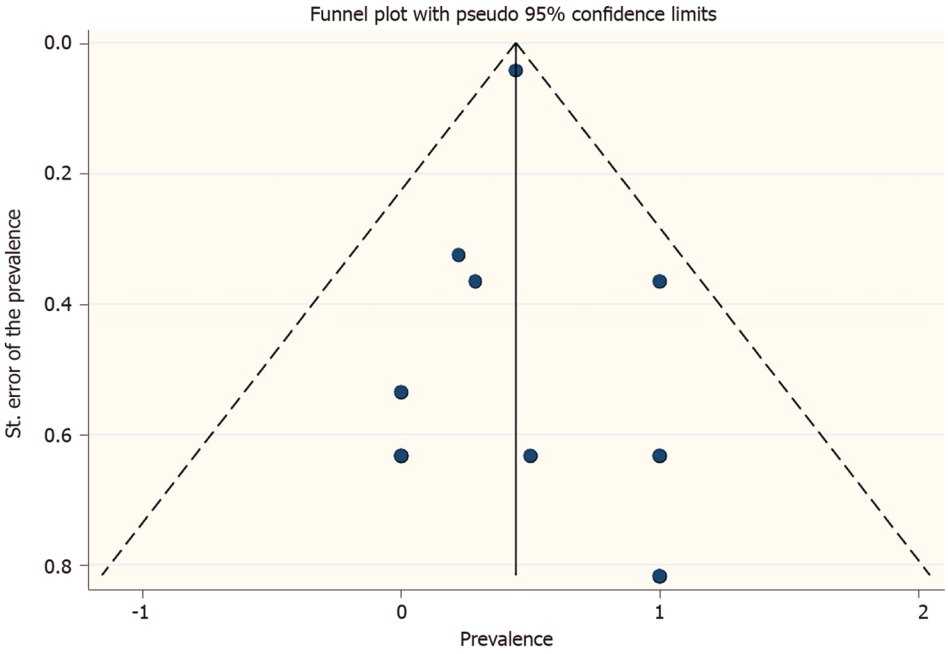

Figure 5 Funnel plot. Outcome: Parent being not being the index case of COVID-19 infected children.

ARTICLE HIGHLIGHTS

Research background

Presently little is known about the global epidemiological burden of parent-to-child coronavirus disease 2019 (COVID-19) disease transmissibility.

Research motivation

As children primarily remain at home with their parents due to the closure of schools across the globe (presumably to prevent community transmission of COVID-19 by children), it is crucial to know their domestic vulnerability to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection from their parents.

Research objectives

This study chiefly aimed to determine the pooled weighted prevalence of COVID-19 infection among children when the parent/s were their index cases.

Research methods

This meta-analysis incorporated articles reporting about the index case of COVID-19 infected ≤ 18 years old children by searching electronic databases. Besides data abstraction and critical appraisal of these studies, using random-effects meta-analysis, the weighted pooled prevalence of parents being and not-being the index case of the COVID-19 infected children was estimated.

Research results

This review included 13 studies published in 2020, reporting about 622 children from Asia, Europe, North America, and South America. Appraisable studies were fair in quality. The crude prevalence of parents being and not being the index cases of COVID-19 infected children were 54% (95%CI: 0.29-0.79; I^2: 62.3%, Chi^2 $P < 0.001$) and 46% (95%CI: 0.21-0.71; I^2: 62.3%; Chi^2 $P < 0.01$), respectively. For the former, on subgrouping by continent, the greatest burden was observed in Asia (75%), and most parents were symptomatic (73%).

Research conclusions

During the ongoing COVID-19 pandemic, a substantial proportion of the COVID-19 infected children acquired the disease from their parents, and the majority of these parents were symptomatic from SARS-CoV-2 infection.

Research perspectives

This research depicts a substantial global burden of parents being the index cases of COVID-19 infected children. It highlights the critical importance of early detection of these index cases.

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ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2021 March 20; 11(2): 15-22

Published by Baishideng Publishing Group Inc

World Journal of Methodology

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Bimonthly Volume 11 Number 2 March 20, 2021

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World Journal of Methodology

Bimonthly Volume 11 Number 2 March 20, 2021

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INDEXING/ABSTRACTING

The *WJM* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Gerhard Litscher

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2222-0682/editorialboard.htm

PUBLICATION DATE
March 20, 2021

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World J Methodol 2021 March 20; 11(2): 15-22

DOI: 10.5662/wjm.v11.i2.15

ISSN 2222-0682 (online)

MINIREVIEWS

Olfactory dysfunction in antineutrophil cytoplasmic antibody-associated vasculitides: A review of the literature

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Author contributions: Pendolino AL designed the study, performed the literature search and wrote the paper; Kaura A prepared the table and reviewed the paper; Navaratnam AV, Pendolino M, Bianchi G, Unadkat S, Randhawa PS and Ottaviano G reviewed the paper; Andrews PJ coordinated the writing of the paper and reviewed the final version.

Conflict-of-interest statement: There is no conflict of interest associated with any of the senior author or other co-authors contributed their efforts in this manuscript.

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Abstract

Olfactory dysfunction (OD) has been described in patients with antineutrophil cytoplasmic antibody-associated vasculitides (AAV), but the underlying mechanisms are not completely understood. The causes of altered smell function can generally be divided into conductive, sensorineural or others. To date no specific treatment is available for AAV-related OD and the efficacy of currently available options has not been explored. The aim of this review is to provide an overview of the causes that may lead to OD in patients with AAV. Current available treatments for OD and possible options in patients with AAV presenting with smell impairment are also mentioned.

Key Words: Smell; Olfactory dysfunction; Antineutrophil cytoplasmic antibody-associated vasculitis diseases; Granulomatosis with polyangiitis; Eosinophilic granulomatosis with polyangiitis; Microscopic polyangiitis

Manuscript source: Invited manuscript

Specialty type: Medical laboratory technology

Country/Territory of origin: United Kingdom

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: December 19, 2020
Peer-review started: December 19, 2020
First decision: December 31, 2020
Revised: January 2, 2021
Accepted: February 19, 2021
Article in press: February 19, 2021
Published online: March 20, 2021

P-Reviewer: Rolla G
S-Editor: Zhang L
L-Editor: A
P-Editor: Yuan YY

Core Tip: Olfactory dysfunction may develop during the course of antineutrophil cytoplasmic antibody-associated vasculitides. Typically, this is caused by a combination of active and chronic sinonasal inflammation causing necrosis and atrophic changes in the nasal mucosa, sensorineural involvement as well as other systemic factors. Systemic treatment of the vasculitis, control of coexisting rhinosinusitis, and management of nasal complications are recommended and could lead to an improvement in olfactory function.

Citation: Pendolino AL, Kaura A, Navaratnam AV, Pendolino M, Bianchi G, Unadkat S, Ottaviano G, Randhawa PS, Andrews PJ. Olfactory dysfunction in antineutrophil cytoplasmic antibody-associated vasculitides: A review of the literature. *World J Methodol* 2021; 11(2): 15-22
URL: https://www.wjgnet.com/2222-0682/full/v11/i2/15.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i2.15

INTRODUCTION

The antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) are multi-system autoimmune disorders, characterised by necrotising inflammation of the small to medium sized vessels with the presence of serum antibodies targeting cytoplasmic components of neutrophils. These antibodies specifically target proteinase 3 (PR3) and myeloperoxidase (MPO), contributing to a cytoplasmic or perinuclear pattern in indirect immunofluorescence staining respectively.

The American College of Rheumatology in 1990 classified granulomatous diseases into either granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA)[1,2]. Further to this, definitions for AAV were formulated at the Chapel Hill Consensus Conference in 1994 and later revised in 2012 and comprise GPA, EGPA and microscopic polyangiitis[3].

Ear, nose and throat involvement has been reported to be the second most common site after the lungs, and often precedes the diagnosis of AAV by many months[4]. The nose and paranasal sinuses, in particular, are the most frequently affected sites in the head and neck, representing 64% to 80% of the cases. Of importance, the nose has been shown to be the only affected site in about 30% of the GPA patients and hence a high index of suspicion is warranted[5].

Common manifestations of AAV in the nose include epistaxis, crusting and nasal polyps, or septal perforation and saddle nose deformity in the more severe cases. Impairment of sense of smell has also been described in patients with AAV, but the mechanisms underlying the olfactory dysfunction (OD) are not completely understood.

The aim of this review is to provide an overview of the causes that can lead to OD in patients with AAV. Current available treatments for OD and possible options in patients with AAV presenting with smell impairment are also mentioned.

CURRENT FINDINGS ON OLFACTORY DYSFUNCTION IN ANCA-ASSOCIATED VASCULITIDES

The involvement of the olfactory system in AAV has been reported by several authors, even if most of the available findings refer to GPA. Subjective assessment of olfaction and taste showed that patients with GPA are aware of their diminished chemosensory functions[6]. Göktas *et al*[7] found that chemical senses (*i.e.* olfactory, gustatory and trigeminal functions) are affected consistently and to a similar extent in GPA patients when they are assessed by means of psychophysical tests (*e.g.* Sniffin' sticks or the University of Pennsylvania Smell Identification Test)[7]. Proft *et al*[8] observed that 75% of their patients with GPA were found to be hyposmic at Sniffin' sticks; a similar prevalence of olfactory impairment (74%) in GPA patients was also reported by Zycinska *et al*[9]. In a retrospective analysis conducted on 230 GPA patients, Kühn *et al*[10] found that the majority of them were hyposmic (62%) with only 18% showing a preserved sense of smell (normosmia) and 20% having a complete smell loss

(anosmia). Fasunla *et al*[6] found that GPA patients were hyposmic with reduced scores in all the olfactory subset tests (identification, discrimination and threshold) when compared to the sex and age-matched healthy control group. These finding were later replicated by other authors[8,9,11].

Very little has been published on olfactory dysfunction in EGPA patients. In 2014 Tallab *et al*[12] reported a case of EGPA presenting with total loss of sense of smell measured by University of Pennsylvania Smell Identification Test, which appeared before the onset of other disease symptoms, suggesting that OD may precede the clinical symptoms[12].

To the best of our knowledge no cases of OD have been described in patients with microscopic polyangiitis.

CAUSES OF OLFACTORY DYSFUNCTION

The lower scores found by several authors in both the identification, discrimination and threshold olfactory subsets tests[8,9] may suggest that mechanisms leading to OD in AAV could be multifactorial[13].

Causes of altered olfactory function can generally be divided into: (1) Conductive, which include recurrent sinonasal inflammation, crusting, granuloma formation, bone structure damage, necrosis or atrophic changes in the mucous membrane; (2) Sensorineural, with a central or a peripheral neural involvement (focal ischemic changes and cranial nerve damage); and (3) Other factors, including systemic factors, concurrent surgery, the use of local (antibiotic ointments) or systemic immunosuppressive drugs[11].

However, and this is probably the most common situation, OD may result as a combination of two or more of the previously described mechanisms. Table 1 summarises the causes and mechanisms involved in the development of AAV-OD.

OLFACTORY DYSFUNCTION DUE TO CONDUCTIVE CAUSES

Sinonasal involvement has been reported to be the most frequent ear, nose and throat manifestation in patients with GPA[14]. Nasal ulceration, crusting and scarring are frequently seen in patients with AAV, especially in GPA[10], and in turn may cause a mechanical obstruction for odorants to reach the olfactory cleft, thus contributing to an altered sense of smell. However, Proft *et al*[8] found no correlation between OD and the aforementioned nasal manifestations, indicating that the decrease in olfactory function could be a consequence of the inflammatory disease in the nose rather than the local manifestation (bloody nasal discharge, crusts, granulomata). Similarly, none of the patients with localised disease had a higher degree of OD[11]. Therefore, active and chronic inflammation (rhinosinusitis) in GPA patients seems to be the more likely cause for the reported OD[9]. Due to the peculiar location of the olfactory fibres in the nose, smell impairment can result as an extension of the mucosal inflammation in the olfactory cleft and to the olfactory receptor cells. Reduction of sense of smell, in fact, is frequently reported in patients with chronic rhinosinusitis (CRS) and it is one of the main symptoms that can aid diagnosis of CRS[14]. As a confirmation of that, OD has been found to be common in GPA patients with increased crusting, pathological granulation but also a higher Lund-MacKay score (a score used for radiologic staging of CRS)[9].

Additionally, the colonisation of the affected nasal mucosa with bacteria such as Staphylococcus aureus may lead to recurrent nasal infections and further nasal inflammation. Having said that, no correlation between olfactory function and colonisation with Staphylococcus aureus was found[15].

Finally, septal perforations (commonly found in the more severe cases of GPA) can potentially cause OD by altering the flow of air and odour molecules into the olfactory cleft. However, Fasunla *et al*[6] did not identify any significant difference in olfactory performance assessed by means of Sniffin' Sticks (TDI score) between patients with or without septal perforation[6].

OLFACTORY DYSFUNCTION DUE TO SENSORINEURAL CAUSES

Cranial nerve involvement in patients with AAV is widely described[16-18]. In particular,

Table 1 Causes and mechanisms involved in the development of antineutrophil cytoplasmic antibody-associated vasculitides olfactory dysfunction

Ref.	Population	Findings
Conductive causes		
Laudien *et al*[11], 2009	76 GPA patients	No correlation between localised disease and a higher degree of olfactory dysfunction
Laudien *et al*[15], 2010	89 GPA patients	No correlation between olfactory function and colonisation with Staphylococcus aureus
Fasunla *et al*[6], 2012	16 GPA patients	No difference in the olfactory performance between patients with and without septal perforation
Proft *et al*[8], 2014	44 GPA patients	Decrease in olfactory function could be a consequence of the inflammatory disease in the nose rather than the local manifestations (bloody nasal discharge, crusting, granulomata)
Zycinska *et al*[9], 2016	43 GPA patients	Active and chronic sinonasal inflammation (rhinosinusitis) in GPA patients seems to be the more likely cause for the reported olfactory dysfunction. Olfactory dysfunction is more common in GPA patients with increased crusting, pathological granulation but also a higher Lund-MacKay score
Sensorineural causes		
Fauci *et al*[17], 1983	85 GPA patients	Cranial nerve involvement reported in 7.4% of GPA patients
Nishino *et al*[18], 1993	324 GPA patients	Cranial nerves involvement reported in 6.5% of GPA patients even if first cranial nerve was rarely involved
Other factors		
Laudien *et al*[11], 2009	76 GPA patients	GPA patients receiving local mupirocin treatment showed no olfactory dysfunction. No correlation between kidney involvement and smell function
Göktas *et al*[7], 2010	9 GPA patients	Neither the disease duration nor the age appear to influence smell function
Fasunla *et al*[6], 2012	16 GPA patients	No correlation between kidney involvement and smell function. GPA patients with and without past history of sinonasal operations did not show any significant difference in sense of smell
Proft *et al*[8], 2014	44 GPA patients	GPA patients with elevated CRP values showed lower scores from smell tests. GPA patients with a higher extent of damage showed a tendency for reduced scores only for the threshold, but not for the identification, the discrimination or the total score (TDI score). No correlation between kidney involvement and smell function. GPA patients under therapy with azathioprine showed significantly lower scores only for odour discrimination. GPA patients undergoing low-dose GC therapy showed a tendency for lower thresholds scores compared to patients without GC therapy
Tallab *et al*[12], 2014	1 EGPA patient	Subjective improvement of smell function after immunosuppressive therapy

GPA: Granulomatosis with polyangiitis; EGPA: Eosinophilic granulomatosis with polyangiitis; CRP: C-reactive protein; GC: Glucocorticoids.

GPA has repeatedly been associated with peripheral and cranial nerve neuropathies[16]. Fauci *et al*[17] found that 7.4% (8/85) of GPA patients had cranial nerve involvement whereas Nishino *et al*[18] reported a lower prevalence of 6.5% in their retrospective series of 324 GPA patients. Nevertheless, involvement of the first cranial nerve seems to be rare[18]. Theoretically, the olfactory nerve may be either involved by (1) Continuity, especially from nasal and skull base granulomas that can influence the neural transduction in afferent pathways; (2) By vasculitic involvement of small vessels surrounding the cranial nerves, resulting in mononeuritis multiplex; and (3) By the adverse influences of the disease on neurotransmitter systems involved in sensory modulation[12,18].

OTHER FACTORS INFLUENCING OLFACTORY FUNCTION

As with other autoimmune diseases, systemic factors could contribute to a lowering of the chemosensory function[19-21]. The active autoimmune processes and the linked cytokine secretion can potentially create a pro-inflammatory environment which can lead to olfactory neuronal damage, subsequent neuronal apoptosis[22] and finally leading to an olfactory function limitation. This hypothesis is supported by previous data from mice models, which showed that proinflammatory cytokines, like the tumour necrosis factor, can directly alter olfactory neuron function[23] and suppress neuroepithelial regeneration[24]. Moreover, lower scores from smell tests have been reported in GPA patients with elevated C-reactive protein values[8]. However, due to the lack of data in GPA patients undergoing olfactory epithelial biopsies, no definitive conclusions can be drawn on the role of immunologic mechanisms in OD in GPA patients or, more generally, in AAV.

Renal insufficiency is known to alter sense of smell[25] and renal function is commonly impaired in patients with AAV. Thus, this could account for OD in AAV patients. However, even though some authors have observed that non-AAV patients with chronic renal failure show OD with lower identification, discrimination and thresholds scores[25,26], no correlation between kidney involvement and smell function was found in GPA patients[6,8,11]. Similarly, no correlation between other organ involvement (eyes, ear, nose, throat, lung, or kidneys) and olfactory function has been reported in the same population[8,11].

The role of disease severity and duration in olfactory impairment is still not clear. Proft *et al*[8] found that GPA patients with a higher extent of damage (measured by the Vasculitis Damage Index score) showed a tendency for reduced scores only for the threshold, but not for the identification, the discrimination or the total score (TDI score) at Sniffin' sticks. Conversely, neither the disease duration nor the age appear to influence smell function[7].

Drug-induced OD has been widely described[27-29]. Damage to the chemosensory functions secondary to immunomodulatory drugs has been reported by different authors although the exact mechanism through which these drugs can induce olfactory damage is unclear. An impairment of fast regenerating tissue (olfactory epithelium) by chemotherapeutic agents has been hypothesised[8,30]. Furthermore, the role of the tumour necrosis factor-alpha-inhibitors or methotrexate on smell was investigated in patients suffering from rheumatoid arthritis but no significant differences in smell between users and non-users of these medications were found[31]. Nevertheless, GPA patients under therapy with azathioprine showed significantly lower scores only for odour discrimination at the Sniffin' sticks subtest[8]. Conversely, Tallab *et al*[12] reported a subjective improvement of smell and taste function after immunosuppressive therapy in a patient with EGPA.

Mupirocin is commonly used as a topical antibiotic in AAV patients presenting with crusting and recurrent nasal infections. In this regard, no olfactory dysfunction was observed by Laudien *et al*[11] in 25 GPA patients receiving local mupirocin treatment.

Oral glucocorticoids (GC) represent a keystone in medical treatment in patients with AAV. While systemic GC improve the sense of smell in patients with rhinosinusitis[28] and high dose GC is used to treat olfactory disorders[32,33], low-dose GC has been shown to probably reduce the peripheral olfactory function[31]. Proft *et al*[8] found that GPA patients undergoing low-dose GC therapy showed a tendency for lower thresholds scores compared to patients without GC therapy. Similarly, low-dose GC in rheumatoid arthritis significantly affects thresholds and TDI scores, but not odour identification and discrimination[31].

Finally, the prolonged and complicated wound healing observed in some AAV patients undergoing nasal surgery could lead to an impairment of the chemosensory function. However, Fasunla *et al*[6] did not find any significant difference in sense of smell when GPA patients with and without past history of sinonasal operations were compared.

CURRENT AVAILABLE TREATMENTS

To date no specific treatment is available for AAV-related OD and the efficacy of treatment options currently adopted for smell impairment has not been explored. Moreover, the lack of a deep understanding of the underlying mechanisms leading to smell loss in this category of patients is limiting the development of more targeted therapies.

In our experience, if a patient presents with nasal crusting or epistaxis, control of the nasal manifestations should be first achieved to rule out any mechanical obstruction (conductive cause) leading to smell impairment. If polyps or mucosal oedema are present, the use of GC, either systemically or topical, should be considered in order to reduce the inflammatory component[33]. However, while indications for oral GC for OD in AAV patients may be reviewed in light of the previously described findings[8,31], the use of topical GC should be weighed up in patients presenting with crusting and epistaxis due to the possibility of an increased risk of bleeding and mucosal dryness with subsequent crusting and local infections. Management of septal perforations, either conservatively or surgically[34,35], should be considered due to the possible relationship between airflow alteration and the consequent obstacle for odour molecules to reach the olfactory cleft. Importantly, if surgical closure of the perforation is planned, it should only be performed when the disease is in remission so as to minimise complications[34].

Smell training can also be considered a potential therapy in AAV patients reporting persistent smell impairment, especially where no other conductive causes can be identified. It is a cost-effective and safe therapy which involves repeated and deliberate sniffing of a set of odorants (commonly lemon, rose, cloves, and eucalyptus) for 20 s each, at least twice a day for a minimum of 3 mo. Smell training is currently recommended in patients with olfactory loss from several aetiologies, even if its effectiveness in patients with AAV has not been explored so far[33].

Other treatments, including phosphodiesterase inhibitors, intranasal calcium buffers, vitamin A, zinc or multivitamins have been described but they are not routinely recommended because of the lack of sufficient evidence[33].

CONCLUSION

This review confirms that OD may develop during the course of AAV. The current literature suggests that olfactory impairment is more frequent in patients with GPA, although a possible bias related to the scarcity of papers about smell loss in patients with EGPA and MPA must be taken into account.

Several mechanisms can lead to smell loss in AAV patients, and in most cases, this arises from a combination of different causes. Systemic treatment of the vasculitis, control of a coexisting rhinosinusitis, and management of nasal complications may improve olfactory function, and should be considered in all patients. To date no specific treatment for olfactory improvement in this particular group of patients has been identified. In the future, cytological and histological studies of the affected olfactory mucosa should be encouraged in order to better understand the mechanisms underlying the OD in AAV.

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ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2021 May 20; 11(3): 23-109

Published by Baishideng Publishing Group Inc

World Journal of Methodology

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INDEXING/ABSTRACTING

The *WJM* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Gerhard Litscher

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2222-0682/editorialboard.htm

PUBLICATION DATE
May 20, 2021

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World J Methodol 2021 May 20; 11(3): 23-45

DOI: 10.5662/wjm.v11.i3.23

ISSN 2222-0682 (online)

REVIEW

Epidemiological link between obesity, type 2 diabetes mellitus and cancer

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Author contributions: Fernandez CJ and George AS performed extensive literature review, drafted the initial manuscript, created the pictures and share the first authorship; Subrahmanyan NA added points with additional literature review, help with the pictures and revision; Pappachan JM conceived the idea, inputted additional scientific points especially the obesity and metabolic aspects of the paper, revised the entire work critically, and approved the final version for publication; all the authors contributed to revision of the paper after peer reviews.

Conflict-of-interest statement: Dr. Pappachan and co-authors have nothing to declare.

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Abstract

There exists a complex interaction between obesity, type 2 diabetes mellitus (T2DM) and cancer, and an increase in the incidence of cancer is expected with the growing obesity-diabetes pandemic. The association of cancer with diabetes mellitus and obesity appears to be site-specific, the highest risk being for post-menopausal breast cancer, endometrial cancer, and colorectal cancer. Moreover, there is worsening of hyperglycaemia with the onset of cancer, evidencing a bi-directional link between cancer and diabetes mellitus and the need for monitoring for diabetes in cancer survivors. In this review, we look at the epidemiological evidence from observational studies and Mendelian randomization studies linking obesity, diabetes, and cancer, as well as the complex pathophysiological mechanisms involved, including insulin resistance with associated hyperinsulinaemia, the effect of chronic low-grade inflammation, and the effect of various adipokines that are associated with obesity and T2DM. Additionally, we describe the novel therapeutic strategies, based on their role on the discrete pathophysiological mechanisms involved in the tumourigenesis.

Manuscript source: Invited manuscript

Specialty type: Medical laboratory technology

Country/Territory of origin: United Kingdom

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

Received: January 13, 2021
Peer-review started: January 13, 2021
First decision: March 1, 2021
Revised: March 2, 2021
Accepted: March 19, 2021
Article in press: March 19, 2021
Published online: May 20, 2021

P-Reviewer: Cao ZF, Ghobadloo SM
S-Editor: FanJR
L-Editor: A
P-Editor: Xing YX

Key Words: Cancer; Obesity; Type 2 diabetes mellitus; Hyperinsulinaemia; Epidemiological link

Core Tip: Cancer is the second most common cause of death globally, and the complex pathogenic mechanisms in the development of cancer are not yet fully elucidated. The interplay between obesity, type 2 diabetes and some forms of cancer are well known for the past few years. With a steady increase in the obesity and diabetes pandemics, the incidence of cancer is expected to increase exponentially in the coming years. This review discusses the complex pathophysiological mechanisms linking these three major disease entities, to enhance clinician awareness across the globe, and proposes emerging potential therapeutic strategies.

Citation: Fernandez CJ, George AS, Subrahmanyan NA, Pappachan JM. Epidemiological link between obesity, type 2 diabetes mellitus and cancer. *World J Methodol* 2021; 11(3): 23-45
URL: https://www.wjgnet.com/2222-0682/full/v11/i3/23.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i3.23

INTRODUCTION

Cancer is the second most common cause of mortality from non-communicable diseases in the world, accounting for nearly 9.6 million deaths in the year 2017[1]. Apart from the excess mortality, cancer also contributed to 233.5 million disability-adjusted life-years in 2017. The incidence of cancer has been rising, due to a rise in the associated risk factors like aging population, obesity, diabetes mellitus, and lifestyle-related factors. Globally, the incidence of obesity has reached pandemic proportions, irrespective of the socioeconomic status and the age group[2]. Rising proportion of individuals with obesity has been the driving force for the diabetes pandemic[3], the incidence/prevalence of which is increasing at a faster rate in low-income and middle-income countries than in high-income countries[4].

There is plenty of evidence supporting the association between cancer and either obesity or diabetes mellitus on an individual basis. A recent study evaluated the impact of combined obesity and diabetes mellitus on cancer risk, by calculating the population attributable fraction (PAF) of incident cancers attributable to obesity and diabetes mellitus[5]. They observed that 5.7% of all incident cancers in 2012 were related to the combined effects of diabetes mellitus and obesity. When they limited their calculation to include only twelve adiposity-related cancers (colorectal cancer, postmenopausal breast cancer, endometrial cancer, gallbladder cancer, pancreatic cancer, liver cancer, kidney cancer, ovarian cancer, gastric-cardia cancer, thyroid cancer, multiple myeloma, and oesophageal adenocarcinoma), and six diabetes-related cancers (colorectal cancer, endometrial cancer, breast cancer, gallbladder cancer, pancreatic cancer, and liver cancer), they observed that 13.5%-15.3% of the cancers were attributable to the combined effects of diabetes mellitus and obesity. The study also observed that nearly one-fourth of the diabetes-related cancers and one-third of the adiposity-related cancers, happened due to a rise in prevalence of these risk factors[5].

As the global burden of obesity and diabetes mellitus is going to rise further, the burden of cancer will continue to increase. Therefore, interventions should be done at multiple levels including individual, community, health-care system, and policy making to prevent the development of cancer from these non-communicable diseases. This review will discuss the epidemiological studies linking obesity and type 2 diabetes mellitus (T2DM) to cancer and will explore the potential pathophysiological mechanisms linking obesity, and T2DM to cancer.

EPIDEMIOLOGICAL STUDIES LINKING OBESITY TO CANCER

The obese population shows an increase in relative risk (RR) for developing various cancers, compared to the non-obese population. A recently published systematic review[6] using the data collected from a meta-analysis of epidemiological studies observed that the RR was highest for endometrial cancer (2.54; 95%CI: 2.11-3.06)[7], followed by renal cancer (1.77; 95%CI: 1.68-1.87)[8]. This was followed by pancreatic cancer (1.48; 95%CI: 1.15-1.92)[9], breast cancer (1.42; 95%CI: 1.30-1.45)[10], liver cancer (1.35; 95%CI: 1.24-1.47)[11], colorectal cancer (1.32; 95%CI: 1.18-1.48)[12], melanoma (1.31; 95%CI: 1.19-1.44)[13], ovarian cancer (1.30; 95%CI: 1.10-1.50)[14], thyroid cancer (1.29; 95%CI: 1.18-1.41)[15], leukaemia (1.26; 95%CI: 1.17-1.37)[13], prostate cancer (1.16; 95%CI: 1.08-1.24)[16], gastric cancer (1.13; 95%CI: 1.03-1.24)[17], and bladder cancer (1.10; 95%CI: 1.06-1.42)[18]. However, a previous study also noted that the obese population has a low RR of getting lung cancer (0.79; 95%CI: 0.73-0.85) compared to the non-obese population, indicating an inverse association[19]. The RR for squamous cell carcinoma, adenocarcinoma, and small cell carcinoma of the lung were 0.68 (95%CI: 0.58-0.80), 0.79 (95%CI: 0.65-0.96), and 0.99 (95%CI: 0.66-1.48) respectively indicating that obesity is protective against all types of lung cancer among both current and former smokers.

Obesity is associated with an increased risk of some cancers and decreased risk of other cancers, suggesting that the association between obesity and cancer clearly depends on the site of the cancer (site-specific association). This suggests that if the epidemiological studies analysing the relationship between obesity and cancer are not adequately stratified for the site of cancer, the associations with less common cancers can be masked. Nearly 4% of all new cancers can be attributed to overweight and obesity (adiposity-related cancers), in which endometrial, postmenopausal breast, and colorectal cancers account for more than 60%[20]. Worldwide, the population attributable fraction (PAF) of cancer related to high body mass index (BMI) was greater among women compared to men (5.4% *vs* 1.9%). Moreover, the countries with very high and high human development index (HDI) had higher PAF (5.3% and 4.8%, respectively), compared to countries with moderate and low HDI (1.6% and 1.0%, respectively)[20]. With increasing rates of obesity at younger age, the adiposity-related cancers are detected at a much younger age.

A dose-response meta-analysis of prospective observational studies reported that each 5 kg weight gain is associated with an increase in the RR for postmenopausal endometrial cancer by 39% among hormone replacement therapy (HRT) non-users (RR 1.39; 95%CI: 1.29-1.49) and by 9% among HRT users (RR 1.09; 95%CI: 1.02-1.16)[21]. Similar weight gain is associated with an increase in RR for postmenopausal ovarian cancer by 13% among HRT non-users (RR 1.13; 95%CI: 1.03-1.23), postmenopausal breast cancer by 11% among HRT non-users (RR 1.11; 95%CI: 1.08-1.13), and colorectal cancer by 9% in men (RR 1.09; 95%CI: 1.04-1.13). Weight gain is also associated with a 42% increase in the RR for renal cancer when the highest and lowest level of adult weight gain are compared (RR 1.42; 95%CI: 1.11-1.81)[21]. However, weight gain is not associated with a rise in colorectal cancer in women, premenopausal breast cancer, postmenopausal breast cancer among HRT users, prostate cancer, and thyroid cancer.

A meta-analysis of one hundred and twenty-six observational cohort studies among breast cancer patients reported that each 5 kg of adult weight gain is associated with a 7% increase in postmenopausal breast cancer (RR 1.07; 95%CI: 1.05-1.09), and each 5 kg/m^2 of gain in BMI is associated with a 17% increase in postmenopausal breast cancer (RR 1.17; 95%CI: 1.11-1.23)[22]. Moreover, each 10 cm increase in waist circumference (WC) and hip circumference (HC), are associated with 11% (RR 1.11; 95%CI: 1.08-1.14) and 6% (RR 1.06; 95%CI: 1.04-1.09) increase in postmenopausal breast cancer, respectively. Furthermore, each 0.1 unit increase in waist-hip ratio is associated with a 10% increase in postmenopausal breast cancer (RR 1.10; 95%CI: 1.05-1.16). The increased risk was noted among hormone receptor positive breast cancers compared to receptor negative breast cancers, and among HRT non-users compared to HRT users. Adult weight gain and BMI gain are not consistently associated with premenopausal breast cancer. Each 5 kg of adult weight loss is associated with a 4% decrease in postmenopausal breast cancer (RR 0.96; 95%CI: 0.88-1.04). The study reported that BMI gain in early adult life (between 18-30 years) is inversely associated with postmenopausal (RR 0.81; 95%CI: 0.75-0.87), and premenopausal (RR 0.86; 95%CI: 0.78-0.96) breast cancer[22].

Another meta-analysis of seven prospective observational studies comprising of 18668 men and 24751 women with a mean age of 62 and 63 years (respectively), with a median follow-up period of 12 years reported 1656 first-incident adiposity-related

cancers including postmenopausal breast, colorectum, lower oesophagus, gastric, liver, gallbladder, pancreas, endometrium, ovary, and kidney cancers[23]. The hazard ratios (HR) for first-incident cancers, *per* standard deviation increment in various adiposity indicators including BMI, WC, HC, and waist-hip ratio (WHR) were calculated. The results were 1.11 (95%CI: 1.02-1.21) for BMI, 1.13 (95%CI: 1.04-1.23) for WC, 1.09 (95%CI: 0.98-1.21) for HC, and 1.15 (95%CI: 1.00-1.32) for WHR. For example, the HR for colorectal cancer for each standard deviation increment in BMI, WC, HC, and WHR are 16%, 21%, 15%, and 20%, respectively. These values are not surprising as WC and WHR are better surrogate markers of visceral fat, than BMI. Moreover, HRT non-users have 20% increased risk *per* standard deviation of BMI, WC, and HC for getting postmenopausal breast cancer, compared to HRT users[23].

A recent prospective study evaluated the effect of weight gain during adult years with or without metabolic dysfunction on the risk of getting adiposity-related cancers[24]. The study reported that, compared to people maintaining a stable weight, those with weight gain of greater than 0.45 kg or 1 pound/year was associated with 38% increase in overall cancer risk (HR 1.38; 95%CI: 1.09-1.76), with women (HR 1.39; 95%CI: 1.03-1.87) having higher risk compared to men (HR 1.32; 95%CI: 0.88-2.00). Compared to weight gain without metabolic dysfunction [metabolically healthy obesity; (MHO)], weight gain with metabolic dysfunction increases the overall risk of cancer risk by 77% (HR 1.77; 95%CI: 1.21-2.59), with men (HR 1.85: 95%CI: 1.00-3.44) having higher risk compared to women (HR 1.74; 95%CI: 1.07-2.82). The study also observed that men and women who gained weight during adult life from non-overweight status at baseline, were associated with 2.18-fold and 1.60-fold overall cancer risk, whereas those who were overweight throughout the study period (from baseline) were associated with statistically non-significant increased cancer risks of 28% (HR 1.28; 95%CI: 0.76-2.14) and 33% (HR 1.33; 95%CI: 0.94-1.88), in men and women, respectively[24].

Nearly 10%-30% of obese individuals are metabolically healthy with lesser visceral and hepatic fat, greater leg fat, expandable subcutaneous fat, preserved cardiorespiratory fitness, insulin sensitivity, and beta cell/adipose tissue function, and lower inflammatory burden[25]. Though there is no standard definition for MHO, presence of obesity with normal glucose and lipid parameters in the absence of hypertension can be used as a criterion to diagnose MHO. Though the risk for getting T2DM and cardiovascular disease is much lower in MHO people compared to people with metabolically unhealthy obesity (MUO), it is still higher than metabolically healthy lean (MHL) people. Moreover, MHO is a transient phenotype that can progress to develop MUO. Hence, MHO should still be considered as an indication for weight loss interventions[25]. A meta-analysis of eight prospective cohort studies comprising of 12542390 participants compared the incidence of any type of cancer in MHO people in comparison to people with metabolically healthy non-obesity (MHNO)[26]. They reported a significantly higher risk of developing cancer with an odds ratio (OR) of 1.14 (95%CI: 1.05-1.23) compared to MHNO people, and 1.17 (95%CI: 1.01-1.35) compared to MHL people. This suggests that all obese individuals, even in the absence of metabolic dysfunction, should be encouraged to lose weight.

A meta-analysis of 230 cohort studies including over 30 million individuals observed that, though overweight and obesity were associated with an increased risk of all-cause mortality, there was a U-shaped association[27]. The concept that cancer patients with elevated BMI might have improved survival compared to cancer patients with normal BMI is known as 'obesity paradox in cancer'. According to many, the term 'obesity paradox' is misleading as the paradox is due to the limitations of BMI, which relies on height and weight without delineating the distribution of adipocytes or distinguishing between adipose tissue and skeletal muscle. According to them, cancer patients with higher BMI might be having higher levels of protective skeletal muscle mass[28]. Others consider that, the paradox is due to methodological flaws including reverse causation, selection bias, and confounding[29]. However, a recent meta-analysis of 203 observational studies including 6320365 participants observed that even though obesity is associated with increased overall mortality, cancer specific mortality, and relapse rate in various cancers, it (obesity) is associated with an apparent protective effect in patients with lung cancer and melanoma[30].

Another meta-analysis of eight population-based cohort studies including 635642 participants who underwent bariatric surgery observed that, bariatric surgery is associated with a significantly reduced incidence of cancer (OR 0.72; 95%CI: 0.59-0.87) overall, and obesity-related cancer in particular (OR 0.55; 95%CI: 0.31-0.96)[31]. However, the reduction in incidence of breast cancer reached statistical significance (OR 0.50; 95%CI: 0.25-0.99), whereas reduction in other cancers did not reach statistical significance. A recent meta-analysis of 21 cohort studies comprising of 304516

participants who underwent bariatric surgery, revealed that bariatric surgery was not only associated with decreased cancer incidence (OR 0.56; 95%CI: 0.46-0.68), but also with decreased cancer mortality (OR 0.56; 95%CI: 0.41-0.75)[32]. The study also observed a significant reduction in breast and endometrial cancers in post-bariatric surgery participants.

Few observational studies reported a controversial observation about an increased incidence of colorectal cancer, in the post-bariatric surgery participants[33]. However, even in these trials, the absolute incidence of colorectal cancer was lower in the bariatric surgery group compared to the obese patients who did not undergo bariatric surgery. The cessation of statin therapy, avoidance of high fibre diet, and changes in colonic microbiome after bariatric surgery could explain a possible increase in the incidence of colorectal cancer in post-bariatric surgery cases.

A large study including 22198 participants who underwent bariatric surgery from the Kaiser Permanente Integrated health data reported a 33% reduction in any cancer incidence (HR 0.67; 95%CI: 0.60-0.74), and 41% reduction in adiposity-related cancer incidence (HR 0.59; 95%CI: 0.51-0.69)[34]. Among the adiposity related cancers, surgery is associated with a statistically significant reduction in postmenopausal breast (HR 0.58; 95%CI: 0.44-0.77), colon (HR 0.59; 95%CI: 0.36-0.97), endometrial (HR 0.50; 95%CI: 0.37-0.67), and pancreatic cancers (HR 0.46; 95%CI: 0.22-0.97), compared to obese patients who did not undergo bariatric surgery. Furthermore, a recent meta-analysis of seven studies including 1213727 participants observed that bariatric surgery reduces colorectal cancer by 36% (RR 0 ·64, 95%CI: 0.42-0.98)[35].

Epidemiological studies linking diabetes to cancer

Observational studies have consistently reported that people with T2DM have an increased risk for several types of cancers including liver, pancreas, endometrium, colorectal, breast, and bladder, and a decreased risk for prostate cancer. The observed association between T2DM and cancer could either be a causal (caused by hyperinsulinaemia or hyperglycaemia), or be a confounder (arising from common risk factors such as adiposity)[36]. The contributions from obesity and T2DM towards tumourigenesis can be independent as exemplified by prostate cancer, the incidence of which is increased with obesity, but decreased with T2DM. Another example is lung cancer, the incidence of which is lower in obesity, but not altered with T2DM. The contributions of obesity and T2DM towards cancer can have an additive (synergistic) effect or an opposing effect, depending on the site of origin of cancer[6].

An umbrella review of 'meta-analyses of observational studies that examined the association between T2DM and cancer' carefully assessed the robustness of the reported associations, considering the quality of the studies and their substantial heterogeneity[36]. The review observed that only a minority of these reported associations have evidence-base without hints of bias. These observed summary associations in the descending order of random effects include endometrial cancer (1.97; 95%CI: 1.71-2.27), intrahepatic cholangiocarcinoma (1.97; 95%CI: 1.57-2.46), colorectal cancer (1.27; 95%CI: 1.21-1.34), and breast cancer (1.20; 95%CI: 1.12-1.28).

A meta-analysis of forty-five observational studies comprising more than eight million participants and 132331 prostate cancer patients revealed a statistically significant inverse association between T2DM and carcinoma of prostate (RR 0.86; 95%CI: 0.80-0.92)[37]. One point supporting the lower incidence of cancer prostate in T2DM is the fact that some men with T2DM with/without obesity have lower androgen levels that results in reduced stimulation of androgen sensitive prostate cancer cells[38]. Another point supporting the lower incidence of cancer prostate is a lower circulating prostate-specific antigen levels seen in men with T2DM with high hemoglobin A1c and fasting blood glucose in the obese, and men with raised alanine transaminase levels which would delay the diagnosis of cancer prostate[39].

Among cancer patients with pre-existing diabetes mellitus, there is a 41% increase in all-cause mortality (HR 1.41; 95%CI: 1.28-1.55)[40]. A subgroup analysis showed increased all-cause mortality with cancers of endometrium (HR 1.76; 95%CI: 1.34-2.31), breast (HR 1.61; 95%CI: 1.46-1.78), and colorectum (HR 1.32; 95%CI: 1.24-1.41). Another meta-analysis on colorectal cancer patients with pre-existing diabetes mellitus observed that the all-cause mortality was increased by 17% (RR 1.17; 95%CI: 1.09-1.25), and cancer specific mortality by 12% (RR 1.12; 95%CI: 1.01-1.24), compared to colorectal cancer patients without diabetes mellitus[41]. Moreover, presence of pre-existing diabetes mellitus was associated with a 51% higher post-operative mortality (OR 1.51; 95%CI: 1.13-2.02) among cancer patients[42]. Cancer patients with pre-existing diabetes mellitus exhibited advanced stage of the disease at the time of diagnosis[43], increased risk of cancer recurrence[44], and decreased disease-free survival (RR 1.27; 95%CI: 1.06-1.52)[41].

However, we ought to bear in mind that observational epidemiological studies are susceptible to certain biases including reverse causality bias, detection bias, and depletion of the susceptible[45]. Mendelian randomization (MR) studies are analytic methods (genetic epidemiological studies) that are used to strengthen the evidence for a causal relationship between an exposure and an outcome. MR studies utilize germline variants obtained from large-scale genome-wide association studies. As these germline variants are determined at the time of birth and remain constant throughout life[46], studies utilizing them will minimize the effects of bias and residual confounding that are observed in observational studies. However, the genetic observational or MR studies have their own strengths and weaknesses. Once the observational and genetic epidemiological studies agree between each other, the results are likely to be more robust.

MR studies have shown that adiposity has a very strong causal association with renal, endometrial, ovarian, oesophageal, pancreatic, and colorectal cancer[46]. Hyperinsulinaemia has a strong association with endometrial, breast, pancreatic and renal cancer risk. Raised circulating insulin-like growth factor-1 (IGF-1) levels have a moderate association with breast and prostate cancer risk. Sex hormone dysregulation and puberty timing have a moderate association with breast and endometrial cancer risk; puberty timing has a moderate association with prostate cancer risk. There is only a weak association between hyperglycaemia and various cancers including those of lung, pancreas, endometrium, kidney, and breast. Finally, no association is observed between T2DM and cancers including pancreatic, endometrial, renal cell, and ovarian cancers[46].

POTENTIAL PATHOPHYSIOLOGICAL MECHANISMS LINKING OBESITY AND DIABETES TO CANCER

Direct effects of hyperinsulinaemia in the pathogenesis of cancer: Insulin receptor/IGF-receptor signalling

The increased incidence of various cancers including breast, endometrial, and colorectal cancers is observed within few months after the diagnosis of T2DM, or even in the prediabetic phase, indicating that in patients with T2DM, it is the endogenous hyperinsulinaemia, rather than hyperglycaemia, that is associated with an increased risk of cancer[47-50]. Moreover, in breast, colorectal and endometrial cancer patients, the endogenous hyperinsulinaemia is associated with cancer progression, recurrence, and excess mortality[51-54]. Compared to normal cells which preferentially rely on mitochondrial oxidative phosphorylation, the cancer cells rely on glycolysis, even in the presence of oxygen (aerobic glycolysis), as a source of energy, possibly due to damaged mitochondria in cancer cells and also as a measure to maximize the available energy sources to support the rapid proliferation. This observation, known as the Warburg effect, suggests an increased glucose uptake and increased reliance on glucose metabolism by the cancer cells[55].

Studies have shown that hyperglycaemia alone may not cause development of cancer in the absence of hyperinsulinaemia, indicating that the key driver of cancer initiation and progression in patients with diabetes, and obesity is hyperinsulinaemia[56]. However, there are multiple other mechanisms involved in cancer initiation and progression. The overall pathophysiological mechanisms linking obesity and diabetes to cancer and the associated intracellular signalling, are illustrated in Figure 1 and the pathophysiological mechanisms linking hyperin-sulinaemia in the tumour microenvironment (TME) to cancer, is represented in the Figure 2.

The insulin/IGF family consists of ligands including insulin, IGF-1, and IGF-2; their tyrosine kinase receptors including insulin receptor-A (IR-A), insulin receptor-B (IR-B), IGF-1 receptor (IGF-1R), IR-A/IGF-1R hybrid, and IR-B/IGF-1R hybrid; and six IGF-binding proteins (IGFBPs) that bind to IGF-1 and IGF-2, but not to insulin. Only the free IGFs, unbound to IGFBPs, are biologically available for binding to their receptors. As the IGFs bound to IGFBPs are protected from degradation, the IGFBPs maintain a stable serum IGF levels. Hyperinsulinaemia decreases IGFBP-1 and IGFBP-2 levels, thus increasing the levels of bioavailable IGF-1 and IGF-2[57]. Moreover, hyperinsulinaemia increases the IGF-1 level by increasing its hepatic production[58]. Apart from the IGFs that circulate in blood, a significant amount of IGF-2 is also secreted by cancer cells and/or tumour stroma to act on IR-A[59].

The IR signalling exerts both metabolic and mitogenic effects. Among the two isoforms that are formed by differential splicing of the insulin receptor gene (splice

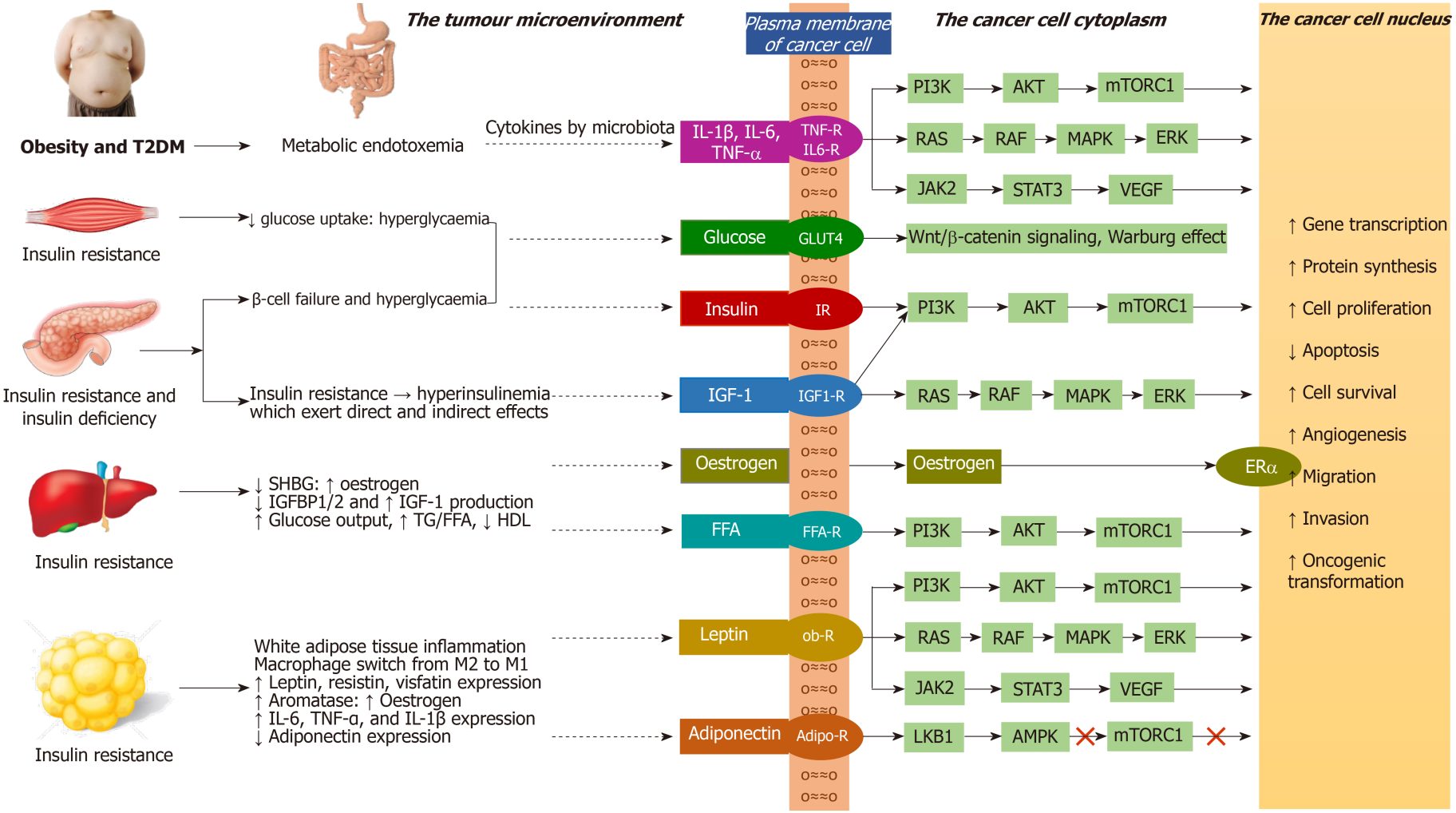

Figure 1 The overall pathophysiological mechanisms linking obesity and diabetes to cancer with associated intracellular signalling. IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumour necrosis factor-α; IR: Insulin receptor; IGF-1: Insulin-like growth factor-1; IGF-1R: Insulin-like growth factor-1 receptor; IGFBP: Insulin-like growth factor binding protein; FFA: Free fatty acid; FFF-R: Free fatty acid receptor; ER-α: Oestrogen receptor-α; Ob-R: Leptin-receptor; Adipo-R: Adiponectin-receptor; SHBG: Sex hormone binding globulin; TG: Triglyceride; HDL: High density lipoprotein; PI3K: Phosphatidyl-inositol-3-kinase; AKT: Protein kinase B; mTORC1: Mechanistic target of rapamycin complex 1 (Mammalian target of rapamycin complex 1); RAS: Rat sarcoma; RAF: Rapidly accelerated fibrosarcoma; MAPK: Mitogen activated protein kinase; ERK: Extracellular-regulated kinase; JAK2: Janus kinase-2; STAT3: Signal transducer and activator of transcription-3; VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia inducible factor-1α; LKB1: Liver kinase B1; AMPK: Adenosine monophosphate-activated protein kinase; T2DM: Type 2 diabetes mellitus.

variants), the IR-B is predominantly expressed in the metabolic tissues including liver, skeletal muscle, adipose tissue, and kidney, whereas IR-A is mainly expressed in the foetal and cancer tissues[60]. IR-B predominantly exerts metabolic effects, whereas IR-A predominantly exerts mitogenic effects. The ratio of IR-A to IR-B in the cell is determined by the expression of certain splicing factors in cells. Insulin, IGF-1, and IGF-2 bind to IR-A, and IR-B with different affinities. Insulin binds to IR-A with a 1.7-fold greater affinity compared to IR-B (only a modest difference in affinity). IGF-2 binds to IR-A with a 40-fold greater affinity compared to IR-B, whereas IGF-1 binds to IR-A with a 10-fold greater affinity compared to IR-B. Insulin binds only to IR-B or IR-A, not to IGF-1R or hybrid receptors. Both IGF-1 and IGF-2 bind to IGF-1R, hybrid receptors, and to IR-A or IR-B. IR-A has 100-fold higher affinity for IGF-2 compared to IGF-1[60]. Thus, IR-A has high affinity for IGF-2 and low affinity for IGF-1, whereas IR-B has a low affinity for IGF-2 and a very low affinity for IGF-1. High IR-A expression, resulting from altered expression of splicing factors in the cell is detrimental in adult life as it is associated with insulin resistance, dysregulated cell proliferation and cancer[61].

While normal cells downregulate the IRs in presence of hyperinsulinaemia, many cancer cells upregulate the IRs and IGF-1Rs in presence of hyperinsulinaemia and associated high IGF-1 levels, leading to mitogenic effects, increased cancer growth and metastasis[62,63]. Cancers that overexpress IR-A include breast, endometrial, lung, colorectal, hepatocellular, prostate, ovary, thyroid, and renal cancers[64-66]. Similarly, the cancers that overexpress IGF-1R include colorectal, breast, hepatocellular, and prostate cancers[67]. The loss of function mutations of tumour suppressor genes including *BRCA1*, *p53*, and *PTEN* lead to high IGF-1R expression[68]. Cancers that overexpress IGF-2 include mesenchymal tumours, breast, oesophageal, ovarian, and hepatocellular; tenosynovial giant cell tumours, Wilms' tumour, and Ewing's sarcoma[69].

Under physiological conditions (in people without hyperinsulinaemia), interaction of insulin and IR-B with subsequent stimulation of phosphatidyl-inositol-3-kinase/protein kinase B/mechanistic target of rapamycin complex 1 (PI3K/AKT/mTORC1) cascade mediate the anabolic effects of insulin including glucose uptake, glycogen synthesis, protein synthesis, and lipid synthesis. In people with hyperinsulinaemia (associated with high IR-A expression) and in cancer cells (associated with high IR-A expression and raised IGF-2), the interaction of insulin and/or IGF-2 with IR-A and the subsequent activation of rat sarcoma/rapidly accelerated fibrosarcoma/mitogen activated protein kinase/extracellular-regulated kinase cascade (RAS/RAF/MAPK/ERK) mediate the mitogenic effects of insulin including cell proliferation, survival, and migration[61]. An imbalance between MAPK and PI3K cascades results in impaired glucose/lipid metabolism in target tissues such as liver, muscle, and adipose tissue with cell proliferation in other tissues[70]. Under physiological conditions the interaction with IR-B is phasic (occurs only in postprandial state) resulting in metabolic effects, whereas under hyperinsulinaemic conditions or in cancer cells the interaction with IR-A is steady or continuous resulting in mitogenic effects[61].

Indirect effects of hyperinsulinaemia in the pathogenesis of cancer: Oestrogen receptor-α/cytokine/reactive oxidative species

Hyperinsulinaemia is associated with increased expression of aromatase enzyme in the TME resulting in increased oestrogen levels. Furthermore, hyperinsulinaemia is associated with decreased sex hormone-binding globulin levels that will increase the levels of bioavailable oestrogens that act on the tumour cells through oestrogen receptor-α, increasing the risk of oestrogen dependent cancers like breast and endometrial cancers[71]. The oestrogen receptor activation augments the insulin/IGF-mediated mitogenic effects in several cancers including that of breast, prostate, neuroblastoma, and pituitary adenoma[72].

Moreover, in carcinoma of prostate, activation of oestrogen receptors and of androgen receptors located at cell membrane induces IGF-1R upregulation to enhance IGF-1 mediated biological effects[73]. Similarly, in breast cancer, activation of IGF-1R and IR upregulate the non-classical or non-genomic membrane oestrogen receptors to potentiate the mitogenic effects[74,75]. Hyperinsulinaemia is also associated with inflammation in the TME leading to cytokine production and activation of the Janus Kinase-2 and Signal Transducer and Activator of Transcription-3 (JAK2-STAT3) and MAPK cascade inside the tumour cells[71]. Insulin upregulates the cellular metabolic activity leading to generation of reactive oxidative species (ROS) and resultant DNA damage, thereby promoting oncogenesis[76].

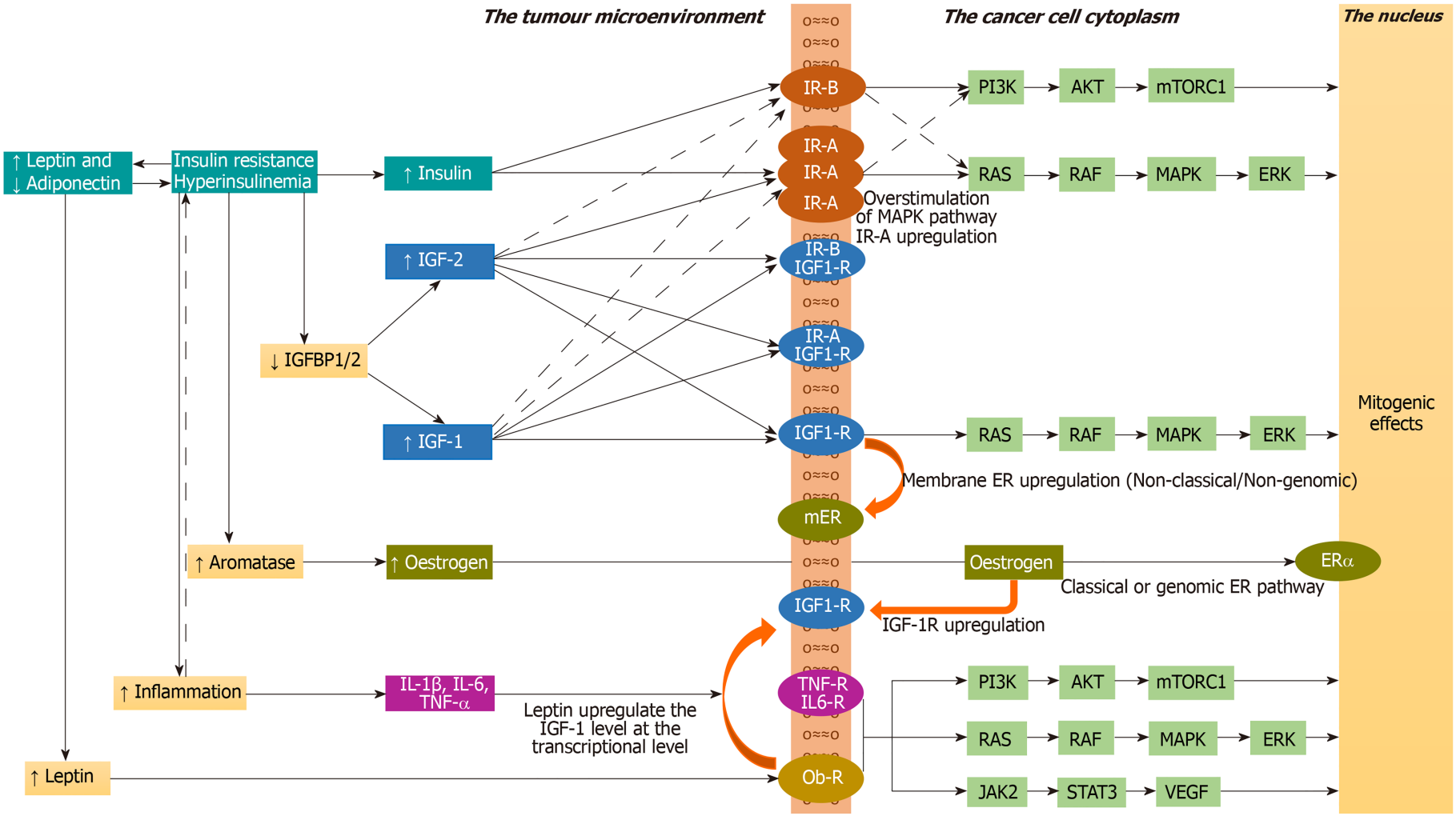

Figure 2 The pathophysiological mechanisms linking the hyperinsulinaemia in the tumour microenvironment to cancer with the associated intracellular signalling. IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumour necrosis factor-α; IR-A: Insulin receptor-A; IR-B: Insulin receptor-B; IGF-1: Insulin-like growth factor-1; IGF-2: Insulin-like growth factor-2; IGF-1R: Insulin-like growth factor-1 receptor; IR-A IGF1-R: Hybrid receptor of IR-A and IGF-1R; IR-B IGF1-R: Hybrid receptor of IR-B and IGF-1R; IGFBP: Insulin-like growth factor binding protein; ER-α: Oestrogen receptor-α; mER: membrane oestrogen receptor; Ob-R: Leptin-receptor; PI3K: Phosphatidyl-inositol-3-kinase; AKT: Protein kinase B; mTORC1: Mechanistic target of rapamycin complex 1 (Mammalian target of rapamycin complex 1); RAS: Rat sarcoma; RAF: Rapidly accelerated fibrosarcoma; MAPK: Mitogen activated protein kinase; ERK: Extracellular-regulated kinase; JAK2: Janus kinase-2; STAT3: Signal transducer and activator of transcription-3; VEGF: Vascular endothelial growth factor.

White adipose tissue remodelling in the pathogenesis of cancer

The white adipose tissue (WAT) comprising of subcutaneous and visceral adipose tissues, act as an energy reservoir for other organs. In response to over-nutrition and obesity, the adipose tissue undergoes dynamic remodelling characterized by alterations in the adipocyte number (adipocyte hyperplasia) in cases of childhood obesity or size (adipocyte hypertrophy) in cases of adult obesity[77]. The potential pathophysiological mechanisms linking obesity to cancer with special emphasis to WAT remodelling is outlined in the Figure 3. The hypertrophic adipose tissue outgrows its blood supply, leading to hypoxia, adipocyte injury/death, adipose tissue macrophage recruitment and a switch from anti-inflammatory to pro-inflammatory macrophages (M2 to M1 switch)[78]. This leads to increased expression of pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β and monocyte chemoattractant protein-1 (MCP-1), and insulin resistance[79].

Pro-inflammatory cytokines propagate the adipose tissue inflammation by recruiting more macrophages (MCP-1)[80]. The macrophages envelope the dead or dying hypertrophic adipocytes to form crown-like structures and these macrophages later become lipid-loaded foam cells[81]. There is increased release of free fatty acids (FFA) from the entrapped adipocytes with subsequent ectopic fat deposition in the liver and skeletal muscle leading to worsening insulin resistance and lipotoxicity[82]. Lipolysis and FFA release from WAT are also stimulated by pro-inflammatory cytokines[83]. The hypertrophic adipocytes exhibit impaired insulin-dependent glucose uptake due to a defect in glucose transporter 4 trafficking, indicating another mechanism for insulin resistance in obese patients with adipocyte hypertrophy, apart from the effect of pro-inflammatory cytokines and ectopic lipid deposition[84].

Pro-inflammatory cytokines generated by chronic low-grade inflammation of WAT can exert direct mitogenic effects *via* cytokine receptors or indirect mitogenic effects *via* increased insulin resistance and resultant hyperinsulinaemia. Moreover, the cytokines can activate androgen receptors to promote survival and proliferation of prostate cancer cells in men[85], and can induce the aromatase enzyme to increase the incidence of oestrogen-dependent tumours in the postmenopausal women[86]. The hyperleptinaemia that accompanies WAT inflammation is another inducer of aromatase enzyme[87].

Obesity and hyperinsulinaemia are associated with raised leptin and reduced adiponectin levels. Similarly, hyperleptinaemia and hypoadiponectinaemia are associated with the development of insulin resistance and hyperinsulinaemia[88,89]. The elevated leptin levels activate various cascades like PI3K, MAPK, and predominantly JAK2/STAT3[90]. Leptin induces IL-6 and TNF-α production, thereby sustaining a chronic inflammatory state[91]. It increases the expression of anti-apoptotic proteins (X-linked inhibitor of apoptosis protein), and pro-angiogenic factors including vascular endothelial growth factor (VEGF), and hypoxia-inducible factor-1α (HIF-1α)[92]. On the other hand, adiponectin, acting *via* liver kinase B1 (LKB1), induces the adenosine monophosphate-activated protein kinase (AMPK) involved in the induction of cell cycle arrest and inhibition of mTOR activity. Elevated leptin and decreased adiponectin levels are known to be associated with proliferation, survival and migration of cancers including that of breast, colon, endometrium, and prostate[92].

Hypoxia and angiogenesis in the pathogenesis of cancer

The hypertrophic adipose tissue outgrows its blood supply and develops hypoxia. HIF expressed in the hypoxic TME is a dimeric transcription factor having inducible subunits (HIF-1α, HIF-2α, or HIF-3α), and a constitutive subunit (HIF-1β)[93]. Hypoxia stabilizes HIF-1α and promotes its association with HIF-1β. The HIFα-HIFβ dimer enters the nucleus leading to activation of the downstream targets. Under hypoxic conditions, HIF-1α promotes tumour angiogenesis by activating the pro-angiogenic genes [(*VEGFA*, *VEGF receptor-1* (*VEGFR1*), *Angiopoietin* (*ANGPT*), and *Ephrin type-A receptor 1* (*EphA1*)], and inhibiting anti-angiogenic genes (*VEGFA*, *VEGFR1*, *ANGPT*, *EphA1*)[93]. Tumour angiogenesis is essential for the survival, growth, invasion, and metastasis of malignant lesions.

Oxidative stress in the pathogenesis of cancer

The metabolically active adipose tissue is a source of ROS/reactive nitrogen species. The adipose tissue from lean individuals expresses antioxidant enzymes including glutathione peroxidase, catalase, and superoxide dismutase 1, whereas these antioxidant enzymes are downregulated in the adipose tissue from obese individuals[83]. The oxidative stress is known to cause DNA double strand breaks and other complex DNA alterations[94]. Low or intermediate levels of oxidative stress

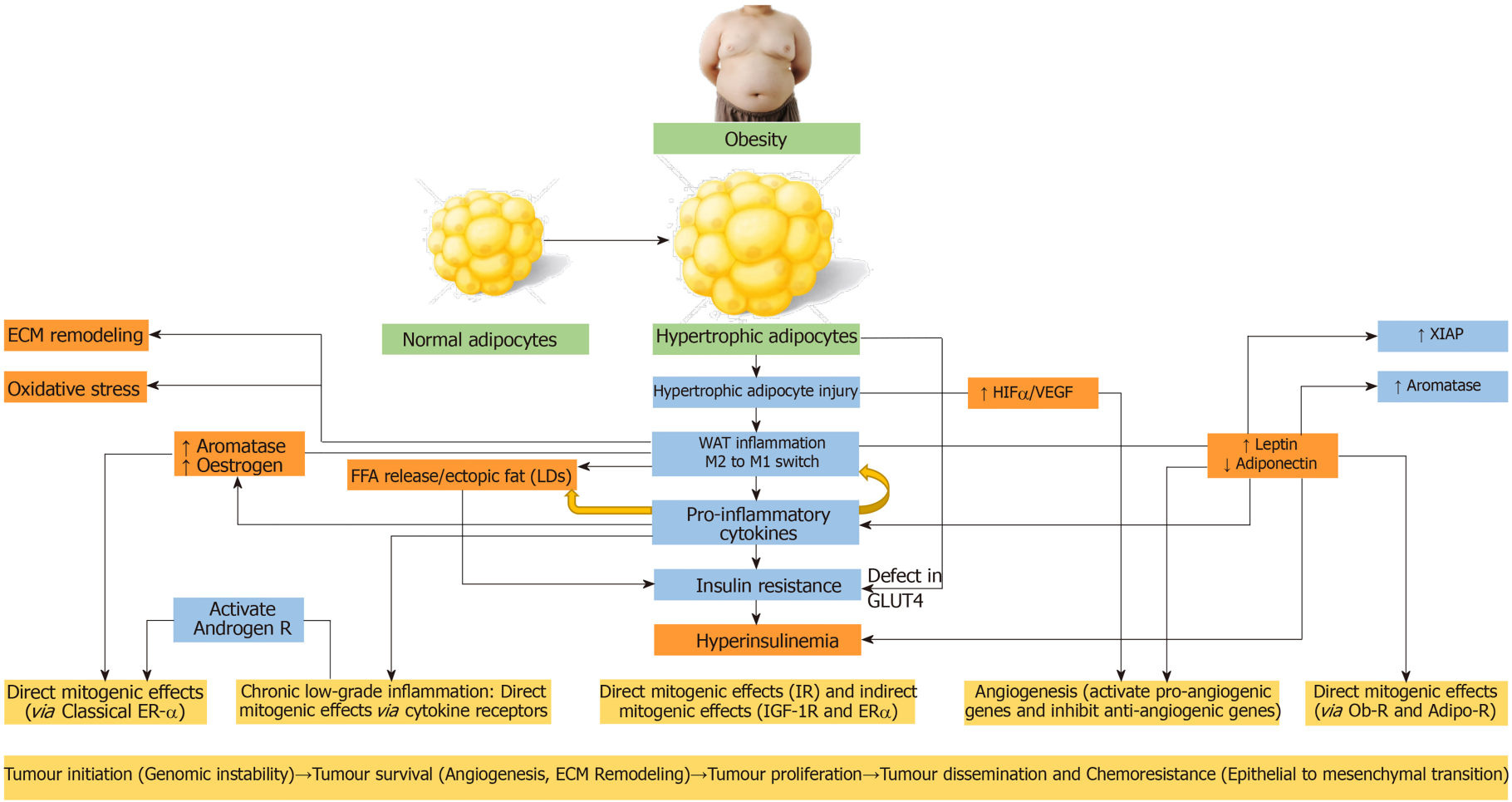

Figure 3 The potential pathophysiological mechanisms linking obesity to cancer with special emphasis to the white adipose tissue remodelling. WAT: White adipose tissue; IR: Insulin receptor; IGF-1R: Insulin-like growth factor-1 receptor; ER-α: Oestrogen receptor-α; Ob-R: Leptin-receptor; Adipo-R: Adiponectin-receptor; FFA: Free fatty acid; LD: Lipid droplets; ECM: Extracellular matrix; XIAP: X-linked inhibitor of apoptosis protein; VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia inducible factor-1α; GLUT4: Glucose transporter 4.

result in genomic instability associated with the activation of oncogenes, inactivation of tumour suppressor genes, angiogenesis, and mitochondrial dysfunction[95]. Obesity *per* se is associated with increased DNA damage and decreased DNA repair. Oxidative

stress can be a consequence of obesity. Moreover, oxidative stress can be the trigger for obesity by altering the food intake and stimulating WAT deposition[96-98].

In obesity associated WAT inflammation, the inflammatory environment increases the oxidative stress to a level that it results in DNA damage, genomic instability, augmented cell survival, and cell proliferation resulting in the development of cancer[99]. Increased ROS production has been observed in various cancers. Tumour cells express high levels of antioxidants to detoxify ROS, to establish a redox balance while maintaining a resistance to apoptosis. Though ROS can be pro-tumourigenic in most, they can also be anti-tumourigenic, initiating tumour cell death, especially when the ROS levels exceed the antioxidant threshold of cancer cells[100].

Extracellular matrix alterations in the pathogenesis of cancer

The TME comprises of a cellular and a non-cellular component. The cellular component includes immune cells, fibroblasts, adipocytes, and endothelial cells, whereas the non-cellular structural component, known as the extracellular matrix (ECM) include a meshwork of polymeric proteins like collagen, elastin, and fibronectin. The ECM provides the biochemical and biomechanical environment within which the cancer cells exist[101]. WAT inflammation induces mechanical changes in the ECM, including myofibroblast enrichment with associated increased stiffness that promote tumourigenesis[102]. Moreover, crosstalk between cancer cells and the microenvironment is an important aspect of tumour progression, as this determines the ability of cancer cells to cross the ECM barrier, access the circulation, and establish metastases[103]. The biochemical and biomechanical properties of the ECM influence the ability of the cancer cells to modify physiological features (plasticity) to survive in the hostile microenvironment, and to resist therapy through acquisition of stemness characteristics and epithelial to mesenchymal or mesenchymal to epithelial transitions[103,104].

Resistin, visfatin, and lipid droplets in the pathogenesis of cancer

The obesity associated chronic low-grade inflammatory state in the adipose tissue, results in genomic instability contributing to tumourigenesis. Moreover, obesity is associated with aggressive cancers, due to the crosstalk between adipose tissue and tumours during cancer progression[105]. The mature adipocytes supply adipokines and lipids to the proliferating cancer cells, whereas the adipose stromal cells, and the immune cells infiltrate the tumour tissue to secrete various paracrine factors within the TME to aid tumour progression. Presence of high levels of leptin and/or leptin-receptor is associated with poor prognosis in several cancers as evidenced by the presence of invasive tumours, lymph node involvement, distant metastasis, and chemoresistance[106]. Elevated leptin levels can upregulate the IGF-1 level acting at the stage of transcription[107]. Resistin and visfatin acts through their receptors to promote tumour cell proliferation, angiogenesis, metastasis, and chemoresistance[108,109].

Obesity is associated with ectopic fat deposition containing FFAs, triglycerides and cholesterol esters in non-adipose tissues. These lipid bodies, known as lipid droplets (LDs), are seen in many cancers, where they are thought to modulate the crosstalk between tumour cells and the cellular component of the TME. LDs are associated with tumour proliferation, chemoresistance, and aggressiveness[110]. Recently, fatty acid receptors with selectivity towards medium-long chain fatty acids (FFAR4 and FFAR1), and towards short chain fatty acids (FFAR2 and FFAR3) are discovered. FFAR4 is associated with proliferation, survival and migration of various cancers including colorectal, pancreatic and bone cancers[111]. The FFAs mediate the proliferation and metastasis of the tumour cells by activating the PI3K-AKT-mTORC1 pathway[112].

Hyperglycaemia in the pathogenesis of cancer

There are many mechanisms that can contribute to high cancer risk in patients with diabetes. The potential mechanisms, with a special emphasis to the Wnt/β-catenin signalling pathway, are portrayed in the Figure 4. These mechanisms can be related to antidiabetic medications[113], hormonal changes (exogenous or endogenous hyperinsulinaemia, raised IGF-1, hyperleptinaemia, and hypoadiponectinaemia), chronic inflammatory state associated with diabetes, oxidative stress associated with diabetes, decreased immunological response to cancer cells arising from competitive impairment of ascorbic acid transport into the immune cells by hyperglycaemia[114], enhanced signalling of epidermal growth factor receptor[115], accelerated cell cycle[116], chemoattractant upregulation, such as glial cell line-derived neurotrophic factor that is involved in the cancer invasiveness and migration[117], cytokine receptor upregulation and ROS generation by the advanced glycation end products

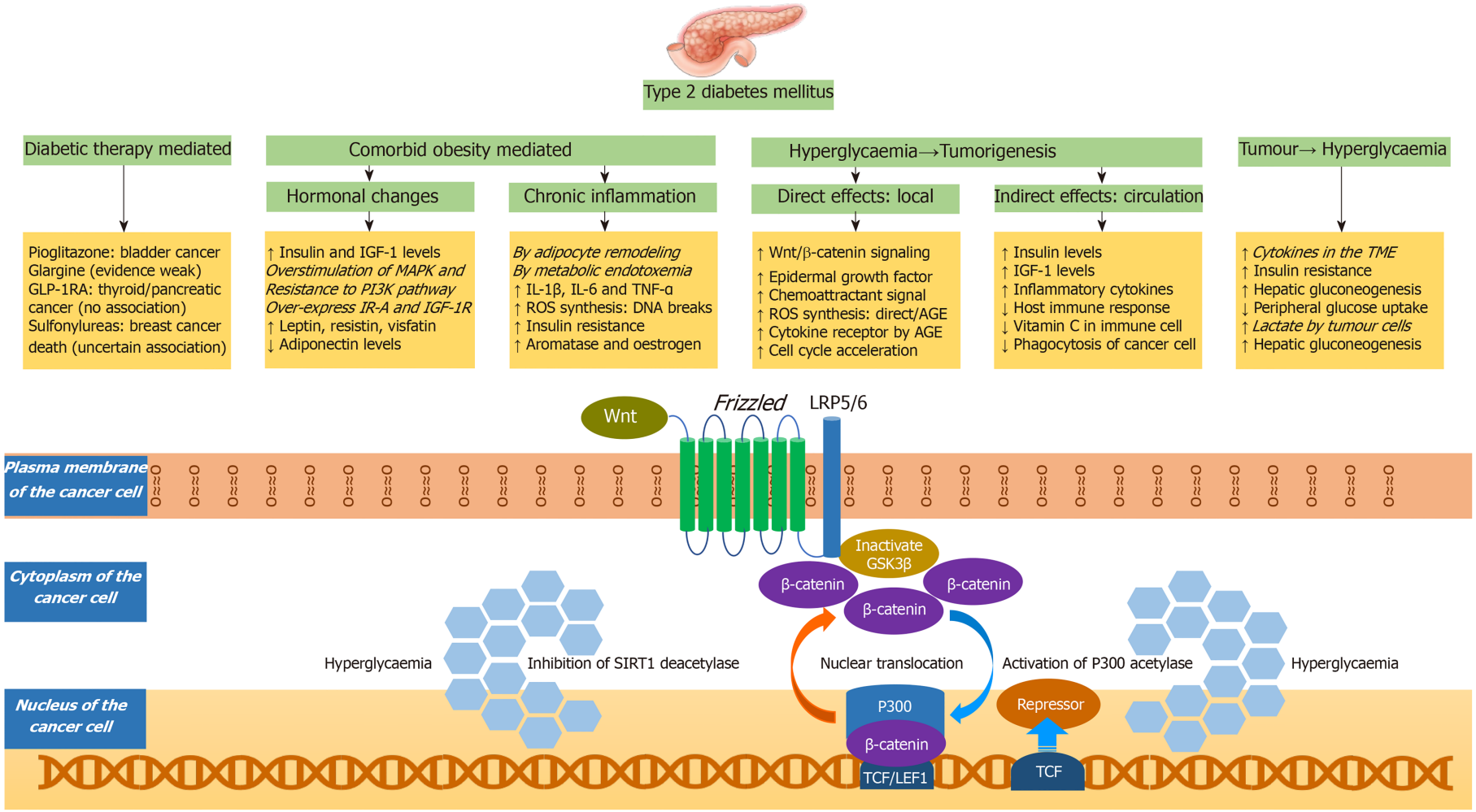

Figure 4 The potential pathophysiological mechanisms linking diabetes to cancer with special emphasis to the Wnt/β-catenin signalling pathway. IGF-1: Insulin-like growth factor-1; IGF-1R: Insulin-like growth factor-1 receptor; IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumour necrosis factor-α; IR-A: Insulin receptor-A; ROS: Reactive oxygen species; GLP-1RA: Glucagon like peptide-1 receptor agonist; MAPK: Mitogen activated protein kinase; PI3K: Phosphatidyl-inositol-3-kinase; AGEs: Advanced glycation end products; TME: Tumour microenvironment; LRP: Lipoprotein receptor-related protein; GSK3β: Glycogen synthase kinase-3β; SIRT1: Sirtuin 1 deacetylase; P300: P300 acetyl transferase; LEF: Lymphoid enhancer factor; TCF: T-cell factor.

(AGEs)[118], and most importantly enhanced Wnt/β-catenin signalling pathway resulting in increased proliferation, survival, invasion, and migration[119]. The raised insulin and IGF-1 levels are associated with overstimulation of MAPK pathway, resistance to PI3K pathway, over-expression of IR-A and activation of IGF-1R[120]. The oxidative stress associated with diabetes can occur through multiple mechanisms: direct effect of hyperglycaemia through glucose metabolism and auto-oxidation, or indirect effect from AGEs, or inflammatory cytokines[120].

Wnt is a family of secreted cysteine-rich glycoprotein ligands that bind to their membrane receptors to activate pathways including non-canonical Wnt-Ca^{2+} pathway, non-canonical planar cell polarity pathway, and canonical Wnt/β-catenin signalling pathway[121]. The classification of Wnt family into canonical or non-canonical is based on the presence or absence of β-catenin. In the canonical Wnt/β-catenin pathway, Wnt binds to its membrane co-receptor having Frizzled and lipoprotein receptor-related protein. This inactivates the Glycogen Synthase Kinase-3β (GSK3β), resulting in β-catenin accumulation in the cytoplasm. GSK3β is an enzyme that phosphorylates the cytosolic β-catenin to trigger degradation of β-catenin by the destruction complex. GSK3β is thereby considered as a tumour suppressor, due to its ability to inhibit the Wnt/β-catenin signalling pathway[122].

In the absence of hyperglycaemia, β-catenin accumulated in the cytoplasm cannot be translocated to the nucleus, to induce the expression of Wnt target genes. However, hyperglycaemia induces p300 acetyl transferase to achieve β-catenin acetylation. Moreover, hyperglycaemia inhibits Sirtuin 1 deacetylase activity. These favour formation of lymphoid enhancer factor 1 (LEF1)/β-catenin/p300 complex and its accumulation inside the nucleus, where it displaces the transcriptional repressor known as T-cell factor (TCF)7L2-corepressor complex, and induce the expression of Wnt target genes (LEF, TCF)[118,123]. These Wnt target genes are involved in initiation, proliferation, senescence bypass, epithelial to mesenchymal transition, and metastasis of tumours[124-127].

Cancer worsens hyperglycaemia

In patients with cancer, the circulating cytokines increases the insulin resistance, decreases the peripheral glucose uptake, increases the hepatic gluconeogenesis, thereby worsens hyperglycaemia. Increased inflammatory cytokines in the TME worsens this hyperglycaemia. Moreover, the product of glycolysis by tumour cells (lactate) stimulates the hepatic gluconeogenesis, further worsening the hyperglycaemia[119]. A recently published study from Korea has shown that cancer can increase the risk of getting subsequent diabetes mellitus in cancer survivors independent of traditional risk factors for diabetes mellitus (HR 1.35; 95%CI: 1.26-1.45)[128]. Though the risk was highest in the first 2 years, it remained high for 10 years following cancer diagnosis (HR 1.19; 95%CI: 1.00-1.43). Though the risk was highest for cancer survivors of pancreatic, kidney, and liver cancers, the risk remained significantly high even for gallbladder, lung, blood, breast, stomach, and thyroid cancers.

Therapeutic strategies for cancer based on the pathophysiological mechanisms

The therapeutic agents based on Wnt/β-catenin signalling include those that act by inhibiting Wnt ligands, inhibiting Wnt receptors/co-receptors, stabilizing the destruction complex, and inhibiting β-catenin-dependent transcriptional pathway[129]. Moreover, GSK3β inhibitors are being developed and entering clinical trials as novel cancer treatments due to their ability to inhibit the Wnt/β-catenin signalling pathway[130]. mTOR participates in multiple signalling pathways to regulate proliferation, autophagy, and apoptosis. Various newly developed mTOR inhibitors are entering clinical studies[112]. The free fatty acid receptors agonists are potential therapeutic agents in the management of cancers of colorectum, and ovary[131,132].

Various inhibitors of MAPK signalling pathway including RAS inhibitors, RAF inhibitors, MAPK inhibitors, and ERK inhibitors have also been recently developed[133]. Three RAF inhibitors and three MAPK inhibitors have received approval for the treatment of late-stage B-RAF harbouring cancers, either alone or in combination with other agents. However, these drugs are associated with intrinsic drug resistance in patients with RAS mutations or acquired drug resistance in patients with B-RAF mutations (after 6-10 mo of treatment). Targeting MAPK and AMPK signalling pathways together represents a promising therapeutic intervention in patients with RAS or RAF mutations[134]. Another promising intervention in patients with B-RAF mutation-associated cancers, is dual inhibition of the MAPK and JAK2/STAT3 pathways using a combination of three MAPK pathway inhibitor types

including BRAF inhibitor, MAPK inhibitor, and ERK inhibitor along with either of JAK2 or STAT3 inhibitor[135].

Though PI3K signalling pathway is important in cell proliferation, and survival, the drugs acting on this pathway, including pan-PI3K inhibitors or dual PI3K/mTOR inhibitors are only modestly effective as monotherapy, with a relatively high incidence of side effects. However, isoform selective PI3K inhibitors are undergoing clinical trials with improved specificity and reduced toxicity[136]. Similarly, several AKT inhibitors are currently in various stages of clinical trials for diverse types of malignancies[137]. AMPK acts as tumour suppressor, as it mediates the effects of the LKB1 tumour suppressor by inhibiting mTORC1 production. Though metformin, and fluoxetine can activate AMPK, several small molecular AMPK agonists are under various stages of development and few of them are expected to enter clinical trials within next few years[138].

As hyperinsulinaemia is the key driver of cancer initiation and progression in patients with diabetes and obesity, drugs that could reduce hyperinsulinaemia could potentially prevent development of cancer. At supra-physiological concentrations, metformin can exert direct anti-proliferative effects. However, at physiological concentrations the anti-proliferative effects are due to its indirect effects including reduction in hyperglycaemia, insulin, IGF-1, and leptin[139]. Clinical trials with the use of metformin in cancer therapy and prevention are ongoing. Peroxisome proliferator-activated receptor gamma (PPARγ) is expressed in cancers including breast, prostate, colon, bladder, and thyroid cancers. Preclinical trials have shown that the PPARγ agonists have tumour suppressor effect as they are pro-apoptotic, induce autophagy, decrease cancer cell invasion and metastatic potential. However, the results of these clinical trials are disappointing due to their side effect profiles[140].

CONCLUSION

Obesity and T2DM are associated with high risk of cancer, and the strongest associations are for postmenopausal breast and endometrial cancers, and colorectal carcinomas. Mendelian randomization studies have shown that obesity and hyperinsulinaemia have very strong associations with cancer, whereas hyperglycaemia and T2DM have either a weak, or no association with cancer. The relationship between T2DM and cancer is bidirectional, as cancer survivors appear to be susceptible to subsequent new onset diabetes mellitus. Optimal screening strategies for diabetes in cancer survivors should be developed. With the increasing global burden of obesity and diabetes mellitus, the burden of cancer will continue to rise in the coming decades. Interventions at all possible levels, should be done to prevent the development of cancer from these common non-communicable diseases. Pathophysiological studies have shown that hyperinsulinaemia has the primary role in tumourigenesis in the setting of obesity and diabetes, associated with chronic inflammation, and elevated adipokines. In addition, patients with diabetes mellitus exhibit enhanced Wnt/β-catenin signalling pathway as one of the possible pathophysiological mechanisms. Newer therapeutic agents based on pathophysiological mechanisms including Wnt/β-catenin, MAPK, PI3K, AMPK and mTOR signalling pathways are undergoing preclinical/clinical trials for the treatment of cancer.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 May 20; 11(3): 46-60

DOI: 10.5662/wjm.v11.i3.46

ISSN 2222-0682 (online)

REVIEW

Molecular diagnosis in cat allergy

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Author contributions: Popescu FD, Ganea CS, Panaitescu C and Vieru M contributed intellectually to this work and have read and approved the final version.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Manuscript source: Invited manuscript

Specialty type: Medical laboratory technology

Country/Territory of origin:

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Abstract

Domestic cats represent one of the most common sources of indoor allergens. All over the world, many households own cats, whose allergens are persistent and widespread. Cat allergy itself is frequent, and its symptoms vary from rhinoconjunctivitis to life-threatening asthma. *In vitro* diagnosis using precision medicine allergy immunoassays is important because natural cat dander extracts may differ in quality and quantity of some of the individual allergen components and other molecules. In the component-resolved diagnosis of cat allergy, singleplex and multiplex specific immunoglobulin (Ig) E assays include use of the cat-specific major allergen, secretoglobin Fel d 1 (as a species-specific molecule), other allergen components (such as lipocalins Fel d 4, cross-reacting with other animal similar molecules, and Fel d 7, present in small quantities in natural extracts), and serum albumin Fel d 2 (related to the cat-pork syndrome). IgA Fel d 5 and IgM Fel d 6 are not available as allergen components in the current commercial IgE immunoassays, but they may impair the *in vitro* diagnostic evaluation of cat allergy because galactose-α1,3-galactose is an IgE-binding epitope of these native feline allergens. The benefits of molecular-based cat allergy diagnosis are continually evaluated, as the role of recombinant allergen components already known is detailed and new other molecules of interest may be discovered in the future.

Key Words: Feline; Allergens; Component-resolved diagnosis; Immunoglobulin E; Immunoassays

Romania

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 23, 2021
Peer-review started: January 23, 2021
First decision: February 14, 2021
Revised: February 22, 2021
Accepted: May 10, 2021
Article in press: May 10, 2021
Published online: May 20, 2021

P-Reviewer: Kang YB
S-Editor: Fan JR
L-Editor: A
P-Editor: Li X

Core Tip: Cats are a common source of allergens for humans, and allergy to these pets are frequent and variable in their clinical manifestations. The benefits of molecular diagnosis in cat allergy include use of the species-specific major allergen Fel d 1, cross-reacting allergen components, including those present in small quantities in natural extracts, while considering molecules that may impair the *in vitro* allergy diagnosis. The identification and characterization of molecular cat allergens with clinical significance has allowed their use in singleplex and multiplex immunoglobulin E immunoassays for a precision diagnostic approach.

Citation: Popescu FD, Ganea CS, Panaitescu C, Vieru M. Molecular diagnosis in cat allergy. *World J Methodol* 2021; 11(3): 46-60
URL: https://www.wjgnet.com/2222-0682/full/v11/i3/46.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i3.46

INTRODUCTION

The domestic cat (*Felis domesticus*, synonym: *Felis catus*) is one of the most common sources of indoor allergens, and allergy to cats in humans is the most common mammalian-origin immunoglobulin (Ig) E-mediated hypersensitivity. Cats have been associated with humans for more than 9500 years and are considered nowadays the most popular pets in the world. In past decades, a high incidence of allergy to these furry animals, especially among children and young adults, has been recorded. Cat allergy is currently estimated to affect approximately 1 in 5 adults worldwide. Many households own cats, indicating that there is a high exposure to their allergens. Moreover, the major and most studied cat allergen, Fel d 1, is persistent and ubiquitously present in indoor habitation spaces, dust samples from homes with or without cats, in public buildings and transportation, making allergen avoidance difficult[1-3]. The symptoms of allergy to cats vary from relatively mild rhinoconjunctivitis to potentially life-threatening asthma exacerbations[2].

Precision medicine allergy immunoassays support the molecular-based diagnosis for cat allergy. Also known as component-resolved diagnostics (CRD), this patient IgE sensitization *in vitro* molecular-level diagnostic approach uses allergenic components.

To date, eight *Felis domesticus* molecular allergens have been recognized as Fel d 1 to Fel d 8 by the World Health Organization/International Union of Immunological Societies (WHO/IUIS)[4]: uteroglobin-like protein Fel d 1, serum albumin Fel d 2, cystatin Fel d 3, lipocalins Fel d 4 and Fel d 7, Igs Fel d 5 and Fel d 6, and latherin-like protein Fel d 8. Cat allergens are involved in the molecular mechanisms underlying IgE-mediated allergic sensitization and different cross-reactivities. Representative isoforms are described for these allergens: Fel d 1.0101, Fel d 2.0101, Fel d 3.0101, Fel d 4.0101, Fel d 5.0101, Fel d 6.0101, Fel d 7.0101, Fel d 8.0101, but none is mentioned as such in the commercial IgE immunoassays. Data on the IgE binding epitopes are scarce, with sequence positions mentioned only for Fel d 1. IgE epitope mapping of this dominant cat allergen revealed five sequential/linear epitopes on chain 1/Fel d 1-A and two on chain 2/Fel d 1-B, in addition to a discontinuous/conformational epitope on chain 1[5], the last one being located on the four helices of the Fel d 1 chain 1 spatially juxtaposed upon protein folding.

Currently, the best characterized and available cat allergenic molecules for commercial IgE assays are Fel d 1, Fel d 2, Fel d 4 and Fel d 7. The two types of such allergen components used in singleplex and multiplex immunoassays are recombinant (r) allergens (produced by recombinant DNA technology) and highly purified natural (n) allergens (purified from natural sources)[6]. All are included in the list of cat allergens presented in the European Academy of Allergy and Clinical Immunology Molecular Allergology User's Guide[7] and in a recent Consensus document on dog and cat allergy[8]. The characteristics of these cat allergens[7-11] are presented in Table 1 together with all other allergenic molecules recognized by the WHO/IUIS database[4].

One major advantage of the CRD is the evaluation of primary sensitization animal source, which is not feasible by using native extracts, and better management of pet

Table 1 Characteristics of cat molecular allergens[7-11] mentioned in the World Health Organization/International Union of Immunological Societies database[4]

Allergen	Biochemical designation	Source of exposure	MW in kDa
Fel d 1[1,2]	Secretoglobin[4]	Saliva, dander	38
Fel d 2[1,3]	Serum albumin	Dander, serum, urine	69
Fel d 3	Cystatin[5]	Dander	11
Fel d 4[1,2]	Lipocalin[5]	Saliva	22
Fel d 5	Immunoglobulin A[4]	Saliva, serum	400
Fel d 6	Immunoglobulin M[4]	Saliva, serum	800-1000
Fel d 7[2]	Lipocalin, von Ebner gland protein	Saliva	17.5
Fel d 8	Latherin-like protein	Saliva	24

The World Health Organization/International Union of Immunological Societies is commonly known by its acronym, WHO/IUIS. Fel d 1, Fel d 2, Fel d 4 and Fel d 7 allergens are available in commercial immunoglobulin E immunoassays:
[1]Available in singleplex immunoassays as recombinant allergen.
[2]Available in multiplex immunoassays as recombinant allergen.
[3]Available in multiplex immunoassays as native purified component.
[4]Presence of glycosylation.
[5]Glycosylation deduced from sequence analysis. MW: Molecular weight.

allergic patients[1]. The importance of molecular-based diagnosis is continually evaluated, as the role of allergen components already identified in cat allergy is detailed, and new molecules of interest may be discovered.

CAT ALLERGEN COMPONENTS FOR MOLECULAR DIAGNOSIS

A deep understanding of the most important cat allergens is crucial for assessing allergen products for *in vitro* molecular diagnosis to evaluate in detail the IgE sensitization profile of patients allergic to furry pets. Other allergen proteins, recently identified and defined, must also be discussed for their potential use in CRD in the future.

Fel d 1

The cat major allergen Fel d 1 is a small tetrameric protein composed of two heterodimers, each containing two distinct chains (chain 1, a polypeptide, and chain 2, a glycopeptide with N-linked oligosaccharide composed of triantennary glycans) linked by disulfide bonds in its native form. This allergen is a secreted globular protein belonging to the secretoglobin family. It is homologous with the human Clara cell 10-kDa phospholipid-binding protein and the progesterone-binding rabbit uteroglobin (uteroglobinlike protein). Fel d 1's biological function for the cat is not clearly established, initially being discussed that it may have a protective role in cat skin[12-16]. Fel d 1 is probably involved in immunoregulation and intra-species chemical communication, binding with good affinity to some fatty acids and steroids, the best ligands being lauric acid (cat pheromone with effects on social interactions) and androsterone (volatile steroid pheromone). Fel d 1 is a thermostable protein produced in various anatomical areas of cats, mainly by the sebaceous glands and anal sacs, but also by salivary and lacrimal glands. Fel d 1 is primarily found in cat skin and hair follicles. As cats groom, Fel d 1 is distributed on the fur, then shed with hair and dander. It is easily airborne and found in various indoor environments, such as homes with and without cats, hotels, schools, buses and trains, occupational and/or leisure environments, including cinemas, animal facilities, pet shops, farms. Pet owner's clothing is a significant source of allergen dispersal. Up to 60% of airborne Fel d 1 molecules are carried by small particles, of which 75% are more than 5 μm in diameter and 25% less than 2.5 μm. This allergen is very pervasive indoors, many airborne Fel d 1 settles out within a couple of days of disturbance, but smaller particles can remain airborne for up to two weeks or even longer. Measurement of this secretoglobin allergen levels in settled dust should not be used as a surrogate for airborne exposure. Moreover, the concept of a specific allergen threshold amount of exposure expected to

provoke respiratory symptoms (such as 8 μg/g of dust) is also probably misleading, mentioning besides that IgE sensitization can occur at much lower Fel d 1 levels [1,3,12,16].

All cats produce Fel d 1 regardless of age, sex, breed, body weight, hair length or housing (indoors *vs* outdoors). Fel d 1 is produced under testosterone control (male cats produce more Fel d 1 than females if uncastrated and 3-5 times less after neutering, while its production could be restored to pre-neutering levels with exogenous testosterone administration)[1]. In the fur of domestic cats, Fel d 1 levels are significantly higher than those of Fel d 4, and cat-to-cat variability was revealed. The quantity of Fel d 1 on cat hair can range from 1 μg/g to more than 1770 μg/g, with high concentrations on hair from the neck region. The hair length does not seem to affect Fel d 1 production. Fel d 1 is also present in cat saliva, but in lower concentrations than Fel d 4. Urine is not a significant source of Fel d 1, but hormonal status affects its urinary levels in male cats, making it possible that litter boxes of intact male cats to be a source of this allergen at home[3,17]. Washing cats is of little benefit, because even if it reduces the amount of Fel d 1 on the skin and fur, the effect does not last long as the amount of Fel d 1 returns to its original level in just 2 d[12]. Feeding cats a diet with an egg product ingredient containing anti-Fel d 1 IgY reduces active Fel d 1 in cat saliva and dander, decreasing the environmental allergen levels[18,19].

The recombinant cat allergen rFel d 1 is produced in an *Escherichia coli* expression system by direct fusion of chain 2 and chain 1. This major allergen accounts for 60%-90% of the total allergenic activity of cat dander extracts, while specific IgE antibodies to rFel d 1 are reported in 90%-98% of European subjects with cat allergy. This is aligned with African data which revealed that nearly 75% of the patients with cat allergy from Zimbabwe have IgE antibodies against rFel d 1[12-15]. Rabbit (*Oryctolagus cuniculus*) Ory c 3 secretoglobin from saliva and dander, belonging to the same secretoglobin family, has very low sequence identity with Fel d 1, with no known IgE cross-reactivity[20]. Sequence similarity of Fel d 1 was reported with the skin brachial gland protein of an arboreal prosimian from Southeast Asia, named low loris (*Nycticebus* spp). Used for communication and defense when mixed with saliva, this gland protein has induced several cases of anaphylaxis in humans, some lethal, reported after the prosimian bites[21,22].

Fel d 1-related epithelial allergens from the majority of "big cats" (Table 2) are cross-reactive with domestic cat Fel d 1[11,23-26]. Sera from cat-allergic patients were analyzed by the first-generation solid-phase isotopic allergosorbent immunoassay using big cat fur extracts, obtained from hair collected by brushing animals (from the Natura Altis Magistra Zoo, Amsterdam, The Netherlands) at the time they were losing their winter fur. All subjects with positive skin test to cat extracts had IgE antibodies reacting with hair extracts from seven Felidae species (lion, Siberian tiger, snow leopard, jaguar, puma, ocelot, serval) but not caracal[23]. Cat-allergic individuals may be uncommonly exposed to such cross-reactive Fel d 1-related allergens in special settings, like zoos, wild parks and circus visits, but only very few cases developed severe allergic reactions upon exposure to lions and tigers in circuses[23-27]. The weight of big cats used in the past in circus entertainment is much greater than that of common domestic cats, and therefore it is likely that they produce large quantities of aeroallergens. Moreover, Siberian tiger hair extract contains 15-times more Fel d 1-like allergens *per* gram than that of lion[23].

rFel d 1 is available in singleplex and multiplex immunoassays, being considered a marker of genuine cat sensitization. It is presented together with other allergens used in singleplex and multiplex IgE assays[7,8,10,28,29] in patients with cat allergy in Table 3.

Fel d 2

The serum albumin Fel d 2 is a minor cat allergen, despite being an important protein in dander. All cats have this allergen. It is an allergen component available as a native purified and recombinant molecule in singleplex and multiplex immunoassays (Table 3). Serum albumin is a large, globular non-glycosylated protein, with α-helical structures stabilized by disulfide bridges. It is synthesized in the liver and represents a main protein constituent of plasma, with important transporter and colloid-osmotic pressure regulating roles. The amino acid identity between cat serum albumin and those of other mammals, such as dog Can f 3, pig Sus s 1, cattle Bos d 6 and horse Equ c 3, is high (75%-85% on average). Fel d 2 is considered a useful biomarker for high risk of cross-reactivity with other serum albumins[29,30-32]. Many patients allergic to cat albumin react to dog and horse albumins. About 15%-25% of cat-allergic patients are sensitized to feline serum albumin. In European allergic patients, monosensitization to Fel d 2 was found in 3.2%-7%[30-33]. There are patients with respiratory

Table 2 Cat Fel d 1 and other cross-reactive Fel d 1-related allergens from big cats (Felidae family)[11,22-25]

Subfamily	Species	Common name	Allergen
Felinae	*Felis domesticus* (*Felis catus*)	Domestic cat	Fel d 1
	Leopardus pardalis	Ocelot	Leo p 1
	Leptailurus serval	Serval	Lep s 1
	Puma concolor	Puma/cougar	Pum c 1
Pantherinae	*Panthera leo*	Lion	Pan l 1
	Panthera onca	Jaguar	Pan o 1
	Panthera pardus	Leopard	Pan p 1
	Panthera tigris longipilis	Siberian tiger	Pan t 1
	Panthera uncia (*Uncia uncia*)	Snow leopard	Unc u 1

Table 3 Allergens used in singleplex and multiplex immunoglobulin E immunoassays in patients with cat allergy[7,8,10,28,29]

Protein family	Allergen	IgE sensitization biomarker
Secretoglobins	rFel d 1	Major cat allergen, species-specific biomarker of primary sensitization to cat, as efficient as or even superior compared to natural cat extract in diagnosis
Lipocalins	rFel d 4	Major cat allergen, biomarker of cross-sensitization to other animal lipocalins, cross-reactive with lipocalins dog rCan f 6, horse rEqu q 1, and mouse nMus m 1
	rFel d 7	Minor cat allergen, biomarker of cross-sensitization to dog lipocalin, cross-reactive with lipocalin dog rCan f 1
Serum albumins	n/rFel d 2	Minor cat allergen, biomarker of sensitization to non-human serum albumin, cross-reactive with pork rSus d1/nSus s1 (cat-pork syndrome) and other serum albumins bovine nBod d 6, dog nCan f 3, and horse nEqu c 3
Immunoglobulins	nFel d 5	Minor cat allergens IgA Fel d 5 and IgM Fel d 6 carry α-Gal epitopes involved in the α-Gal syndrome and in impairing cat allergy *in vitro* diagnostics in parasite-infected patients; α-Gal biomarker: nBos d TG

Major cat allergen: Allergen recognized by immunoglobulin E antibodies of > 50% of cat allergic patients; Minor allergen: Allergen recognized by < 50% of the allergic population; IgA: Immunoglobulin A; IgM: Immunoglobulin M; α-Gal: Galactose-α-1,3-galactose; TG: Thyroglobulin, bovine.

allergy who present exclusive IgE sensitization to many serum albumins of furry animals. Regarding the clinical relevance, Fel d 2 sensitization is associated with moderate/severe rhinitis and diagnosis of asthma; it is also associated with severity of respiratory symptoms and with FeNO, as a type 2 biomarker, in young asthmatics. Moreover, high levels of IgE against Fel d 2 are associated with atopic dermatitis in children with cat allergy[34-38]. Fel d 2 is also important in relation to food allergy[1].

Cat-pork syndrome, described below[39-41], is the main food allergy phenotype in cat-allergic patients and it is secondary to the cross-reactivity of Fel d 2 with other albumins from mammals. This entity consists primarily of IgE-mediated respiratory symptoms following exposure to cats, and secondarily of food allergy symptoms after the ingestion of pork meat; therefore, the term "cat-pork syndrome" seems to be appropriate, although it is also frequently referred to as "pork-cat syndrome". The clinical picture varies from oral itching and urticaria to anaphylaxis. Fatal anaphylaxis after eating wild boar meat has also been reported. Symptoms usually occur within 30-45 min after eating pork meat, and it is not related to tick bites. Although most of the patients report reactions only to pork, some (10%-20%) report reactions to beef as well, including broiled beef intestines, but no one to cow's milk. Because albumin is a heat-labile protein, fresh meat, undercooked or dried and smoked pork are more consistent elicitors. Pork grilled meat, ribs, ham, sausages and hamburger have been mentioned as triggers. Only 1%-3% of patients who are allergic to cats seem to be at risk for allergic reactions to pork consumption, keeping in mind that only one-third of subjects who are IgE-sensitized to porcine serum albumin are likely to present food allergy to pork meat. Identification of the component-specific sensitivity pattern related to cat-pork syndrome allowed use of the cat albumin Fel d 2 and swine serum albumin nSus s 1 as markers for CRD in this clinical entity. Domestic pig (*Sus scrofa domesticus*) components nSus s 1 and rSus d 1 are available for IgE singleplex and multiplex immunoassays. These serum albumin molecules also cross-react with dog serum albumin nCan f 3 and bovine serum albumin (BSA) nBos d 6[29,42-45].

A new subphenotype of cat-pork syndrome was recently reported as anaphylaxis to BSA-containing surgical tissue adhesive (45% BSA) used as an adjunct for achieving hemostasis during cardiovascular surgery in a patient with asymptomatic long-term home exposure to cat and IgE sensitization to rFel d 1 and nFel d 2, but not to galactose-α1,3-galactose (α-Gal) containing bovine thyroglobulin. As Fel d 2 sensitization may predict cross-reactivity to nonhuman mammalian serum albumins, preoperative assessment of IgE sensitization to rFel d 2 in cat-allergic patients could be meaningful to avoid bovine and porcine surgical products[46]. BSA contained in culture media used in artificial insemination is an important anaphylaxis risk factor in patients allergic to cats, with sensitization to BSA being another possible cause of allergic reactions to some vaccines[47-49]. Moreover, equine serum albumin (also presenting high sequence identity with Fel d 2) is a causative factor of anaphylaxis to horse serum-based snake antivenom[50].

Fel d 3

Fel d 3 cystatin is a minor allergen, unavailable in commercial immunoassays. The prevalence of IgE reactivity to rFel d 3 is about 10%. It belongs to the cystatin superfamily of cysteine protease inhibitors (CPIs), being part of the stefin family. It is a small acidic protein, without cysteine residues or disulfide bonds, and having 80% sequence identity to bovine cystatin. Another animal cystatin with similar low molecular mass is Can f 8[51]. Besides Fel d 3 from cat dander, IgE-reactive cystatins have been identified in the kiwi fruit *Actinidia deliciosa* (Act d 4), *Ambrosia artemisifolia* weed pollen (Amb a CPI), and the parasitic nematode *Anisakis simplex* (Ani s 4). The sequence similarity between phytocystatin Act d 4 and other cystatins is only 13% to Fel d 3, 27% to Ani s 4, and 40% to Amb a CPI[52].

Fel d 4

The lipocalin Fel d 4 is a major allergen synthesized in cat salivary glands and found primarily in saliva in higher concentrations compared with Fel d 1. This cat allergen is involved in feline chemical communication, serving as a kairomone by eliciting defensive behavior in mice. Cat saliva is the main source of this allergen, which is deposited through grooming on the fur. Fel d 4 levels have no relation to hair length and its salivary levels appeared to be greater in neutered than intact female cats due to hormonal influences[6,8,17].

Fel d 4 is available as a recombinant molecule[10,28] in singleplex and multiplex immunoassays (Table 3). Lipocalins constitute the largest mammalian allergen family and, despite their highly conserved structure, they have variable sequence identities and cross-reactivities. The Fel d 4 cat allergen molecule has sequence identity of 67% with dog lipocalin Can f 6 and similar to horse lipocalin Equ c 1, which explains the moderate-high risk of cross-reactivity with these clinically significant allergen molecules. This is an argument for using such cross-reactive animal allergen molecules in CRD. Although Equ c 1 was regarded as a horse allergen marker, it should be considered as a highly cross-reactive molecule with the cat and dog lipocalins Fel d 4 and Can f 6. Specific IgE antibodies to Can f 6 are present in nearly 40% of patients sensitized to dogs; however, they are present in 60% of patients sensitized to both cats and dogs, which could be related to sequence identity with Fel d 4. There are patients with selective IgE reactivity to Fel d 4 but not to Equ c 1, and patients with IgE reactivity to Fel d 4 but not to Can f 6. Other major lipocalins, rabbit Ory c 4, domestic guinea pig Cav p 6, rat Rat n 1, and mouse Mus m 1, show identities between 47% and 52%. Fel d 4 shows weak cross-reactivity with the other dog lipocalin Can f 2, having less than 22% of their sequences being identical[29,53-55].

It is generally accepted that Fel d 4 lipocalin is the second most frequent sensitizing feline allergen. IgE reactivity to Fel d 4 is found in up to 63% of cat-sensitized subjects. The majority of children sensitized to Fel d 4 are also sensitized to Fel d 1 but not *vice versa*. In Central European cat-allergic patients, the sensitization rate to Fel d 4 is inferior to Fel d 1 but higher compared to Fel d 2, while monosensitization to Fel d 4 is scarce. Sensitization to this allergen molecule has relevance to the clinical presentation, as Fel d 4 is associated with the presence of asthma symptoms. Moreover, high levels of IgE to Fel d 4 are also associated with atopic dermatitis in children with cat allergy[29,33-35,56].

Fel d 5 and Fel d 6

IgA Fel d 5 and IgM Fel d 6 are present in high concentrations as Igs in cat saliva[56] and serum, and also in natural cat dander extracts, but are not used as molecular allergen components in the commercial IgE immunoassays. The IgE reactivity was found to be directed at carbohydrates of these Igs (lack of activity to deglycosylated cat

IgA) and to IgM from other animal species (rabbit, mouse, dog, pig, cow and horse) but not to human Igs. α-Gal is an IgE-binding epitope of both cat allergen Igs Fel d 5 and Fel d 6, which are cross-reactive with each other[57-59]. Serum specific IgE antibodies to the α-Gal carbohydrate epitope cause impaired *in vitro* diagnostic evaluation of cat allergy. These specific Igs may be present in patients with cat sensitization but they are not associated with rhinitis or asthma[15,29].

The glycosylated allergen component nFel d 5 present in cat dander extracts is recognized by nearly 40% of cat-sensitized European patients. Less than 20% of African patients with cat allergy have IgE against Fel d 5 compared with 66% among parasite-infected subjects without reported symptoms of cat allergy; of note, the majority (85%) of nonallergic Zimbabwean subjects with schistosomiasis and/or geohelminth infections showed anti-α-Gal IgE antibodies. The greater IgE binding to α-Gal *vs* Fel d 5 is explained by the lower number of α-Gal epitopes in nFel d 5. There is a strong correlation reported for the IgE antibody levels and cat dander extract, Fel d 5 and α-Gal specifically but not rFel d 1. The α-Gal epitope on IgA Fel d 5 is responsible for IgE anti-α-Gal reactivity to cat epithelia in parasite-infected patients[15]. Moreover, serum IgE antibodies to cat dander extract were detected among African children from rural Kenya without positive skin tests to cat epithelia extract[60], and no significant relationship was found between IgE and positive skin prick test responses to cat among South African children[61]. The α-Gal epitope is present not only on Fel d 5 and Fel d 6 but also on parasites. In addition, IgE antibodies against α-Gal are induced by tick bites. Therefore, nFel d 5 and nFel d 6 are not good markers for cat allergy diagnosis[15].

In order to decipher the problem of α-Gal cross-sensitivity in the cat IgE sensitization *in vitro* assessment, it is recommended to use at least the reliable rFel d 1 and the α-Gal biomarkers from a molecular perspective[15,29,62]. α-Gal-bearing glycoproteins are used in solid-phase immunoassays as biomarkers. Besides α-Gal coupled to human serum albumin and beef (Bos domesticus) carbonic anhydrase nBos d CA, the most widely used α-Gal markers are the recombinant human/murine chimeric monoclonal antibody cetuximab (2.04 μg α-Gal *per* mg) and the beef thyroglobulin (5.6 μg of α-Gal *per* gram). The performance characteristics in immunoassays of the last two biomarkers are relatively similar[63-67]. The bovine thyroglobulin (Bos d) α-Gal carrying molecule is commonly used in the singleplex fluorescence enzyme immunoassay with capsulated cellulose polymer as solid-phase[6,28,68]. Regarding the induction of IgE antibodies against α-Gal in humans, bites of hard ticks from the Ixodidae family are the most important primary sensitization source. The prevalence of α-Gal IgE sensitization depends on the degree of exposure to ticks[69,70]. Individuals from rural areas or with forest-related jobs have higher risk of such but only less than 10% of them present features of α-Gal syndrome[63,71-73].

The α-Gal syndrome consists of IgE-mediated allergy to α-Gal presenting as late-onset anaphylaxis after ingestion of pig, beef or lamb meat/viscera, or immediate-onset anaphylaxis to parenteral exposure to drugs containing α-Gal, such as cetuximab, snake antivenom, gelatin in plasma volume substitutes, and some vaccines[67,70]. In the α-Gal syndrome, most patients experience a decline in α-Gal-specific IgE titers by avoiding tick bites; as such, these levels should be reassessed at regular intervals[74]. The mechanisms by which parasites also induce α-Gal-specific IgE antibodies in subjects with no history of cat allergy are not elucidated but mucosal blood feeding may be involved, such as for urinary blood fluke (*Schistosoma haematobium*) or intestinal blood-feeding hookworms (*Ancylostoma duodenale*, *Necator americanus*)[15]. Keeping in mind that the human blood group B antigen represents a fucosylated α-Gal structure, some studies have revealed that individuals with blood groups AB and B may present a reduced susceptibility to IgE sensitization to α-Gal[63,73].

An association of α-Gal syndrome with anaphylaxis to pork kidney and allergic rhinoconjunctivitis with cat sensitization, presenting serum IgE to cat extract but no specific IgE to Fel d 1, has been reported[75]. Although patients allergic to red meat with specific IgE response against α-Gal are considered not to have IgE antibody responses to plant-derived cross-reactive carbohydrate determinants[67], this association is also possible[67,76]. Interestingly, α-Gal and cross-reactive carbohydrate determinants among the N-glycans of salivary glands of ticks were also reported recently[29,77].

Fel d 7

Fel d 7 is available as recombinant cat lipocalin in the singleplex fluorescence enzyme immunoassay with capsulated cellulose polymer solid-phase and the new generation

macroarray nanotechnology-based multiplex immunoassay[10,28] (Table 3). It was reported to bind IgE in approximately 40% of subjects with rhinoconjunctivitis and/or asthma exposed to cats. Almost 20% of patients with Fel d 7-specific IgE do not have detectable IgE against Fel d 1. Fel d 7 is present in small quantities in natural extracts. The concentration of this lipocalin in cat hair extracts is approximately 0.24 μg/mL. Fel d 7 is a von Ebner gland protein isolated from the posterior region of the cat tongue, known to contain lingual salivary glands. It shares a high sequence identity (62%) with the major dog allergen Can f 1, giving it high potential for cross-reactivity with Can f 1. Thus, Fel d 7 may contribute to respiratory allergy symptoms not only in cat but also in dog-allergic patients. Because the concentration of Fel d 7 in cat saliva is about 4 mg/mL, it is plausible that cat licking may be a route for the sensitization to Fel d 7 along with the inhalation of aerosolized allergen[29,78-81].

Fel d 8

Fel d 8 is a distinct latherin-like protein. The frequency of IgE binding of sera from patients with respiratory cat allergy to rFel d 8 is nearly 20%. The IgE binding to Fel d 8 is highly correlated with binding to Fel d 1. Fel d 8 is not usually detected in natural cat dander extracts, being found in the saliva of cats and isolated from their submandibular salivary gland[78]. It has a high degree of homology to horse Equ c 4 and Equ c 5. Equ c 5 is an allergen that binds IgE in 77% of horse-allergic patients, and rEqu c 4 is available in the new macroarray multiplex immunoassay[10,80,81]. Fel d 8 belongs to the lipopolysaccharide-binding protein/bactericidal permeability-increasing family[81] and it is not yet available in the commercial IgE immunoassays.

Other cat allergens

Fel d S100, a calcium-binding protein detected in cat saliva, and Fel d Hp, a haptoglobin detected in blood, are two additional allergens mentioned in the Allergome database[11,81], also not currently available in commercial immunoassays. S100A12 and haptoglobin are undenominated IgE binding proteins. The IgE antibody response to S100A12 is of low prevalence, but the specific IgE titer could be high in some individuals. This is of interest as it suggests inhalation of this calgranulin inflammatory mediator, known to have interspecies activity. IgE binding to plasma haptoglobin is infrequent, but significantly more IgE binding was found in subjects with cat-allergy than in those without allergy. The likely source of exposure to this acute phase protein is saliva from cats with poor gingival hygiene[81].

Because a frequent association between cat and dog sensitization is known for several decades, and a common question is whether this is due to co-sensitization to different allergen components or cross-reactivity between cat and dog allergenic molecules, a short presentation of additional allergens related to this aspect is needed.

Cross-reactivity between cat and dog allergens is usually explained by high-sequence homologies or structural similarities between lipocalins Fel d 4 and Can f 6, albumins Fel d 2 and Can f 3, as mentioned above, but recently a cat Niemann-Pick type C2 (Cat-NPC2) allergenic protein, a homologue of Can f 7, was also detected in cat dander extracts. Can f 7 shares 78% sequence identity with Cat-NPC2, and this clearly indicates the possible cross-reactivity between them. rCat-NPC2 can bind specific IgE in at least 14.5% of cat-allergic subjects[82]. This newly identified and characterized animal allergen has the potential of becoming a useful tool for CRD, but it is not yet available in commercial IgE immunoassays. Interestingly, cross-reactivity was observed also between Cat-NPC2 and Der f 2 (also belonging to the NPC2 family of proteins) indicating a possible association between IgE sensitizations to cat, dog and house dust mites[82].

Moreover, a previous report demonstrated the presence of a Fel d 1-like allergen with a molecular weight of 20 kDa in dog dander extracts, which may be responsible for *in vitro* double positivity to cat and dog. The clinical significance of this cross-reactivity is not clear since no patients with IgE cross-reactivity to this Can f CRA (Fel d 1 cross-reactive allergen) revealed clinical symptoms to dogs[83].

Regarding kallikrein allergens, no patterns of cross-reactivity of cat allergens with male dog prostatic kallikrein Can f 5 have been identified to date. Therefore, even if there are few case reports of human seminal plasma allergy in women sensitized to Can f 5 from dog urine and dander[29,84-86], no such cross-reactivity reactions have been published in cat allergic patients.

MOLECULAR APPROACH TO CAT ALLERGY

The molecular approach to cat allergy involves allergen components used in singleplex and multiplex immunoassays for *in vitro* diagnosis, presented in Table 3. The designation of allergen names is derived from the source, the first three letters of the genus, the first letter of the species, and a number indicating the chronology of the discovery, for example, Fel d 1 is the first allergen from the domestic cat *Felis domesticus*[87]. The common exposure to these allergen molecules includes different indoor settings, such as homes with cats as pets, but also in schools, daycare centers, public buildings, workplaces, and public transport vehicles, particularly if pet ownership is more prevalent in the area[88] because of their transportability on clothing[89]. A popular misconception persists regarding cat allergy related to the belief that certain cat breeds produce less allergens and are 'hypoallergenic' due to their fur type[90]. The major allergen Fel d 1 is produced by the cat's sebaceous glands, and, together with Fel d 4, is detected in the saliva and distributed on the fur by grooming. In common neutered domestic cats, fur length and color or body size did not relate Fel d 1 levels in reservoir dust from homes. Fel d 4 levels are also not related to hair length, however, neutered female cats have higher levels compared to unneutered ones[17,86,91]. There have been attempts to obtain so-called 'allergy-free' transgenic cats characterized by the absence of Fel d 1, by disrupting the coding sequence of the target gene with a specialized construct[92] or by CRISPR-Cas9-mediated genomic editing of Fel d 1[93]. To date, there are no hypoallergenic or allergen-free cats[1].

The diagnosis of cat allergy may seem uncomplicated at first glance, since most patients react to the main allergen molecule Fel d 1, but it is important to keep in mind that the natural cat dander extracts used for diagnosis, while containing this allergen mainly, differ in the quality and quantity of cat individual allergens and other molecules. Moreover, contamination of commercially available animal dander extracts with house dust mite allergens is possible and may induce *in vivo* false-positive responses. CRD using individual allergenic proteins can improve the diagnosis of mammalian pet allergy[56,94-96].

Recombinant and well-defined allergen components have great advantages for CRD immunoassays used to assess IgE sensitization patterns to cat allergen components; these include primary sensitization and presence of allergy, polysensitization and presence of severe allergy, secondary sensitization, cross-reactivity to other furry animals, and irrelevant sensitization[1,86]. In patients suspected of cat allergy, Fel d 1, Fel d 2 and Fel d 4 seem to be the most important allergen components to assess. IgE sensitization to more than three cat allergen molecules in children is superior in predicting future cat symptoms than sensitization to cat extract, and sensitization to the major species-specific allergen is a predictor of cat allergy at adult age[9,29]. Sensitization to Fel d 1 is associated with asthma, and polysensitization (Fel d 1, Fel d 2 and Fel d 4) is associated with both clinical reactivity to cat and also bronchial responsiveness and increased FeNO as a type 2 inflammation biomarker. Asthmatic children with cat allergy have higher Fel d 1-specific IgE levels than children with rhinitis only. Asthma symptoms to cat exposure are associated with specific IgE antibodies to cat allergens Fel d 1 and Fel d 4 in cat-allergic children. Moreover, IgE sensitization to Fel d 2 and Fel d 4 is associated with atopic dermatitis in children with cat allergy[86].

CONCLUSION

The benefits of molecular diagnosis in cat allergy involve the use of the cat-specific major allergen as a species-specific molecule, cross-reacting allergen components, including those present in small quantities in natural extracts, while considering those impairing the *in vitro* allergy diagnosis. Identification and characterization of molecular cat allergens allowed their use in singleplex and multiplex immunoassays for a precision diagnostic approach, with assessing their clinical significance and the association with cat allergy phenotypes and severity[29].

The manifestations of cat allergy vary widely, from rhinitis and conjunctivitis to severe asthma. Other than respiratory and ocular allergy, cat licks can cause contact urticaria upon exposure to the saliva, while cat bites can cause anaphylaxis in patients sensitized to cats[2,97,98]. IgE sensitization to cat epithelia increases the risk of patients to develop asthma or rhinitis. In addition, persistent atopic dermatitis lesions occur more often in patients sensitized to cat dander. There is also clear evidence for the clinical importance of assessing cat allergen components in relation to both α-Gal and

cat-pork syndrome[29].

Allergenic molecules induce specific IgE sensitization of mast cells and trigger type 2 allergic inflammation upon re-exposure. The availability of natural purified or recombinant allergens improved the understanding of the molecular mechanisms leading to these immune responses, which vary depending on several structural and biological characteristics of these allergens. In addition, other pro-inflammatory properties of some allergens must be mentioned, including late-phase allergic inflammation induced by non-IgE reactive peptides of Fel d 1 *via* major histocompatibility complex-restricted T cell activation[99-101].

The molecular approach for cat allergy allows a better understanding of the exposure and immune response to feline allergens, the relationship of these specific IgE responses to symptoms, and their clinical relevance[29].

Identification of cat allergen-specific IgE antibodies, either bound to mast cells by skin prick tests or in serum by immunoassays, detects IgE sensitization, a condition necessary but not sufficient to make the definitive diagnosis of cat allergy[100]. CRD, with or without *in vivo* tests, must be used within the framework of a detailed clinical history, because IgE sensitization does not necessarily imply clinically relevant allergy[86,99,100]. A deeper *in vitro* analysis with the help of IgE immunoassays using molecular allergens creates the bigger picture of the patient IgE sensitization profile in order to assess genuine sensitization, primary sensitization source, co-sensitization, cross-reactivity and allergy risks, including prediction of allergy severity[1,86].

Precision allergy molecular diagnostic applications (PAMD@) in cat allergy involve several molecular allergens used in commercial singleplex and multiplex IgE immunoassays, Fel d 1, Fel d 2, Fel d 4 and Fel d 7, these being the allergenic components currently available on the market[100]. For other native or recombinant allergenic components to be included in such immunoassays used in clinical practice, they must not only be well characterized and experimentally validated, but must also be clinically validated and available from their production point of view. Moreover, the characteristics of the solid-phase of the immunoassay and the manner by which allergenic molecules are coupled are important to reflect their biochemical properties and specific requirements for stability, preserving epitope complexity. Regarding native IgA Fel d 5 and IgM Fel d 6 allergen components with α-Gal IgE-binding epitopes, their use may be associated with analytical errors and impaired *in vitro* diagnostics in some patients, in such cases bovine thyroglobulin being a good molecular biomarker for α-Gal IgE sensitization[5,15,28,29,86]. Although α-Gal is present on cat Igs, cross-sensitization between cat allergens and the oligosaccharide antigen is not considered clinically relevant[100].

Concerning cat allergen immunotherapy, although some patients may likely benefit more from it, particularly those with moderate-to-severe disease, monosensitized to Fel d 1[102], and a good immune and clinical response to subcutaneous immunotherapy is associated with high doses of major allergens in the cat allergen extracts[103], more data are required from large trials to obtain more definitive conclusions. Summing-up, cat allergy CRD, recently proposed to be termed as PAMD@ by the updated World Allergy Organization consensus document[100], allows for an accurate and detailed assessment of patients' IgE sensitization profiles and may facilitate individualized management options[88,100].

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 May 20; 11(3): 61-74

DOI: 10.5662/wjm.v11.i3.61

ISSN 2222-0682 (online)

MINIREVIEWS

Concise review of stereotactic irradiation for pediatric glial neoplasms: Current concepts and future directions

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Author contributions: Sager O, Dincoglan F, Demiral S, Uysal B, Gamsiz H, Colak O and Ozcan F played significant role in data acquisition, interpretation of data, reviewing and writing of the manuscript; Gundem E, Elcim Y and Dirican B worked on checking the manuscript for important intellectual content; Beyzadeoglu M took part in designing, reviewing and writing of the manuscript and checking the manuscript for important intellectual content; all authors have read and approved the final manuscript.

Conflict-of-interest statement: The authors state that they have no conflicts of interest.

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Abstract

Brain tumors, which are among the most common solid tumors in childhood, remain a leading cause of cancer-related mortality in pediatric population. Gliomas, which may be broadly categorized as low grade glioma and high grade glioma, account for the majority of brain tumors in children. Expectant management, surgery, radiation therapy (RT), chemotherapy, targeted therapy or combinations of these modalities may be used for management of pediatric gliomas. Several patient, tumor and treatment-related characteristics including age, lesion size, grade, location, phenotypic and genotypic features, symptomatology, predicted outcomes and toxicity profile of available therapeutic options should be considered in decision making for optimal treatment. Management of pediatric gliomas poses a formidable challenge to the physicians due to concerns about treatment induced toxicity. Adverse effects of therapy may include neurological deficits, hemiparesis, dysphagia, ataxia, spasticity, endocrine sequelae, neurocognitive and communication impairment, deterioration in quality of life, adverse socioeconomic consequences, and secondary cancers. Nevertheless, improved understanding of molecular pathology and technological advancements may pave the way for progress in management of pediatric glial neoplasms. Multidisciplinary management with close collaboration of disciplines including pediatric oncology, surgery, and radiation oncology is warranted to achieve optimal therapeutic outcomes. In the context of RT, stereotactic irradiation is a viable treatment modality for several central nervous system disorders and brain tumors. Considering the importance of minimizing adverse effects of irradiation, radiosurgery has attracted great attention for clinical applications in both adults and children. Radiosurgical applications offer great potential for improving the toxicity profile of radiation delivery by focused and precise targeting of well-defined tumors under stereotactic immobilization and image guidance. Herein, we provide a concise review of stereotactic irradiation for

Manuscript source: Invited manuscript

Specialty type: Medical laboratory technology

Country/Territory of origin: Turkey

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: November 17, 2020
Peer-review started: November 17, 2020
First decision: March 31, 2021
Revised: April 7, 2021
Accepted: April 14, 2021
Article in press: April 14, 2021
Published online: May 20, 2021

P-Reviewer: Nath J
S-Editor: Gao CC
L-Editor: A
P-Editor: Yuan YY

pediatric glial neoplasms in light of the literature.

Key Words: Radiosurgery; Stereotactic irradiation; Stereotactic radiosurgery; Pediatric glioma; Gamma knife; Linear accelerator

Core Tip: Pediatric gliomas comprise the majority of brain tumors in children. Radiotherapeutic management of pediatric gliomas poses a formidable challenge considering the adverse effects of irradiation for this vulnerable patient population. In this context, efforts have been focused on improving the toxicity profile of radiation delivery. Stereotactic irradiation with stereotactic radiosurgery or stereotactic radiotherapy in a single or few treatment fractions may serve as a viable radiotherapeutic approach to achieve this goal given the high conformality along with steep dose gradients around the target volume allowing for reduced normal tissue exposure under precise immobilization and image guidance.

Citation: Sager O, Dincoglan F, Demiral S, Uysal B, Gamsiz H, Colak O, Ozcan F, Gundem E, Elcim Y, Dirican B, Beyzadeoglu M. Concise review of stereotactic irradiation for pediatric glial neoplasms: Current concepts and future directions. *World J Methodol* 2021; 11(3): 61-74
URL: https://www.wjgnet.com/2222-0682/full/v11/i3/61.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i3.61

INTRODUCTION

Brain tumors, which are among the most common solid tumors in childhood, remain a leading cause of cancer-related mortality in pediatric population[1-3]. Gliomas, which may be broadly categorized as low grade glioma (LGG) and high grade glioma (HGG), account for the majority of brain tumors in children[4]. Expectant management, surgery, radiation therapy (RT), chemotherapy, targeted therapy or combinations of these modalities can be used to manage pediatric gliomas. Several patient, tumor and treatment-related characteristics including age, lesion size, grade, location, phenotypic and genotypic features, symptomatology, predicted outcomes and toxicity profile of available therapeutic options should be considered in decision making for optimal treatment[4-6]. Management of pediatric gliomas poses a formidable challenge to the physicians owing to concerns about treatment induced toxicity. Adverse effects of therapy for this vulnerable patient population may include neurological deficits, hemiparesis, dysphagia, ataxia, spasticity, endocrine sequelae, growth abnormalities, audiovisual toxicity, neurocognitive and communication impairment, deterioration in quality of life, adverse socioeconomic consequences, and secondary cancers[7-10]. Nevertheless, improved understanding of molecular pathology and technological advancements may improve management of pediatric glial neoplasms. Multidisciplinary management with close collaboration of disciplines including pediatric oncology, surgery, and radiation oncology is warranted to achieve optimal therapeutic outcomes[11-14].

In the context of RT, stereotactic irradiation represents a viable treatment modality for several central nervous system disorders (CNS) and brain tumors[15-19]. Considering the importance of minimizing adverse effects of irradiation, radiosurgery has attracted critical attention for clinical applications in both adults and children. Radiosurgical applications offer great potential for improving the toxicity profile of radiation delivery by focused and precise targeting of well-defined tumors under stereotactic immobilization and image guidance. Herein, we provide a concise review of ste-reotactic irradiation for pediatric glial neoplasms in light of the literature.

STEREOTACTIC IRRADIATION FOR PEDIATRIC HGG

Based on the classification of World Health Organization (WHO) in 2016, HGG comprises glioblastoma, anaplastic astrocytoma, and diffuse midline glioma including

diffuse intrinsic pontine glioma (DIPG)[20]. Pediatric HGG accounts for approximately 8%-12% of all childhood CNS tumors and it is the leading cause of cancer-related mortality in children under 19 years of age[21-24]. Pediatric HGG usually follows an aggressive disease course which results in morbidity and mortality, however, there are several distinctive features of pediatric HGG regarding natural history, causative genetic mutations, response to treatment, and tumor localization within the brain[6,22,25-28]. While HGG frequently arises from LGG with malignant transformation in adults, this is very uncommon in pediatric patients with differences in genetic and epigenetic features. Similar to adult HGG, surgery is the primary treatment modality for management of pediatric HGG, and the extent of resection is a significant prognostic factor[29-34]. Surgery alone may be insufficient for optimal management, and adjunctive therapies including RT and chemotherapy are recommended. Gross total resection of HGG is usually difficult owing to the infiltrative nature of the disease and the risk of excessive toxicity particularly when the lesions are located in close vicinity of critical neurovascular structures[22,35,36]. Microscopic tumor cells may still remain even after gross total resection with potential for subsequent recurrence. Due to the increased vulnerability of younger children to adverse effects of ionizing radiation and the relatively favorable disease course, RT is typically deferred for this subgroup of patients under 3 years of age by considering other therapeutic options[37-39]. Never-theless, older children are frequently referred for postoperative RT with concurrent and adjuvant chemotherapy[6,22,29,40]. In the context of RT for pediatric HGG, con-ventional fractionation is common practice owing to lack of superiority of altered fractionation regimens[41-44]. Of note, several series investigated the utility of hypo-fractionated RT regimens especially for DIPG[44-47]. Compared to conventionally fractionated RT delivered over 5 wk to 6 wk, hypofractionated RT schedules may offer reduction in number of anesthesia administrations for patients treated under anesthesia and less burden on patients, parents, and treatment centers.

Radiation dose escalation strategies, combined modality treatment approaches, and incorporation of contemporary RT techniques such as radiosurgery are being investigated to improve the therapeutic ratio for HGG in view of the aggressive disease course and poor treatment outcomes despite intensive management. Stereotactic irradiation is a common RT technique for treatment of adult HGG and several studies support its use for this indication either as part of initial management or as salvage therapy[18,19,48-51]. Data on stereotactic irradiation of HGG have been mostly extracted from the literature including adult patients considering that there is paucity of data about pediatric HGG. Survival after hypofractionation (including radiosurgical treatments) in glioblastoma has been assessed in a recent meta-analysis and systematic review[52]. Meta-analysis of eleven comparative studies regarding first line management of glioblastoma with hypofractionated *vs* conventionally fractionated irradiation revealed no significant difference between the two fractionation schemes, and hypofractionation has been suggested as a reasonable alternative fractionation scheme for selected patients[52]. In the context of radiosurgery, a phase III randomized trial conducted by the Radiation Therapy Oncology Group reported no survival advantage with the addition of stereotactic irradiation to conventional external beam RT[53]. Nevertheless, there is active investigation on the utility of stereotactic irradiation for achieving improvements in treatment outcomes of patients with HGG. Stereotactic irradiation is an extreme form of focal RT which is used to deliver high doses of radiation in a single or a few fractions to well-defined lesions. Minimal exposure of normal tissues due to steep dose gradients around the target volume may be achieved with radiosurgery. While several studies have reported improved treatment outcomes with incorporation of stereotactic irradiation for adult HGG, there is paucity of data on the utility of radiosurgery for pediatric HGG[54-61].

Giller *et al*[58] reported outcomes of robotically guided radiosurgery for pediatric brain tumors. Twenty-one patients aged between 8 mo and 16 years received Cyberknife radiosurgery for pilocytic astrocytomas, anaplastic astrocytomas, ependymomas, atypical teratoid/rhabdoid tumors, medulloblastomas, cranio-pharyngiomas, and other pathologies which were deemed unresectable[58]. Local control was achieved in patients with anaplastic astrocytoma, and the authors concluded that Cyberknife radiosurgery could be used for achieving local control of selected pediatric brain tumors with elimination of the requirement for rigid head fixation[58]. In another series of 90 children receiving stereotactic radiosurgery (SRS) for brain tumors at the Joint Center for Radiation Therapy during a 10-year period between 1987 and 1997, 20% of the patients (18 patients) had pediatric HGG[59]. Out of the total 90 patients, 10 patients (11%) had glioblastoma and 8 patients (9%) had anaplastic astrocytoma[59]. Median progression free survival (PFS) was 12 mo (range: 3-119 mo)

and median 3-year actuarial local control rate was 50% for the 18 patients with glioblastoma and anaplastic astrocytoma[59]. Four patients receiving SRS as part of initial management were alive and free of progression at 50, 62, 66, and 119 mo, respectively[59]. Baumann *et al*[60] reported their experience with pediatric radiosurgery in a series of 52 patients. Local control was worse in patients with HGG compared to LGG[60]. Grabb *et al*[61] assessed the role of SRS in 25 pediatric patients with surgically incurable glial tumors treated between 1988 and 1994. Twelve patients had malignant astrocytomas or ependymomas. While 7 children died of disease with a median survival of 6 mo after SRS, 5 children were alive at 12, 45, 50, 72, and 72 mo after radiosurgical management[61].

In summary, stereotactic irradiation may be considered as a viable therapeutic strategy for management of adult HGG particularly in the recurrent disease setting. There is scarce literature regarding the utility of stereotactic irradiation for HGG in children, however, this advanced radiotherapeutic technology may offer benefits for pediatric patients and deserves further investigation to improve normal tissue sparing through precise stereotactic localization under image guidance.

STEREOTACTIC IRRADIATION FOR PEDIATRIC LGG

Pediatric LGG is the most common CNS neoplasm among children[5,30]. Most common subtype of pediatric LGG is pilocytic astrocytoma, and other subtypes are diffuse astrocytoma (fibrillary, gemistocytic, or protoplasmic), subependymal giant cell astrocytoma, pilomyxoid astrocytoma, pleomorphic xanthoastrocytoma, gangliglioma, gangliocytoma, desmoplastic infantile ganglioglioma, and dysembryoplastic neuroepithelial tumor[5,23]. Prognosis for these heterogeneous group of tumors is usually favorable, thus toxicity profile of management is very important[62-65]. Location and extent of disease are critical factors which should be considered in decision making for treatment of pediatric LGG. Other important factors include age, symptomatology, phenotypic and genotypic features, predicted outcomes and toxicity profile of available therapeutic options. Optimal care of patients with pediatric LGG may require incorporation of multimodality management with close collaboration of pediatric oncology, surgery, and radiation oncology disciplines[64,65]. Surgical resection is the principal mode of management for tumors which are amenable to surgery. Observation may be considered after surgical removal of the tumor to spare pediatric patients from potential toxicity of adjunctive therapies. Previous data on pediatric and adult patients have shown improvements in treatment outcomes with incorporation of RT in management of LGG[66,67]. There have been significant advances in the disciplines of pediatric neurosurgery and radiation oncology over the years[12-14]. Despite advances in therapy, irradiation for pediatric brain tumors still remains to be a challenge given the vulnerability of children to adverse RT effects such as neuroendocrine and neurocognitive deficits, growth abnormalities, audiovisual toxicity, deterioration in quality of life, adverse socioeconomic consequences, and secondary cancers[7-10,68]. Nevertheless, optimal surgical management may not be feasible for tumors at critical locations such as the optic pathway, brainstem, basal ganglia, thalamus, hypo-thalamus, and other eloquent brain areas. Therefore, irradiation in the form of radiosurgery or conventionally fractionated RT may be considered in the presence of surgically inaccessible tumors, incomplete excision, or recurrence. Conformal RT techniques, particle therapy, and radiosurgical treatments may offer reduced normal tissue exposure in management of pediatric LGG[68-72]. Among the radiotherapeutic options for treatment of pediatric LGG, stereotactic irradiation offers a viable RT technique. Radiosurgery is a very highly focused form of therapeutic irradiation with the potential of improving the toxicity profile of radiation delivery through steep dose gradients around the target volume. Pilocytic astrocytomas, the most common of pediatric LGG, are typically visualized as well-defined lesions on neuroimaging which renders them more suitable for radiosurgical management. While infiltrative nature of the disease comprises a challenging aspect in radiosurgery for HGG, most LGG lesions with well-defined borders are suitable for treatment with stereotactic irradiation. Several studies including pediatric patients have addressed the utility of stereotactic irradiation in LGG management either as primary, adjuvant, boost or salvage therapy[73-88]. Table 1 shows summarized data from selected series of stereotactic irradiation for LGG including pediatric patients.

Barcia *et al*[73] reported their experience with SRS for deeply seated inoperable LGG in a series of 16 patients including 8 children. Histological confirmation of LGG was available for 7 patients, and 12 patients had received prior irradiation. Median age was

Table 1 Selected series of stereotactic irradiation for low grade glioma including pediatric patients

Ref.	Study period	Number of patients	Proportion of pediatric patients (%)	Histology	Setting	Treatment	Dose (Gy)	Age (yr)	Tumor size	Prior irradiation	Follow-up duration	Tumor control or PFS (%)
Barcia *et al*[73], 1994	1978-1991	16	50	LGG	Primary or boost therapy	SRS by use of a cobalt source and stereoguide	Mean margin dose 21.7 Gy	Median age 20 yr (range: 4-68 yr)	-	12 patients	Median 50 mo	Tumor control 81
Somaza *et al*[74], 1996	1990-1993	9	100	Pilocytic astrocytoma	Adjuvant or salvage therapy	GKSRS	Median margin dose 15 Gy	Mean age 8.6 yr (range: 4-17 yr)	Mean tumor diameter 16 mm	2 patients	Median 19 mo	Tumor control 100
Kida *et al*[75], 2000	2000	12 (total number of patients in the study is 51)	100	WHO Grade I low grade astrocytoma	As part of initial management or salvage therapy	GKSRS	Mean margin dose 12.5 Gy	Mean age 9.8 yr	Mean tumor diameter 25.4 mm	-	Mean 27.6 mo	Tumor control 91.7
Boëthius *et al*[76], 2002	1978-1997	19	84.2	Pilocytic astrocytoma	Adjuvant therapy	GKSRS	Median margin dose 10 Gy	Mean age 10.6 yr (range: 2-60 yr)	Median 2.2 cc	2 patients	Median radiological follow-up 4.7 yr	Tumor control 94.7
Hadjipanayis *et al*[77], 2003	1987-2000	49	59	Pilocytic astrocytoma (37 patients) and WHO Grade II fibrillary astrocytoma (12 patients)	As part of initial management or salvage therapy	GKSRS	Median margin dose 15 Gy	Median age 14 yr (range: 3-52 yr) for patients with pilocytic astrocytoma and median age 25 yr (range: 5-57 yr) for patients with WHO Grade II fibrillary astrocytoma	Median 3.3 cc	13 patients	Median 32 mo after SRS	Tumor control 67
Saran *et al*[78], 2002	1994-1999	14	100	LGG	As part of initial management or salvage therapy	LINAC-based SRT	Total dose 50-55 Gy	Median age 8 yr (range: 5-16 yr)	Median 19.5 cc	0 patient	Median 33 mo	PFS 87 at 3 yr
Marcus *et al*[79], 2005	1992-1998	50	-	WHO Grade I-II astrocytoma	Salvage therapy	LINAC-based SRT	Mean total dose 52.2 Gy	Median age 9 yr (range: 2-26 yr)	≤ 5 cm in maximal dimension in all patients	0 patient	Median 6.9 yr	PFS 82.5 at 5 yr, PFS 65 at 8 yr
Wang *et al*[80], 2006	1993-2003	21	-	LGG	Primary, boost, adjuvant or salvage therapy	GKSRS	Median margin dose 14.5 Gy	Median age 20 yr (range: 6-70 yr)	Median 2.4 cc	7 patients	Median radiological follow-up 49 mo	Tumor control 67
Kano *et al*[81], 2009	1987-2006	50	100	Pilocytic astrocytoma	As part of initial management or salvage therapy	GKSRS	Median margin dose 14.5 Gy	Median age 10.5 yr (range: 4.2-17.9 yr)	Median 2.1 cc	5 patients	Median 55.5 mo	PFS 70.8 at 5 yr

Henderson *et al*[82], 2009	1997-2004	12	-	WHO Grade I LGG (10 patients), WHO Grade II LGG (2 patients)	As part of initial management or salvage therapy	GKSRS	Median margin dose 13 Gy	Median age 17.4 yr (range: 5.9-63 yr)	Median 4.4 cc	4 patients	Median 48.2 mo	PFS 75 at 4 yr
Weintraub *et al*[83], 2012	1989-2011	24	100	LGG	As part of initial management or salvage therapy	GKSRS	Median margin dose 15 Gy	Median age 11 yr (range: 4-18 yr)	Mean 2.4 cc	3 patients	Median imaging follow-up 74 mo	Tumor control 83
Hallemeier *et al*[84], 2012	1992-2005	18	33	Pilocytic astrocytoma	As part of initial management or salvage therapy	GKSRS	Median margin dose 15 Gy	Median age 23 yr (range: 4-56 yr)	Median 9.1 cc	10 patients	Median 8 yr	PFS 41 at 5 yr
Lizarraga *et al*[85], 2012	1995-2010	12	41.7	Pilocytic astrocytoma	Salvage therapy	LINAC-based SRS or SRT	Median dose 18.75 Gy for SRS and median dose 50.4 Gy for SRT	Median age 21 yr (range: 5-41 yr)	Median 6.5 cc for SRT and median 1.69 cc for SRS	0 patient	Median 37.5 mo	PFS 73.3 at long term
Simonova *et al*[86], 2016	1992-2002	25	100	Pilocytic astrocytoma	As part of initial management or salvage therapy	GK-based SRS or SRT	Median margin dose 16 Gy for patients receiving single fraction, median dose 25 Gy for SRT	Median age 13 yr (range: 3-17 yr)	Median 2.7 cc	2 patients	Median 15 yr	PFS 80 at 10 yr
Trifiletti *et al*[87], 2017	1990-2015	28	-	Pilocytic astrocytoma	As part of initial management or salvage therapy	GK-based SRS or SRT	Median margin dose 16 Gy	Median age 17.4 yr (range: 2-70.3 yr)	Median 1.84 cc	4 patients	Median 5.4 yr	PFS 96 at 6 yr
Gagliardi *et al*[88], 2017	2001-2014	39	23.8	LGG	As part of initial management or salvage therapy	GKSRS	Median margin dose 15 Gy	Median age 31 yr (range: 9-72 yr)	Median 1.24 cc	8 patients	Median 54.5 mo	PFS 52.8 at 5 yr

LGG: Low grade glioma; SRS: Stereotactic radiosurgery; GKSRS: Gamma knife stereotactic radiosurgery; WHO: World Health Organization; PFS: Progression free survival; LINAC: Linear accelerator; SRT: Stereotactic radiotherapy.

20 years (range: 4-68 years). Cobalt source and stereo guide were used for either primary or boost therapy with a mean margin dose of 21.7 Gy. Complete response was achieved for 8 patients (50%), and tumor shrinkage or stabilization was detected in 5 patients (31%) corresponding to a tumor control rate of 81%. Three patients (19%) who had brainstem glioma succumbed to their disease with no response to SRS. The authors concluded that radiosurgery could serve as an effective therapeutic modality for management of deeply seated LGG[73].

Somaza *et al*[74] from Pittsburgh University investigated the role of gamma knife SRS (GKSRS) in adjuvant treatment of 9 children with deeply seated, growing and unresectable pilocytic astrocytomas. Lesions had a mean diameter of 16 mm and were localized at cerebellar peduncle, dorsolateral pons, midbrain, thalamus, hypothalamus, caudate nucleus, and temporal lobe. Mean margin dose was 15 Gy. At a mean follow-up duration of 19 mo, tumor control was achieved in all patients with

significant tumor shrinkage in 5 patients and no further growth in 4 patients. No patients suffered from early or late toxicity. The authors concluded that GKSRS proved to be safe and effective for management of deeply seated and small volume pilocytic astrocytomas[74].

Kida *et al*[75] reported long term outcomes of GKSRS in the management of low grade astrocytomas in a large series of 51 patients from Japan. The study included 12 pediatric patients with a mean age of 9.8 years. Tumor control rate was 91.7% for WHO grade I astrocytomas and 87.2% for WHO grade II astrocytomas. Mean margin dose was 12.5 Gy for WHO grade I astrocytomas and 15.7 Gy for WHO grade II astrocytomas. Higher treatment response was achieved in patients ≥ 10 years of age with WHO grade I astrocytomas and for those with follow-up duration exceeding 2 years. The authors concluded that radiosurgery could play an important role in management of low grade astrocytomas and complete cure could be expected at least for some patients[75].

Boëthius *et al*[76] from Sweden reported outcomes of 19 patients receiving GKSRS for pilocytic astrocytoma at Karolinska Hospital. Mean age was 10.6 years (range: 2-60 years) and the study group included 16 pediatric patients. Median tumor volume was 2.2 cc. A median marginal dose of 10 Gy was used since majority of tumors were located within or in close vicinity of the brainstem. At a median radiological follow-up duration of 4.7 years and median clinical follow-up duration of 7 years, a satisfactory tumor control rate of 94.7% was achieved despite the relatively lower GKSRS dose[76].

Hadjipanayis *et al*[77] assessed outcomes of 49 patients (including 29 children) receiving GKSRS at the Pittsburgh University for LGG. Involved locations included the brainstem in 22 patients, thalamus in 6 patients, temporal lobe in 5 patients, cerebellum in 4 patients, frontal lobe in 4 patients, parietal lobe in 3 patients, insular cortex in 1 patient, hypothalamus in 1 patient, third ventricle in 1 patient, corpus callosum in 1 patient, and optic tract in 1 patient. Median age was 14 years (range: 3-52 years) for the 37 patients with pilocytic astrocytoma including 25 children aged ≤ 18 years. Median age was 25 years (range: 5-57 years) for the 12 patients with WHO Grade II fibrillary astrocytoma including 4 children aged ≤ 18 years. Median margin dose was 15 Gy and 16 Gy for pilocytic astrocytomas and WHO Grade II fibrillary astrocytomas, respectively. Overall, serial neuroimaging after GKSRS revealed complete tumor resolution in 11 patients, reduced tumor volume in 12 patients, stable tumor volume in 10 patients, and delayed tumor progression in 16 patients. Out of the 37 patients with pilocytic astrocytoma, tumor control was achieved in 25 patients (68%). Out of the 12 patients with WHO Grade II fibrillary astrocytoma, tumor control was achieved in 8 patients (67%). The authors concluded that SRS offers a safe and promising therapeutic modality for management of selected patients with pilocytic astrocytomas or WHO Grade II fibrillary astrocytomas[77].

Saran *et al*[78] from Royal Marsden Hospital reported outcomes of stereotactically guided conformal radiotherapy (SCRT) in the management of progressive or inoperable pediatric LGG. Median age was 6 years (range: 5-16 years). Fourteen patients received linear accelerator (LINAC)-based SCRT in 30-33 daily fractions, and the total dose was 50-55 Gy. Lesion locations included the optic chiasm in 9 patients, third ventricle in 2 patients, pineal region in 1 patient, craniocervical junction in 1 patient, and hypothalamus in 1 patient. Median tumor volume was 19.5 cc (range: 7.5-180 cc). Median follow-up duration was 33 mo. The 3-year local PFS and overall survival rate following SCRT was 87% and 100%, respectively. The authors concluded that SCRT offers a feasible and high precision technique for stereotactic irradiation of pediatric LGG[78].

Marcus *et al*[79] from Dana-Farber Cancer Institute assessed the efficacy of LINAC-based stereotactic radiotherapy (SRT) for management of small, localized, pediatric brain tumors. Their prospective study included 50 patients with LGG. Out of the 50 patients, 35 patients had WHO grade I astrocytoma and 15 patients had WHO grade II astrocytoma. Median age was 9 years (range: 2-26 years). Out of the 50 patients, 38 patients had progression after surgery and 12 patients had progression after chemotherapy. Mean total dose for SRT was 52.2 Gy delivered in 1.8-Gy daily fractions. With a median follow-up duration of 6.9 years, PFS rate was 82.5% at 5 years and 65% at 8 years. Overall survival was 97.8% and 82% at 5 and 8 years, respectively. There were 6 cases of local progression all within the primary tumor bed. There was no marginal failure. The authors concluded that SRT offers excellent local control for small, localized LGG in children and limited margins with stereotactic immobilization and planning techniques could be considered to minimize late sequelae in view of no marginal failures in the study[79].

Wang *et al*[80] reported outcomes of GKSRS for 21 patients with 25 histologically proven low grade astrocytomas treated at the Taipei Veterans General Hospital.

Median age was 20 years (range: 6-70 years). Median margin dose was 14.5 Gy. With a median radiological follow-up duration of 49 mo and median clinical follow-up duration of 67 mo, all patients with pilocytic astrocytoma were free from tumor progression. Complete tumor remission was achieved in 3 patients. PFS rate was 65% at 10 years. The authors suggested reduction in GKSRS dose to prevent excessive toxicity in the setting of combined use of GKSRS and RT. The authors concluded that GKSRS may be utilized for management of selected patients with low grade astrocytomas to achieve durable long term local tumor control rates with acceptable toxicity[80].

Kano *et al*[81] from Pittsburgh University assessed GKSRS outcomes for management of newly diagnosed or recurrent juvenile pilocytic astrocytomas. Their series included 50 pediatric patients with a median age of 10.5 years (range: 4.2-17.9 years). Lesion locations included the cerebellum in 20 patients, brainstem in 13 patients, cerebral hemispheres in 7 patients, basal ganglia in 6 patients, and ventricles in 4 patients. Out of the total 50 patients, only 5 patients had received prior fractionated RT ± chemotherapy. Median margin dose was 14.5 Gy. Median follow-up duration was 55 mo. For the entire series, PFS after GKSRS (including tumor growth and cyst enlargement) was 91.7%, 82.8% and 70.8% at 1, 3 and 5 years, respectively. Univariate analysis revealed that solid lesion, target volume < 8 cc, newly diagnosed disease, and no brainstem involvement were prognostic factors for improved PFS with statistical significance. The authors concluded that treatment response was better in small volume residual solid juvenile pilocytic astrocytomas and GKSRS should be considered if resection is not feasible or in the presence of early recurrence[81].

Henderson *et al*[82] reported the Indiana University experience with GKSRS for low grade astrocytoma management in a series of 12 patients. Median age was 17.4 years (range: 5.9-63 years). A total of 13 lesions were treated using a median margin dose of 13 Gy. With a median follow-up duration of 48.2 mo, 2- and 4-year tumor control rates were 84.6% and 76.9, respectively. Overall survival and PFS rates were 83.3% and 75% at 4 years, respectively. The authors concluded that GKSRS could provide local control for carefully selected patients with unresectable or recurrent low grade astrocytomas[82].

Weintraub *et al*[83] from Virginia University reported outcomes of GKSRS for management of 24 pediatric patients. Median age was 11 years (range: 4-18 years). Out of the 24 patients, 15 patients were diagnosed with WHO grade I astrocytoma and 4 patients were diagnosed with WHO grade II LGG by histopathological assessment. Mean tumor volume was 2.4 cc and median margin dose was 15 Gy. Median radiological follow-up duration was 74 mo and median clinical follow-up duration was 144 mo. Complete resolution of tumor was achieved in 5 patients (21%) and ≥ 50% reduction in tumor size was achieved in 18 patients (75%). The authors concluded that GKSRS offers good clinical control of residual or recurrent gliomas in pediatric patients[83].

Hallemeier *et al*[84] reported outcomes of 18 patients (including 6 children) treated with GKSRS for recurrent or unresectable pilocytic astrocytoma at the Mayo Clinic. Median age was 23 years (range: 4-56 years). One or more prior surgical resection was performed in 13 patients (72%). Ten patients (56%) had received previous conventionally fractionated external beam RT and 4 patients (22%) had received prior systemic chemotherapy. Median treatment volume for GKSRS was 9.1 cc. Median margin dose was 15 Gy for previously irradiated patients and 16 Gy for patients without prior RT. Median follow-up duration was 8 years. PFS rates were 65%, 41%, and 17% at 1, 5, and 10 years, respectively. Overall survival rates were 94%, 71%, and 71%, at 1, 5, and 10 years after GKSRS, respectively. Prior external beam RT was found to be associated with inferior overall survival and PFS outcomes. The authors concluded that GKSRS could serve as a meaningful therapeutic option for management of recurrent or unresectable pilocytic astrocytomas when surgery and/or external beam RT fails[84].

Lizarraga *et al*[85] from the University of California reported outcomes of LINAC-based stereotactic irradiation for progressive/residual pilocytic astrocytomas in a series of 12 patients (including 5 children < 18 years of age). Median age at the start of stereotactic irradiation was 21 years (range: 5-41 years). All patients had undergone upfront partial surgical debulking as initial management without adjuvant chemotherapy or RT. Salvage stereotactic irradiation was considered in the setting of local progression. LINAC-based SRS was used to treat a median target volume of 1.69 cc in 3 patients with a median dose of 18.75 Gy. LINAC-based SRT with a median total dose of 50.4 Gy was used to treat a median target volume of 6.5 cc in 9 patients. No radiation induced adverse effects were observed in the study, and probabilities of long term PFS and disease specific survival were 73.3% and 91.7%, respectively[85].

Simonova *et al*[86] from Prag assessed long term outcomes of GK-based SRS or SRT for pilocytic astrocytomas in a series of 25 pediatric patients. Median age was 13 years (range: 3-17 years)[86]. Selection of single fraction or fractionated stereotactic irradiation was based on lesion size, location and proximity to surrounding critical structures. Median target volume was 2.7 cc (range: 0.2-25 cc). Five patients (20%) received single fraction radiosurgery with a median dose of 16 Gy. Twenty patients (80%) received stereotactic irradiation in 5 or 10 fractions using a median dose of 25 Gy. The 10-year overall survival and PFS rates were 96% and 80%, respectively. A significantly better PFS was observed in patients with a planning target volume of 2.7 cc or less. The authors concluded that radiosurgery offers an alternative therapeutic modality for management of small residual or recurrent pilocytic astrocytomas providing long term local control[86].

Trifiletti *et al*[87] reported outcomes of 28 patients receiving GK-based stereotactic irradiation for management of pilocytic astrocytomas at the University of Virginia. Median age was 17.4 years (range: 2-70.3 years). Single fraction GKSRS was performed in 27 patients, and 1 patient received stereotactic irradiation in 3 fractions. Median tumor volume was 1.84 cc and median margin dose was 16 Gy. Median clinical follow-up duration was 5.2 years and median radiological follow-up duration was 4.6 years. Local tumor control rate was 93% without adverse radiation effects. Actuarial PFS rates were 96%, 96%, 96%, and 80% at 1, 3, 6, and 12 years, respectively. The authors concluded that SRS offers an appropriate technique for management of pilocytic astrocytomas in the primary or recurrent disease setting with favorable tumor control rates and infrequent clinical toxicity[87].

Gagliardi *et al*[88] assessed long term outcomes of GKSRS for LGG. Their series of 39 patients included 10 pediatric patients. Median age was 31 years (range: 9-72 years). Most common histology was pilocytic astrocytoma. Median tumor volume was 1.24 cc. Median margin dose was 15 Gy. Median follow-up duration was 54.5 mo. Actuarial PFS rates at 1, 5, and 10 years were 74.9%, 52.8%, and 39.1%, respectively. Assessment of patients' quality of life and functional performance was performed by utilization of standardized functional performance scores and validated subjective health survey questionnaires. Clinical improvement and Karnofsky Performance Status improvement were observed in 52.4% and 45.5% of the patients, respectively. The authors concluded that GKSRS may serve as a viable therapeutic modality for management of LGG which may provide tumor growth control and improve patients' functional performance and quality of life with optimization of social functioning and minimization of disease-related psychological impact[88].

In summary, stereotactic irradiation has been more frequently incorporated into management of pediatric LGG as compared to adult HGG. Pilocytic astrocytoma accounts for the majority of pediatric LGG and may be considered as suitable for radiosurgical treatment with its well-defined borders on neuroimaging. Clearly, several other factors are critical in decision making for stereotactic irradiation of a pediatric patient with LGG. Stereotactic irradiation has been used as primary therapy in the presence of deeply seated lesions at eloquent brain areas, or as a boost treatment in conjunction with conventionally fractionated external beam RT, and more frequently to treat progressive or recurrent pediatric LGG (Table 1)[73-88]. Overall, these series reported favorable tumor control rates with stereotactic irradiation. Improvements have been observed in clinical symptoms, functional performance and quality of life parameters with low rates of severe toxicity. However, there is still room for improvement with the need for accumulation of further robust and high level evidence to consider stereotactic irradiation as a standard part of management for pediatric LGG.

CONCLUSION

Pediatric brain tumors are the most common solid tumors in children which may lead to morbidity and mortality. Gliomas comprise the majority of brain tumors in children. Radiotherapeutic management of gliomas in children poses a formidable challenge considering the adverse effects of irradiation for this vulnerable patient population. In this context, efforts have been focused on improving the toxicity profile of radiation delivery. Stereotactic irradiation with SRS or SRT in a single or few treatment fractions may serve as a viable radiotherapeutic approach to achieve this goal. High conformality along with steep dose gradients around the target volume allows for reduced normal tissue exposure under precise immobilization and image guidance. While conventionally fractionated RT regimens administered over 5 wk to 6 wk may lead to

substantial burden on children particularly when daily anesthesia is needed, radiosurgical approaches allow for abbreviated treatment courses. Also, margin-free strategies may be considered in the setting of stereotactic irradiation with precise immobilization and image guidance for management of well demarcated lesions such as pilocytic astrocytomas[89].

Overall, stereotactic irradiation has been utilized less frequently for HGG and more commonly for LGG in children[58-61,73-89]. Some of the studies reporting data on stereotactic irradiation of pediatric gliomas also included adult patients. Drawing firm conclusions may be confounded by diversities in patient, tumor, and treatment characteristics in studies with limited number of patients and inherent limitations. Nevertheless, available limited data on stereotactic irradiation of pediatric gliomas suggest potential utility of this contemporary approach as part of initial management or for treatment of progressive or recurrent lesions despite the need for further supporting evidence.

In the context of future directions, immunotherapy, identification of driver alterations and introduction of effective targeted therapies may pave the way for innovatory treatment strategies for children with pediatric glial neoplasms[90-93]. There is need for active investigation on development of safe and efficacious therapeutic approaches for management of pediatric glial neoplasms.

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World Journal of Methodology

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World J Methodol 2021 May 20; 11(3): 75-80

DOI: 10.5662/wjm.v11.i3.75

ISSN 2222-0682 (online)

MINIREVIEWS

Rationalising animal research synthesis in orthopaedic literature

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Author contributions: Tsikopoulos K was involved in the study conceptualization and writing of the original draft; Sidiropoulos K contributed to provision of resources and revising of the article; Kitridis D was involved in the generation of figures and project administration; Drago L, Ebenzar R and Lavalette D supervised this project and contributed to revisions of this paper; all authors reviewed and approved the final version of the manuscript.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

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Abstract

Systematic reviews in orthopaedic literature are frequently criticised for offering inconsistent conclusions. On top of that, high-quality randomized human evidence on crucial orthopaedic topics is more often than not lacking. In this situation, pooling animal literature could offer an excellent insight into unanswered critical clinical questions, thus potentially improving healthcare. In this paper, we sought to present the rationale and basic principles governing meta-analysis of animal research. More specifically, we elaborated on the available evidence-based methods to achieve a scientifically sound animal data synthesis. In addition, we discussed result interpretation, strength of recommendations and clinical implications based on the results of these meta-analytic modalities.

Key Words: Meta-analysis; Animal research; Evidence synthesis; *in vivo*; Orthopaedics

Core Tip: Relying on the findings of properly conducted meta-analyses of animal research is crucial, particularly in the paucity of human evidence on crucial orthopaedic topics. It is an undeniable fact that authors tend to encounter a great many challenges when conducting this type of research as they have to address several potential sources

Manuscript source: Invited manuscript

Specialty type: Orthopedics

Country/Territory of origin: United Kingdom

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 2, 2021
Peer-review started: January 2, 2021
First decision: January 25, 2021
Revised: February 5, 2021
Accepted: March 12, 2021
Article in press: March 12, 2021
Published online: May 20, 2021

P-Reviewer: Pérez-Cabezas V
S-Editor: Liu M
L-Editor: A
P-Editor: Yuan YY

of bias. For that reason, we advocate that readers should critically appraise the findings of animal syntheses papers.

Citation: Tsikopoulos K, Sidiropoulos K, Kitridis D, Drago L, Ebnezar R, Lavalette D. Rationalising animal research synthesis in orthopaedic literature. *World J Methodol* 2021; 11(3): 75-80
URL: https://www.wjgnet.com/2222-0682/full/v11/i3/75.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i3.75

INTRODUCTION

Given the nature and rarity of many orthopaedic diseases, conducting high-quality double-blind randomized control trials is not always feasible. This is particularly true when it comes to addressing a particular orthopaedic surgical intervention. Hence, crucial research questions remain unanswered due to the fact that safe conclusions cannot be drawn purely based on a few underpowered and low-quality individual studies. In this situation, animal evidence could offer valuable information towards delineating the potential of a prevention and/or therapeutic orthopaedic intervention.

The rationale behind synthesizing animal literature is to avoid the potential bias which is commonly detected in narrative literature reviews. To elaborate, selective presentation of individual study findings and incorrect weighting of conclusions can exert a negative impact on the credibility of a systematic review. Rather, by summarizing the results of multiple individual studies a researcher could potentially produce more valid results provided that guidelines governing meta-analyses of animal papers are respected. In this paper, we sought to present the key elements for conducting a high-quality meta-analysis of animal research which could provide a useful insight into unanswered clinical questions in orthopaedics.

PROSPECTIVE ANIMAL REVIEW REGISTRATION AND REPORTING GUIDELINES. ARE THEY NECESSARY?

Regardless of the nature of the subjects utilised in an *in vivo* evidence synthesis, it is strongly advocated that systematic reviews be prospectively registered with a valid database (*e.g.*, PROSPERO). The main reason behind this protocol registration is to increase transparency in reporting and prevent selective outcome reporting issues.

On top of that, abiding by published guidelines for systematic reviews (*e.g.*, Preferred Reporting Items for Systematic Reviews and Meta-Analyses) is of utmost importance given the fact that poor reporting diminishes accuracy and potential usefulness of an animal meta-analysis[1].

CONTROLLED *VS* UNCONTROLLED DATA SYNTHESIS: IS THERE ANY DIFFERENCE?

From a methodological standpoint, if properly controlled homogenous groups are available, then standard head-to-head meta-analysis can be safely undertaken by using a readily available piece of statistical software [*e.g.*, Review Manager (RevMan)][2]. However, synthesising uncontrolled research represents a different task which can be achieved by means of proportional meta-analysis[3]. It is underlined that although indirect comparisons could be made by comparing overlapping of confidence intervals in the aforementioned type of meta-analysis, safe conclusions on the comparative efficacy of interventions cannot be reached and therefore this approach is not generally recommended.

LUMPING INTERVENTION GROUPS IN META-ANALYSES OF ANIMAL RESEARCH

One frequently encountered methodological issue in pair-wise meta-analyses is the limited statistical power precluding reliable conclusions to be drawn[4]. To address this issue, lumping intervention groups into valid subgroups with respect to literature classifications[5,6] is recommended. By and large, a crucial point authors need to pay attention to when they elect for the subgroup pathway is the trade-off between statistical power and precision in reporting. We advocate that as long as published guidelines have been followed prior to creating subgroups and sensitivity analysis has been conducted to investigate the impact of subgrouping on the data synthesis, the validity of the findings is not severely compromised.

POOLING DICHOTOMOUS AND CONTINUOUS DATA MEASURING THE SAME OUTCOME. IS IT POSSIBLE?

Encountering a situation where information for the same outcome is presented in some studies as dichotomous data and in other papers by means of a continuous variable is a common phenomenon in animal research. To address this issue, re-expressing standardized mean differences to odds ratios (or the *vice versa*) is recommended[7]. Subsequently the generic inverse variance model in RevMan can be utilised to pool those converted data together[7] (Figure 1). Although we recognise this could be a challenging task for a researcher to accomplish, the problem of missing information which may compromise the validity of the meta-analysis results can be overcome[5].

FEASIBILITY OF EXTRACTING QUANTITATIVE DATA FROM GRAPHICAL PRESENTATIONS

Meticulous data extraction is a crucial element in performing a satisfactory systematic review and meta-analysis. It is a common phenomenon in original papers published a long time ago to present their findings in a graphical manner with no corresponding numerical data. In this situation, taking advantage of the use of an appropriate software tool (*e.g.*, Plot Digitizer and Getdata Graph Digitizer)[8] which allows for reliable digitization of graphs and/or plots is recommended to abstract and subsequently synthesise the required information.

QUALITY ASSESSMENT IN SYSTEMATIC REVIEWS OF ANIMAL PAPERS

Quality appraisal of individual animal studies performed by means well-established tool such as the SYRCLE's Risk of Bias tool[9], ensures consistency and prevents discrepancies in assessing risk of bias in systematic reviews of animal intervention studies. SYRCLE's Risk of Bias tool is an adaptation of the Cochrane Risk of Bias tool which could potentially facilitate transition of animal research into clinical practice. On top of that, due to the relatively standardised use of this instrument in the existing literature, the necessity of improving particular methodological aspects of animal studies can be easily stressed[9]. It should be noted that a graphical quantification of the risk of bias summarising the assessments for each domain could be of essence (Figure 2)[5].

IS PUBLICATION BIAS A COMMON THREAT TO VALIDITY IN LABORATORY ANIMAL RESEARCH?

It is an undeniable fact that "negative" laboratory animal results more often than not remain unpublished[10]. Therefore, exploration of selective reporting in animal papers appears to be critical. In other words, merely relying on statistical significance may introduce bias in the results of the statistical analysis and potentially threaten the validity of the meta-analysis findings.

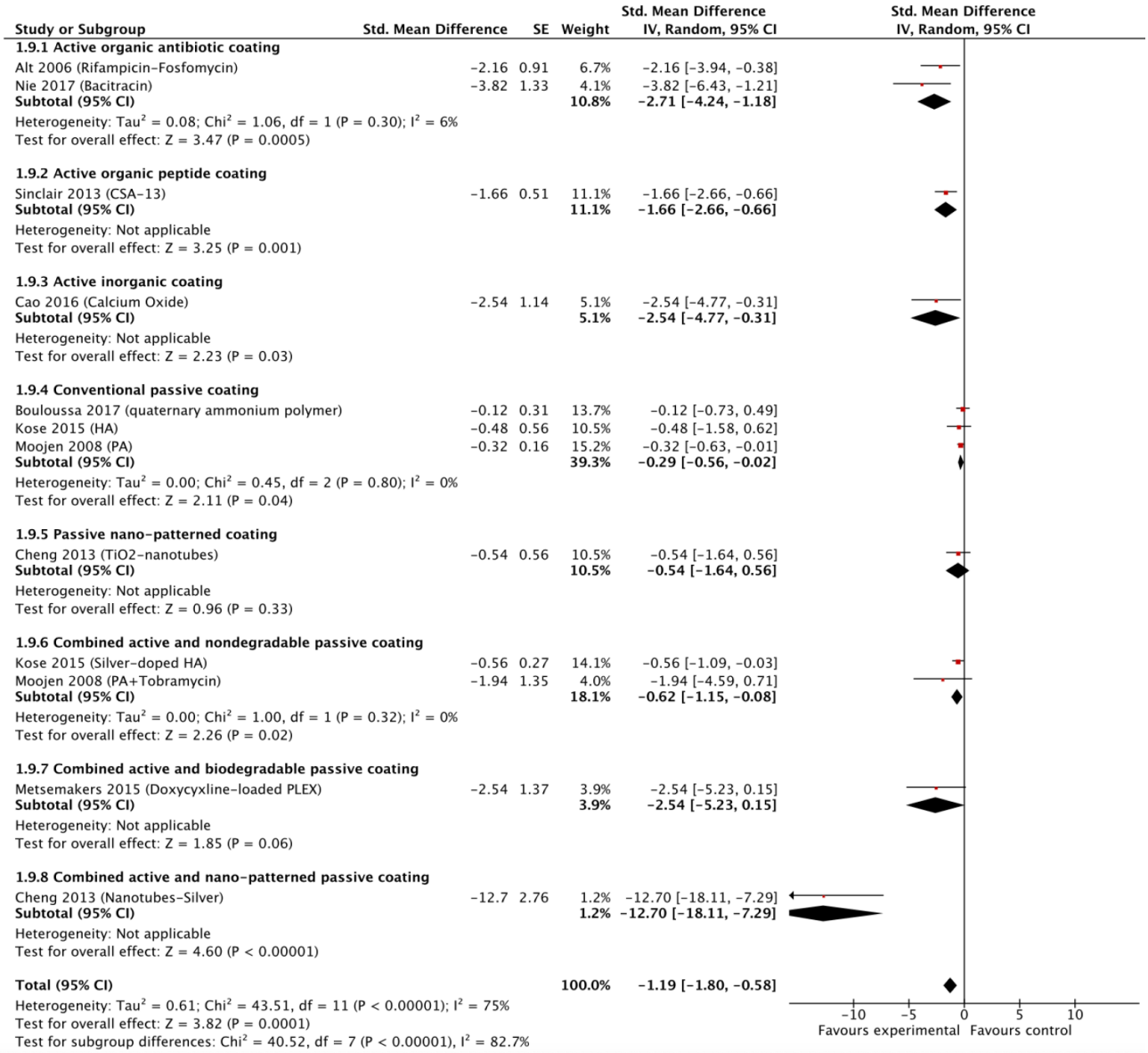

Figure 1 Forest plot of standardised mean differences with multiple subgroup analyses is demonstrated. The methicillin-resistant *Staphylococcus aureus* infection prevention potential is assessed by means pair-wise meta-analysis in inverse variance mode to consider not only continuous but also dichotomous data in the analysis. CI Confidence interval; CSA: Cationic steroidal antimicrobial; HA: Hydroxyapatite; SMD: Standardised mean difference; IV: Inverse variance; PA: Periapatite; PLEX: Polymer-lipid encapsulation matrix; SE: Standard error; TiO2 = Titanium dioxide. Citation: Tsikopoulos K, Sidiropoulos K, Kitridis D, Hassan A, Drago L, Mavrogenis A, McBride D. Is coating of titanium implants effective at preventing *Staphylococcus aureus* infections? A meta-analysis of animal model studies. *Int Orthop* 2020. Copyright© The Author(s) 2020. Published by Springer Nature Publishing Group[5]. The authors have obtained the permission for figure using from the Springer Nature Publishing Group (Supplementary material).

HIERARCHY OF EVIDENCE-BASED MEDICINE AND BIAS ASSESSMENT

It is highlighted that while a systematic review is generally better than an individual study, a meta-analysis of animal studies should not be placed at the top of the hierarchy in a pyramid that depicts validity[11]. This is because a meta-analysis is as good as the studies identified and included[12]. Nevertheless, in the absence of high-quality evidence, relying on the results of a meta-analysis of animal models is advisable provided that caution is exercised due to potential bias.

INTERPRETING RESULTS AND DRAWING CONCLUSIONS

It is worthy of note that prior to drawing meta-analysis conclusions, sample size of the included comparison groups, quality rating of the involved studies, effect sizes, and statistical heterogeneity should be taken into account. On top of that, investigating the

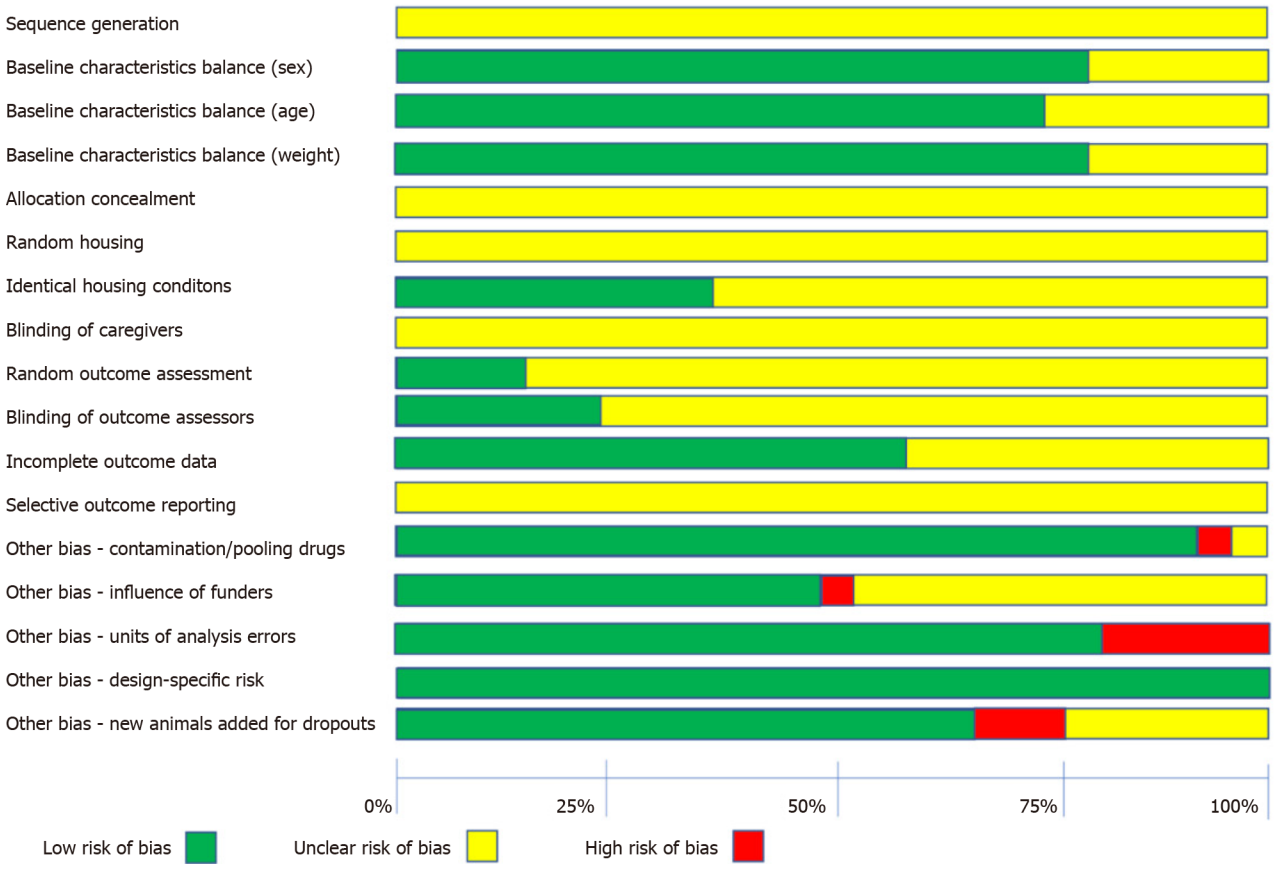

Figure 2 Quantification of risk of bias assessment enables not only summarising quality appraisal results but also making judgments as to what the future studies should look at. Citation: Tsikopoulos K, Sidiropoulos K, Kitridis D, Hassan A, Drago L, Mavrogenis A, McBride D. Is coating of titanium implants effective at preventing *Staphylococcus aureus* infections? A meta-analysis of animal model studies. *Int Orthop* 2020. Copyright© The Author(s) 2020. Published by Springer Nature Publishing Group[5]. The authors have obtained the permission for figure using from the Springer Nature Publishing Group (Supplementary material).

impact of various sources of clinical heterogeneity by means of a sensitivity analysis (*i.e.*, exclusion of one or more papers from the analysis to assess the impact of a particular confounding factor on the findings of the study) with a view to verify the meta-analysis results is strongly advocated.

CONCLUSION

Despite the abundance of literature on developing meta-analytic skills relating to human data, methodological papers dealing with animal data synthesis are lacking. In the current article, we focused on the technicalities and implications of pooling animal literature which could be particularly useful when investigating the results of orthopaedic surgical interventions in the absence of human evidence. It is worthy of note that due to the experimental nature of animal papers, a certain amount of uncertainty in the meta-analysis conclusions is anticipated. For that reason, we advise caution when it comes to extrapolating the results of this type of research back to human biology.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 May 20; 11(3): 81-87

DOI: 10.5662/wjm.v11.i3.81

ISSN 2222-0682 (online)

MINIREVIEWS

Bowel intussusception in adult: Prevalence, diagnostic tools and therapy

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: All the authors are aware of the content of the manuscript and have no conflict of interest.

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Abstract

Intussusception is defined as invagination of one segment of the bowel into an immediately adjacent segment. The intussusception refers to the proximal segment that invaginates into the distal segment, or the intussusception (recipient segment). Intussusception, more common occur in the small bowel and rarely involve only the large bowel. In direct contrast to pediatric etiologies, adult intussusception is associated with an identifiable cause in almost all the symptomatic cases while the idiopathic causes are extremely rare. As there are many common causes of acute abdomen, intussusception should be considered when more frequent etiologies have been ruled out. In this review, we discuss the symptoms, location, etiology, characteristics, diagnostic methods and treatment strategies of this rare and enigmatic clinical entity in adult.

Key Words: Adult intussusception; Bowel invagination; Bowel obstruction; Computed tomography; Laparoscopic surgery; Endoscopy

Manuscript source: Invited manuscript

Specialty type: Medical laboratory technology

Country/Territory of origin: Italy

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 18, 2021
Peer-review started: January 18, 2021
First decision: February 14, 2021
Revised: February 15, 2021
Accepted: March 18, 2021
Article in press: March 18, 2021
Published online: May 20, 2021

P-Reviewer: Zhang L
S-Editor: Gao CC
L-Editor: A
P-Editor: Yuan YY

Core Tip: Intussusception in adult is rare, but its onset is often tumor-related. The diagnosis of intussusception in adult is challenging as a result of the nonspecific signs and symptoms. We herein discuss the epidemiology and the clinical features of bowel intussusception in adult and the role of radiology and surgery in the management of this insidious condition.

Citation: Panzera F, Di Venere B, Rizzi M, Biscaglia A, Praticò CA, Nasti G, Mardighian A, Nunes TF, Inchingolo R. Bowel intussusception in adult: Prevalence, diagnostic tools and therapy. *World J Methodol* 2021; 11(3): 81-87
URL: https://www.wjgnet.com/2222-0682/full/v11/i3/81.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i3.81

INTRODUCTION

The term intussusception refers to the invagination of a segment of the gastrointestinal tract into the lumen of an adjacent segment[1]. This condition lead to a transient or permanent bowel obstruction that can evolve even to intestinal ischemia. Intussusception is much more prevalent in children rather than in adult with an overall incidence in the second group of around 2-3 cases per million of the general population per-year[2].

Adult intussusceptions often onsets as an intermittent cramping abdominal pain associated with signs of bowel obstruction[3]. Diagnosis of intussusception in adult is challenging since the acute abdominal pain is at the same time a non-specific symptom and one of the most frequent complaint reported in the setting of emergency medicine.

Past medical history, physical exam and laboratory test can aid to increase the level of suspicion, but imaging is almost always needed to make diagnosis of bowel intussusception. Although abdominal computed tomography (CT) scan is useful in this setting, it has low specificity in differentiate malignant, benign or idiopathic lead points[4-6].

The optimal management for adult intussusception is still controversial, nevertheless its definitive treatment consists in surgical intervention with appropriate approach depending on the underlying etiology and location.

ETIOLOGY

Any perturbation of the normal pattern of intestinal peristalsis increase the risk of intussusception[7]. As opposed to the pediatric population, adult intussusception is commonly caused by a pathologic lead point; it can be located in the lumen of the bowel, inside the wall or extramural[8], and its occurrence is associated to an identifiable cause in 80%-90% of symptomatic cases[7,9,10]. The causes of adult intussusception are summarized in Table 1.

Malignant and benign neoplasms account for 60% of cases with a lead point; the remaining non-idiopathic cases are usually caused by postoperative adherences, Crohn's disease, infections, intestinal ulcers, and Meckel diverticulum[7,11].

In a recent systematic review and meta-analysis from Hong *et al*[12] 1229 adults with intussusception were identified from 40 retrospective case series: Pooled rates of malignant and benign tumors and idiopathic etiologies were 32.9%, 37.4% and 15.1%, respectively.

According to several reports[7-9,11], when dividing etiologies by enteric and colonic location, the small bowel intussusception is more often caused by benign lesions. In contrast, colonic intussusception is more likely to have an underlying malignant lead point (often a colonic adenocarcinoma). When the small bowel intussusception is induced by malignant lesions these are often metastatic disease (*i.e.*, carcinomatosis).

Notably the ileocolic location in adult intussusception is a variant in which almost the totality of cases has a malignant lead point involving the ileocecal valve[9] (Table 2).

Table 1 Causes of adult intussusception

Benign	Malignant
Enteric	
Adherences, coeliac disease, Crohn's disease, endometriosis, hamartoma, infections, Kaposi sarcoma, lipoma, Meckel diverticulum, neurofibroma, polyps (inflammatory, adenomatous), stromal tumor, tubercolosis	Adenocarcinoma, carcinoid tumors, leiomyosarcoma, lymphoma, malignant gastrointestinal stromal tumor, metastatic carcinoma, neuroendocrine tumor
Colonic	
Adherences, inflammatory pseudopolyp, lipoma, polyps (inflammatory, adenomatous)	Adenocarcinoma, lymphoma, sarcoma

Table 2 Frequent causes of adult intussusception located to ileocolic site

Ileocolic	
Malignant	Adenocarcinoma, metastatic carcinoma, lymphoma, gastrointestinal stromal tumor

PREVALENCE

As previously mentioned, bowel intussusception afflicts children more than adults with an approximate ratio of 20 to 1. In fact intussusception in adult account for < 5% of all cases of intussusception and is found in 1% of patients with bowel obstruction[7], with a surgical report of less than 1 in 1300 abdominal operations[13]. Usually it involves adults, after the fifth decade, with no difference among male and female[8].

The bowel intussusception is commonly classified in four types according to the land-marks of its origin and extension: (1) Enteric type: the intussusception is limited to the small intestine; (2) Ileocolic type: the ileum passes the ileocolic segment, but the appendix does not invaginate; (3) Ileocecal type: the ileocecal portion invaginates into the ascending colon; and (4) Colocolonic type: the intussusception is limited to the colon and rectum (no anal protrusion).

Small bowel is more often involved by intussusception rather than large bowel. Based on the systematic review of Hong *et al*[12] the pooled rates of enteric, ileocolic, and colonic location types account for 49.5%, 29.1%, and 19.9%, respectively.

This predominance of enteric intussusception has its exception in the populations of the central and western Africa in which is most common the cecocolic intussusception (tropical intussusception)[14] probably for the interaction of dietary habits (high-fiber diet), genetics and gut microbiome features.

PATHOPHISIOLOGY AND CLINICAL PRESENTATION

The most common locations involved in intussusception are at the junctions between mobile and fixed segments of the bowel, such as between the freely-moving ileum and the retroperitoneal cecum[8].

Part of a proximal segment of the bowel slides into the next distal section. This event can lead to bowel obstruction and intestinal ischemia. The compromised blood flow to the affected segment can cause necrosis of the intestinal wall with bacterial translocation, peritonitis, sepsis and even perforation. The clinical scenario can be variable but usually characterized by acute intermittent or constant crampy abdominal pain, vomiting and bloating[15,16].

Pain is the most common symptom reported at a rate of up to 80% in several series[11,12,17,18].

The patient present abdominal tenderness and signs of systemic inflammatory response syndrome (*i.e.*, hypothermia or hyperthermia, hypotension, and tachycardia). Fever is usually a sign of the onset of intestinal necrosis. Decreased or absent bowel sounds can be present as well as signs of parietal peritoneal irritation, failure to gas passage, abdominal masses, and diarrhoea even with bloody stool. Laboratory tests usually document increase of leukocytes count and inflammatory markers such as polymerase chain reaction.

DIAGNOSTIC TOOLS

As already extensively presented in previous studies, the preoperative diagnosis of bowel intussusception raises several questions to the doctor and in this regard, a paper published by Reijnen *et al*[19] report a preoperative diagnostic rate of 50%.

Intestinal intussusception presents considerable variability in the patient's clinical presentation (abdominal pain, vomiting, nausea) and shows signs of palpable abdominal masses on objective examination.

To make a correct differential diagnosis with other similar intestinal pathologies, it is therefore useful to use radiodiagnostic instruments: abdomen X-ray, small bowel series with barium, abdominal ultrasound, abdominal CT.

Intussusceptions are classified according to location (enteroenteric, ileocolic, ileocecal, or colo-colic) and cause (benign, malignant, or idio-pathic).

The abdomen X-ray (Figure 1) may reveal signs of intestinal obstruction (hydro-air levels, distension of the intestinal tract upstream, unexplained masses) which can occur in different abdominal quadrants depending on the level of obstruction (high or low)[20].

An upper gastrointestinal contrast entero-X-ray may show a "stacked coin" or "coil-spring" appearance, while the lower gastrointestinal contrast entero-X-ray, useful in patients with colic or ileus-colic obstruction, may show a “cup-shaped” filling defect or “spiral” or “coil-spring” appearances[20].

Another useful tool is ultrasound, a methodical operator dependent, which can show signs such as the “target” or “doughnut” in the transverse scans (Figure 2), or the "pseudo-kidney" sign or "hay-fork" sign in the longitudinal view[21].

CT is currently considered the gold standard for the intussusception diagnosis. Very sensitive, it can highlight the position, the nature of the mass and the relationship with the surrounding tissues[20].

The CT scan may help to find a lead-point intussusception that can be localized in the all bowel tract. The CT scan can also demonstrate some pathognomonic radiological aspects as target-like and the sausage-shaped soft tissue mass. These specific findings can be clearly visible or they can remain undetected due to edema; in these cases the classic three-layer appearance and anatomic detail are often lost and so an irregularity mass can show the intussusception. Mesenteric fat and blood vessels are barely visible.

THERAPY

As previously reported, adult’s intussusception is frequently cause by a pathologic lead point. For those reasons, treatment of bowel intussusception causing obstruction has typically involved surgery, often with bowel resection, as opposed to the pediatric population. The attempt of hydrostatic reduction in the adult population is not indicated; on the contrary, in the pediatric population this is the treatment of choice in the majority of cases; in fact, in this latter group of age the percentage of surgical treatment is so far less the 10% of the reported cases[22].

In recent series and retrospective review articles[23-26], the evidence that the increased use of cross sectional imaging such as CT has resulted in increment of the radiological diagnosis of intussusception, with a successful nonoperative management in many cases, has led to some degree of controversy regarding optimal management of these patients.

The main issues in the management of adult intussusception are: (1) When proceed with surgical exploration; (2) Once the surgical approach is the treatment of choice, whether attempt intraoperatively reduction or proceed direct to resection of the affected segments; and (3) Once the surgical approach is the treatment of choice, it should be performed open or laparoscopically.

In the most recent review article is reported that surgical exploration is the treatment of choice in case of: (1) Patients with signs and symptoms of acute abdomen; in this scenario abdominal exploration is the gold standard when symptoms of clinical obstruction are reported in association with radiological signs of obstruction, dehydration and increase of white blood cells along with inflammatory markers at laboratory tests; emergency exploration is mandatory in presence of signs of septic shock and peritonism (conditions almost always suggestive of intestinal ischemia); (2) Patients with diagnosis of intussusception with a mass visible on CT scan, also in the absence of clear clinical signs of acute abdomen; and (3) Patients with diagnosis of colonic or ileocolic intussusception, usually associate with neoplasm, also in the

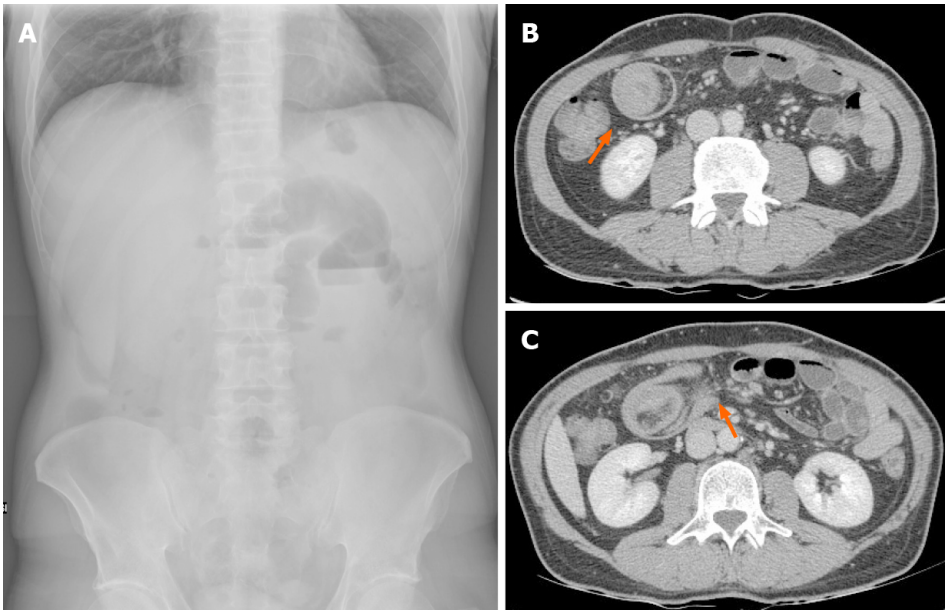

Figure 1 Inflammatory fibroid polyp of the small intestine. A 43-year-old male presenting with abdominal pain and vomiting. A: Abdomen X-ray showed signs of intestinal obstruction with hydro-air levels in the upper quadrants; B: Computed tomography scan confirmed bowel obstruction with presence of "target sign" (orange arrow); C: Mesenteric fat and blood vessels are visible (orange arrow). Surgical resection revealed an inflammatory fibroid polyp of the ileum.

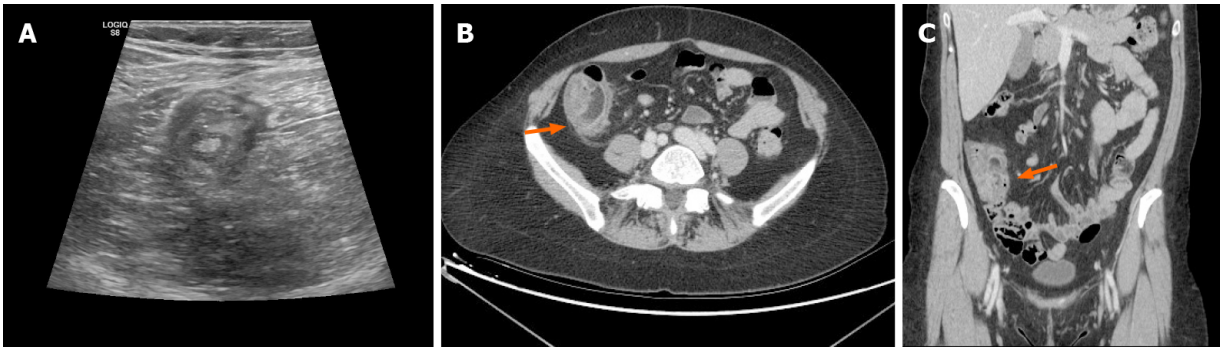

Figure 2 Ileocecal valve adenocarcinoma. A 56-year-old female presenting with right iliac fossa pain. A: Ultrasound scan revealed "target sign"; B and C: Computed tomography scan confirmed ielo-colic intussusception, with no signs of bowel obstruction [orange arrow, horizontal (B) and coronal (C)]. Surgical resection revealed an ileocecal valve adenocarcinoma (pT2 N0).

absence of clear clinical signs of acute abdomen. In these setting, preoperative endoscopy can be done in order to confirm the presence of pathology and/or cancer[8].

On the other side, many reports suggest a "wait and see" strategy, with serial clinic and imaging evaluation to ensure spontaneous resolution in entero-enteric intussusceptions without lead point mass and short affected segment (< 3.5 cm)[24-26]. Based on the systematic review of Hong *et al*[12], it is important to remark that the pooled rate of patients that received this type of conservative treatment is less of 5% and is limited to patient with entero-enteric locations.

Undoubtedly, other controversy remains as to whether reduction of the intussusception should be attempted intraoperatively[27,28].

This controversy is related to the consideration that reducing the intussusception before resection carries risks of perforation and the theoretical possibility of dissemination of malignant cells during the attempt. The other theoretical risks of preliminary manipulation and reduction of an intussuscepted bowel is related to the endangerment of anastomotic complications of the manipulated friable and edematous bowel tissue[16,20].

On the other hand, the reduction of bowel intussusception is useful both to preserve important lengths of small bowel and to prevent possible development of short bowel syndrome, especially when the small bowel is the only tract involved because of its lower rate of association to malignancy[29,30].

On this point, we suggest that simple reduction is acceptable in post-traumatic or idiopathic intussusceptions, where no pathological cause could be identified, obviously after the exclusion of bowel ischaemia or perforation, especially in case of small bowel intussusception. Considering the high rate of primary adenocarcinoma, colonic intussusception should be resected *en bloc* without reduction to avoid potential intraluminal seeding or venous tumor dissemination: a formal resection using appropriate oncologic techniques are recommended, with the construction of a primary anastomosis between healthy and viable tissue. Finally, a selective approach seems appropriate for ileocolic adult intussusception because of its intermediate nature between enteric and colonic sites[11,12,31].

The choice of preforming laparoscopic rather than open procedure depends both on the clinical condition of the patient and on surgeon's laparoscopic experience[8,27].

A standardized laparoscopic technique to approach intussusception is not available, due to the all different possible causes and locations, some tips and tricks are reported in literature[8,32,33]: the pneumoperitoneum establishment must be performed with open laparoscopy at the umbellicum because of the high risk of bowel lesions with the Verres technique. Due to the rarity of the left-side's intussusception is recommend to place the two additional 5-mm ports one in the left lower quadrant and the other suprapubically. If needed, other ports can be placed depending on the location of the pathology. During laparoscopy all four quadrants of the abdomen and the pelvis must be thoroughly explored; once the pathologic segment is found, it can either be resected or eviscerated and dealt with extracorporeally using small incision, depending on surgeon skill and severity of the occlusive syndrome related to intussusception. It is recommended to sample suspected fluid collections for culture as well as to biopsy suspected lesions.

CONCLUSION

Bowel intussusception in adult is a rare condition with acute onset or seldom-elusive progress. Clinicians and surgeons are not supported by designated scoring systems in this challenging diagnosis because of non-specific symptoms, and its preoperative identification is often missed or delayed. On the other hand, intussusception is a surgical emergency associated to high rates of mortality in case of delayed treatment, therefore it is important to think about this less common diagnostic possibility when facing an acute abdominal pain with sign of bowel obstruction.

The management of bowel intussusception in adult remains mainly surgical. The timing and type of approach depends on several factors such as the underlying causes, the severity of clinical presentation, the site and the length and vitality of the bowel segment involved.

Anyway, the increased use of cross sectional imaging has increased the early-diagnosis of intussusception, in many cases with a successful nonoperative management; such findings led to some questioning about the optimal management of these conditions.

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World Journal of Methodology

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World J Methodol 2021 May 20; 11(3): 88-94

DOI: 10.5662/wjm.v11.i3.88

ISSN 2222-0682 (online)

ORIGINAL ARTICLE

Randomized Clinical Trial

Comparison of lag screws and double Y-shaped miniplates in the fixation of anterior mandibular fractures

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Author contributions: Melek L solely contributed to the design of the study, implementation of the clinical trial, writing and revision of the manuscript.

Institutional review board statement: This study was approved by the Institutional Review Board (IRB) of the Faculty of Dentistry, Alexandria University, Egypt, and the protocols used in the study were approved by the Research Ethics Committee. Human Subjects Review: Approval Number: IRB 00010556-IORG 0008839 Board Name: Research Ethics Committee, Alexandria Faculty of Dentistry Board Affiliation: Faculty of Dentistry, Alexandria University, Egypt Phone: (+203) 4812201.

Clinical trial registration statement: The study was registered on clinicaltrials.gov (ClinicalTrials.gov ID: NCT04396054).

Informed consent statement: A written informed consent was signed by each patient before the operation.

Conflict-of-interest statement: The author declares no conflict of interest.

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Abstract

BACKGROUND

Mandibular fractures constitute about 80.79% of maxillofacial injuries in Alexandria University, either as isolated mandibular fractures or as a part of panfacial fractures. The combination of symphyseal and parasymphyseal fractures represent 47.09% of the total mandibular fractures.

AIM

To compare the effectiveness of lag screws *vs* double Y-shaped miniplates in the fixation of anterior mandibular fractures.

METHODS

This study is a prospective randomized controlled clinical trial, performed on sixteen patients with anterior mandibular fractures. Patients were divided equally into two groups, each consisting of eight patients. Group 1: Underwent open reduction and internal fixation using two lag screws. Group 2: Underwent open reduction and internal fixation using double Y-shaped plates. The following parameters were assessed: operating time in minutes, pain using a visual analog scale, edema, surgical wound healing for signs and symptoms of infection, occlusion status and stability, maximal mouth opening, and sensory nerve function. Cone beam computed tomography was performed at 3 and 6 mo to measure bone density and assess the progression of fracture healing.

RESULTS

The study included 13 males (81.3%) and 3 females (18.8%) aged 26 to 45 years (mean age was 35.69 ± 6.01 years). The cause of trauma was road traffic accidents in 10 patients (62.5%), interpersonal violence in 3 patients (18.8%) and other causes in 3 patients (18.8%). The fractures comprised 10 parasymphyseal fractures (62.5%) and 6 symphyseal fractures (37.5%). The values of all parameters were comparable in both groups with no statistically significant difference except for the mean bone density at 3 mo postoperatively which was 946.38 ± 66.29 in group

Data sharing statement: No additional data are available.

CONSORT 2010 statement: The guidelines of the CONSORT 2010 statement have been adopted.

Manuscript source: Invited manuscript

Specialty type: Dentistry, oral surgery and medicine

Country/Territory of origin: Egypt

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: October 13, 2020
Peer-review started: October 13, 2020
First decision: December 21, 2020
Revised: January 2, 2021
Accepted: March 11, 2021
Article in press: March 11, 2021
Published online: May 20, 2021

P-Reviewer: Fuentes R, Miyamoto I
S-Editor: Zhang L
L-Editor: Webster JR
P-Editor: Yuan YY

1 and 830.36 ± 95.53 in group 2 (P = 0.015).

CONCLUSION

Both lag screws and double Y-shaped miniplates provide favorable means of fixation for mandibular fractures in the anterior region. Fractures fixed with lag screws show greater mean bone density at 3 mo post-operation, indicative of higher primary stability and faster early bone healing. Further studies with larger sample sizes are required to verify these conclusions.

Key Words: Anterior mandibular fractures; Symphyseal fracture; Parasymphyseal fracture; Miniplates; Lag screws; Double Y-shaped plates

Core Tip: The aim of this study is to compare the effectiveness of lag screws *vs* double Y-shaped miniplates in the fixation of anterior mandibular fractures in terms of fracture stability and progression of bone healing.

Citation: Melek L. Comparison of lag screws and double Y-shaped miniplates in the fixation of anterior mandibular fractures. *World J Methodol* 2021; 11(3): 88-94
URL: https://www.wjgnet.com/2222-0682/full/v11/i3/88.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i3.88

INTRODUCTION

Mandibular fractures constitute about 80.79% of maxillofacial injuries in Alexandria University, either as isolated mandibular fractures or as a part of panfacial fractures. The combination of symphyseal and parasymphyseal fractures represent 47.09% of the total mandibular fractures[1]. However, this percentage of anterior mandibular fractures in relation to other mandibular fractures is variable among different studies and locations[2].

Lag screws have been described as a reliable, stable and safe method of internal fixation for anterior mandibular fractures. The absence of anatomical hazards, thickness of the bone cortices and curvature of the anterior mandible are all factors contributing to the suitability and success of using lag screws in that region[3].

Miniplates have been widely used for decades for the fixation of mandibular fractures owing to their easy handling and adaptation, in addition to providing functionally stable fixation[4]. Different designs of miniplates, varying from the conventional form by Champy, have been proposed to provide extra stability of the fracture. A biomechanical study has shown that double Y-shaped miniplates provide greater resistance to displacement in comparison to conventional straight miniplates[5].

The aim of this study is to compare the effectiveness of lag screws *vs* double Y-shaped miniplates in the fixation of anterior mandibular fractures.

MATERIALS AND METHODS

Ethic statements

This study is a prospective randomized controlled clinical trial. It was performed on sixteen patients with anterior mandibular fractures, selected from those admitted to the Emergency Department of Alexandria University Hospital. This study followed the Declaration of Helsinki with regard to medical protocol and ethics, and the regional Ethical Review Board of the Faculty of Dentistry, Alexandria University approved the study (Approval Number: IRB 00010556-IORG 0008839). The study was registered on clinicaltrials.gov (ClinicalTrials.gov ID: NCT04396054). A written informed consent was signed by each patient before the operation.

Patients

The patients were divided equally into two groups, each consisting of eight patients. Assignment of each patient into one of these two groups was carried out using computer random numbers: Group 1: Underwent open reduction and internal fixation using two lag screws; Group 2: Underwent open reduction and internal fixation using double Y-shaped plates.

Inclusion criteria

Patients of both genders aged from 25 to 45 years, suffering from anterior fractures of the mandible (symphyseal or parasymphyseal) were included. Those with old fractures, infected or comminuted fractures were excluded from the current study.

Study design

A thorough clinical examination was performed preoperatively on all patients, in addition to panoramic radiographs. All patients were operated by the same surgeon under general anaesthesia with nasotracheal intubation. Complete disinfection of the oral cavity and face was performed using povidone iodine solution, followed by draping with sterile towels exposing the surgical site. Maxillomandibular fixation was carried out to adjust the occlusion using arch bars and eyelet wiring. After that, an intraoral mandibular vestibular incision was made exposing the fracture line where reduction of the two segments was carried out under direct vision.

In the first group, fixation of the reduced segments was achieved using 2 lag screws (O and M Medical GmbH Eschenweg, Germany). The diameter of the screws was 2.7 mm and the length ranged from 18 to 24 mm. Screw fixation was performed by passage of the screw through a larger gliding hole into a smaller traction hole on each side of the fracture (Figure 1). In the second group, fixation of the reduced segments was achieved using double Y-shaped plates (Stryker-Leibenger, Germany) with 6 monocortical 2.0 mm diameter screws (Figure 2).

After direct fixation was performed in both groups, the incision was closed using layered suturing and the maxillomandibular fixation was removed. Postoperative care for all patients included the following: (1) Each patient received intravenous Cefotaxime 1 mg/12 h (Cefotax, by EIPICO) for one day postoperatively followed by Amoxicillin clavulanate (Augmentin, manufactured by MPU) 1 mg given orally twice daily for the next 5 d; (2) An analgesic anti-inflammatory drug in the form of Diclofenac Sodium (Rheumafen, by GlaxoSmithKline) 75 mg vial up to the second postoperative day was given followed by Diclofenac Potassium (Rheumafen tablets, by GlaxoSmithKline) 50 mg tablets three times daily for the next 5 d; (3) All patients were instructed to use chlorohexidine mouth wash (Hexitol, by an Arabic drug company) for maintenance of good oral hygiene; and (4) Instructions for a soft high calorie diet was given to all patients to be followed for 4 wk postoperatively.

Postoperative follow-up: Patients were followed up on the second, third postoperative days, first and second weeks, then after one, 3 and 6 mo. The following parameters were assessed: operating time in minutes, pain using a visual analog scale, edema, surgical wound healing for signs and symptoms of infection, occlusion status and stability, maximal mouth opening, and sensory nerve function using a dental probe to assess sensory changes along the mental nerve distribution and comparing it to the contralateral side. Cone beam computed tomography was performed at 3 and 6 mo to measure bone density and assess the progression of fracture healing.

Statistical analysis

Data were fed to the computer and analyzed by the appropriate statistical tests using the IBM Statistical Package for Social Science software version 21.0. Significance of the obtained results was set at the 5% level. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean and standard deviation. The independent samples *t*-test was used to compare the means of quantitative data.

RESULTS

This study was conducted on 16 patients suffering from anterior mandibular fractures. The study included 13 males (81.3%) and 3 females (18.8%) aged 26 to 45 years (mean age was 35.69 ± 6.01 years). The cause of trauma was road traffic accidents in 10 patients (62.5%), interpersonal violence in 3 patients (18.8%) and other causes in 3

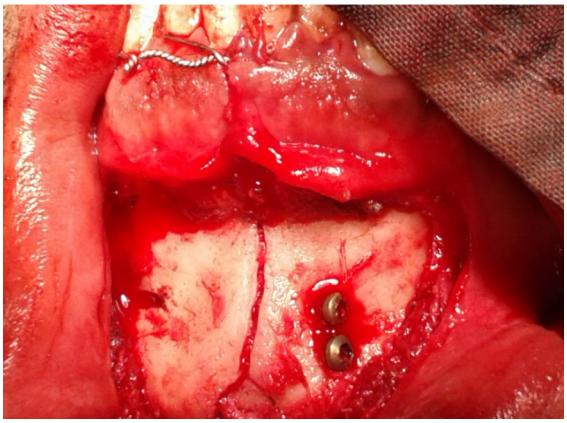

Figure 1 Symphyseal fracture fixed with two lag screws.

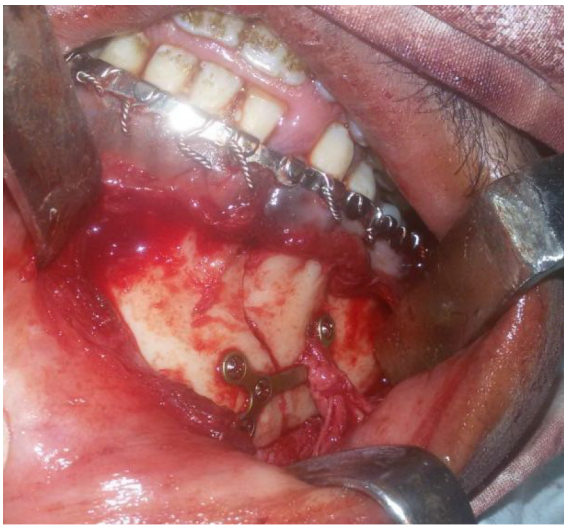

Figure 2 Parasymphyseal fracture fixed with double Y-shaped miniplate.

patients (18.8%). The fractures comprised 10 parasymphyseal fractures (62.5%) and 6 symphyseal fractures (37.5%).

In group 1, patients were treated with open reduction and internal fixation using lag screws and the mean operating time from start of hardware application to end of fixation was 14.38 ± 1.92 min. In group 2, patients were treated with open reduction and internal fixation using double Y-shaped miniplates and the mean operating time was 15.63 ± 1.53 min. The difference between the two groups regarding the mean operating time was statistically insignificant ($P > 0.05$) (Table 1).

With regard to postoperative edema, only 2 patients in the study sample showed severe edema (12.5%), while all other patients demonstrated mild to moderate edema (87.5%) on the second postoperative day. By the end of the first week, the edema has resolved completely in all patients.

The mean pain intensity in the first postoperative week was 4.125 ± 1.25 in group 1 and 4.75 ± 1.04 in group 2 with no statistically significant difference ($P = 0.294$). Pain was completely resolved by the end of the second week.

The mean maximal mouth opening measured two weeks after surgery was 38.25 ± 2.38 mm in group 1, and 37.63 ± 2.92 mm in group 2 with no statistically significant difference ($P = 0.646$).

The surgical wounds healed uneventfully in all patients in both groups except for one patient in group 2 who had wound dehiscence that was managed conservatively using irrigation and antiseptic mouth washes until secondary intention healing was achieved. No sensory nerve impairment was detected postoperatively in any of the patients in either group. Satisfactory occlusion and normal inter-cuspal relation were evident in all patients except for one patient in group 1 who had slight malocclusion postoperatively, that was managed by selective grinding.

Table 1 Mean operating time

	Group	*n*	mean ± SD	*t*	Significance (2-tailed)
Operating time	1	8	14.3750 ± 1.92261 min		0.172
Operating time	2	8	15.6250 ± 1.52947 min	-1.439	

The mean bone density at the fracture line [measured in grey scale using the CBCT OnDemand3D™ software (310 Goddard Way, Suite 250 Irvine, CA, United States, https://www.ondemand3d.com)] at 3 mo postoperatively was 946.38 ± 66.29 in group 1 and 830.36 ± 95.53 in group 2. The difference between the two groups was statistically significant (P = 0.015). At 6 mo postoperatively, the mean bone density in group 1 was 1062.66 ± 63.89 and in group 2, it was 1083.86 ± 82.83, with no statistically significant difference between the 2 groups (Table 2).

DISCUSSION

The current study compared the use of lag screws *vs* double Y-shaped miniplates in the fixation of anterior mandibular fractures and comparable results were found in most evaluated parameters except for a statistically significant higher mean bone density in the lag screw group at 3 mo postoperatively.

The male to female ratio in the study sample showed a marked male predilection (4.33: 1) in agreement with other studies[1,6]. It is suggested that high-speed driving and greater participation in outdoor activities are probably more characteristic in men rather than women in our society, which renders them more susceptible to accidents in that age group. Moreover, in accordance with previous studies, road traffic accidents were the major cause of trauma followed by personal violence and other causes[1,7].

The present study demonstrated a comparable mean operating time in both groups with no statistically significant difference, starting from hardware application to the end of fixation. This is in contrast to other studies which have shown shorter time for lag screw fixation in comparison to miniplates[8,9].

Mean pain score at the end of the first week was numerically (but not statistically) lower in the first group. Bhatnagar *et al*[10] obtained similar results with less pain in the lag screw group, and they explained their findings by the higher stability of the fracture line provided by lag screws in comparison to miniplates and less hardware applied leading to reduced persistent postoperative pain.

No postoperative sensory nerve impairment was detected in either group after fracture fixation, owing to the gentle fracture manipulation, careful dissection of the mental nerve and cautious application of screws in close proximity to the nerve. This is concordant with the results of the study by Agarwal *et al*[11] who did not observe any postoperative nerve deficit and stressed the importance of skills and patience during hardware application in anterior mandibular fractures.

The difference in mean bone density was statistically significant between the two groups at 3 mo post-operation suggestive of early bone healing. This is consistent with previous studies[9,12] using lag screws in fractures of the anterior mandible. This may be due to their compressive effect on the fracture segments, facilitating the progression of primary bone healing. However, by the end of the follow-up period, both groups had comparable mean bone density values indicative of adequate fracture healing and stability. Double Y-shaped miniplates with their special design have shown predictable biomechanical behavior with greater resistance to displacement when compared with straight miniplates[5].

To our knowledge, this is the first clinical trial comparing lag screws to double Y-shaped miniplates in the fixation of anterior mandibular fractures. This special design of miniplates provides better stability than straight miniplates and easier application/adaptation than 3-dimensional miniplates in the anterior region. However, the main limitation of the current study is the small sample size, which in some way, might have affected interpretation of the results. The small number of patients included is attributed to the meticulous case selection to meet all the inclusion criteria and minimize the variability between cases as much as possible.

Table 2 Mean bone density in the 2 groups at 3 and 6 mo postoperatively

	Group	*n*	mean ± SD	*t*	Significance (2-tailed)
Bone density 3 mo post-operation	1	8	946.3825 ± 66.29304	2.822	0.015[a]
	2	8	830.3625 ± 95.52573		
Bone density 6 mo post-operation	1	8	1062.6575 ± 63.88916	-0.573	0.576
	2	8	1083.8550 ± 82.82562		

[a]$P < 0.05$.

CONCLUSION

Both lag screws and double Y-shaped miniplates provide favorable means of fixation for mandibular fractures in the anterior region. Fractures fixed with lag screws show greater mean bone density at 3 mo post-operation, indicative of higher primary stability and faster early bone healing. Further studies with larger sample sizes are required to verify these conclusions.

ARTICLE HIGHLIGHTS

Research background

Several methods of fixation are available for the management of anterior mandibular fractures.

Research motivation

It is important to find the most suitable method to provide optimal fixation and stability against torsional forces in these fractures.

Research objectives

The effectiveness of lag screws and double Y-shaped miniplates in the fixation of anterior mandibular fractures was compared.

Research methods

Sixteen patients divided into 2 equal groups were included in the study.

Research results

The values of all parameters were comparable between the 2 groups except for the mean bone density which was significantly higher in the lag screw group at 3 mo post-operation.

Research conclusions

Both methods provide favorable fixation for anterior mandibular fractures with lag screws apparently leading to higher primary stability and faster healing.

Research perspectives

Further studies to confirm this conclusion and to compare with other methods of fixation are recommended.

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World Journal of Methodology

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World J Methodol 2021 May 20; 11(3): 95-109

DOI: 10.5662/wjm.v11.i3.95

ISSN 2222-0682 (online)

SYSTEMATIC REVIEWS

Tocilizumab as treatment for COVID-19: A systematic review and meta-analysis

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Author contributions: All authors made equal contribution to this manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest.

PRISMA 2009 Checklist statement: The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines were followed for evaluating records identified during the literature search.

Manuscript source: Invited manuscript

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Abstract

BACKGROUND

The majority of patients with coronavirus disease 2019 (COVID-19) have good prognoses, but some develop a critical illness that can lead to death. Evidence shows severe acute respiratory syndrome is closely related to the induced cytokine storm. Interleukin-6 is a key player; its role in systemic inflammation is well known.

AIM

To evaluate the effect of tocilizumab (TCZ), an interleukin-6 receptor antagonist, on the outcomes for patients with COVID-19 pneumonia.

METHODS

PubMed, EMBASE, SCOPUS, Web of Science, MedRxiv, Science Direct, and the Cochrane Library were searched from inception to 9th June 2020 for observational or prospective studies reporting results of hospitalized adult patients with COVID-19 infection treated with TCZ. Effect sizes were reported as odds ratios (ORs) with 95% confidence intervals (CIs), and an OR less than 1 was associated with a better outcome in those treated with TCZ.

RESULTS

Overall 13476 patients (33 studies; n = 3264 received TCZ) with COVID-19 pneumonia and various degree of severity were included. Outcome was improved with TCZ. In the primary analysis (n = 19 studies reporting data),

Specialty type: Infectious diseases

Country/Territory of origin: Italy

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: November 1, 2020
Peer-review started: November 1, 2020
First decision: November 30, 2020
Revised: December 2, 2020
Accepted: March 28, 2021
Article in press: March 28, 2021
Published online: May 20, 2021

P-Reviewer: Fan Y
S-Editor: Zhang L
L-Editor: A
P-Editor: Xing YX

mortality was reduced in patients treated with TCZ (OR = 0.64, 95%CI: 0.47-0.87; $P < 0.01$). In 9 studies where risk of death with TCZ use was controlled for other variables mortality was reduced by 57% (OR = 0.43, 95%CI: 0.27-0.7; $P < 0.01$). Intensive care need (mechanical ventilation) was also reduced (OR = 0.36, 95%CI: 0.14-0.89; $P = 0.02$).

CONCLUSION
In COVID-19-infected patients treated with TCZ, outcome may be improved compared to those not treated with TCZ.

Key Words: Tocilizumab; COVID-19; Pandemic; Treatment; Meta-analysis; Review

Core Tip: Coronavirus disease 2019 (COVID-19) infection is associated with a citokine storm during acute phase. Interleukin-6 is a key player in this systemic inflammation. We evaluated the effect of tocilizumab (TCZ) on the outcomes of COVID-19 pneumonia. Mortality was reduced in patients treated with TCZ (Odds ratio =0.64, 95% confidence intervals: 0.47-0.87; $P < 0.01$). We conclude that TCZ may improve outcome of COVID-19 infected patients.

Citation: Petrelli F, Cherri S, Ghidini M, Perego G, Ghidini A, Zaniboni A. Tocilizumab as treatment for COVID-19: A systematic review and meta-analysis. *World J Methodol* 2021; 11(3): 95-109
URL: https://www.wjgnet.com/2222-0682/full/v11/i3/95.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i3.95

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 emerged in Wuhan, China in December 2019 and a pandemic was declared by the World Health Organization on March 11, 2020. The pandemic rapidly became a major global health concern. The vast majority of patients with coronavirus disease 2019 (COVID-19) have good prognoses, but some develop a critical illness that can lead to death. The data show that approximately 20% become severe or critical and require hospitalization[1]. Evidence shows that severe deterioration following severe acute respiratory syndrome coronavirus 2 infection is closely related to the associated cytokine storm[2]. Tocilizumab (TCZ) is an immunomodulatory therapeutic, an interleukin (IL)-6 receptor antagonist approved by the United States Food and Drug Administration and the European Medicine Agency for treating cytokine release syndrome. One of the key cytokines described in the cytokine storm induced by COVID-19 is IL-6, and its role in systemic inflammation is well known. Following an intriguing biological rationale, several institutions have proposed using TCZ off-label to treat COVID-19[3]. Thus far, randomized controlled trials have not been reported in the literature, but observational studies and case reports describe the compassionate use of TCZ. Results leave the efficacy of TCZ controversial. We performed a meta-analysis of the studies available to date.

MATERIALS AND METHODS

Literature search and selection criteria

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines were followed for evaluating records identified during the literature search[4].

The search included MEDLINE, EMBASE, Scopus, the medRxiv preprint server, Science Direct, Web of Science, and the Cochrane Controlled Register of Trials for articles published up to June 9, 2020 describing trials or observational series about the efficacy of TCZ in patients with COVID-19 pneumonia. Search terms were tocilizumab and COVID-19. The inclusion criteria were: (1) Randomized or single-arm prospective

studies, observational or retrospective case series of patients with COVID-19 and treated with TCZ outside of clinical trials; (2) written in the English language; (3) reporting patient clinical characteristics; and (4) including at least 5 patients. Animal studies, case reports, editorials, commentaries, and clinical or pharmacological reviews were excluded. If multiple studies reported on the same population and met the inclusion criteria, the newest study was selected unless different endpoints or subgroup analyses were performed or updated.

Data extraction and endpoints

Two authors (Ghidini A, Petrelli F) determined article eligibility based on the abstracts. A third (Zaniboni A) independently read the articles, and agreement for trial inclusion was reached. Two authors (Petrelli F, Ghidini A) independently extracted data to a standard form constructed using Microsoft Word and compared results for agreement. Extracted data were author, publication year, number of participants treated, study design, patient group demographics and clinical characteristics (*e.g.*, median age, sex, country, comorbidities), median follow-up, laboratory and clinical parameters (symptoms) of participants, rate of admission to the intensive care unit (ICU) before and after TCZ use, associated drugs, imaging (baseline and improvements shown in imaging), number of cycles with TCZ and resulting adverse events, death rate, median hospitalization time, rate of discharge from the ICU and/or hospital, and hazard ratios for mortality or other events associated with TCZ use.

Eligible studies were critically appraised by two independent reviewers at the study level for methodological and reporting bias by adapting the ROBIN-I tool[5] for assessing risk of bias in selected observational studies. By definition, single-arm or observational trials have a high risk of bias due to the absence of a control group and randomization. Otherwise, the Nottingham-Ottawa-Scale was used as a quality check for retrospective studies.

Statistical analysis

The primary endpoints were mortality (%) and ventilatory improvement (defined as the proportion of participants relieved from ICU admission or from non-invasive ventilation defined at the time from initiation of the study treatment) among those treated with TCZ. The outcome data extracted for each study were analyzed using random-effects models and were reported as weighted measures of any event. Event rates reported in individual studies were aggregated into pooled rates. All other continuous variables were analyzed using descriptive statistics. We used the procedures of the comprehensive meta-analysis (CMA) software to calculate the effect size using dichotomous outcomes; and if these were not available either, we used other statistics (such as *t*-value or *P* value) to calculate the effect size. A random-effects meta-analysis of odds ratios (ORs) was used to aggregate efficacy outcomes reported across trials. A meta-analysis of adjusted ORs attained from multivariate analysis only was also provided.

Heterogeneity was assessed using the χ^2 test. Statistical significance and the magnitude of I^2 were considered. When I^2 was less than 50%, low to moderate heterogeneity was assigned; otherwise, substantial heterogeneity was assigned. A significance threshold of $P < 0.05$ was adopted. All analyses were performed using CMA software version 2.2 (Biostat).

We tested publication bias by inspecting the funnel plot on primary outcome measures and by Duval and Tweedie's trim and fill procedure yields an estimated effect size after publication bias has been taken into account (as implemented in CMA). We also conducted Egger's test of the intercept to quantify the bias captured by the funnel plot and to test whether it was significant.

RESULTS

Thirty-three studies met inclusion criteria among 604 retrieved (Figure 1). The demographic and clinical characteristics of included studies are reported in Tables 1-3 (references reported in Supplementary material). Overall 13476 patients (n = 3264 received TCZ) with COVID-19 pneumonia and various degree of severity were included. The median age was 62 years. Almost all received treatments consisting of antibiotics (*e.g.*, azithromycin), antivirals, steroids plus or minus hydroxychloroquine. Mortality was 22.4% [95% confidence intervals (CIs): 17.9%-26.8%]. Ventilatory status improved in 63.9% (95%CI: 50.4%-75.6%).

Table 1 Baseline characteristics of tocilizumab treated patients

Ref.	Country	Type of study	No. of pts	Median follow up (d)	Male/Female, %	Median age (yr)	CV Comorbities, %	Respiratory/diabetes %	Other/cancer, %	Other medications, %	Ventilatory status (Baseline to end of follow up, %)	ICU admission %/time to ICU admission (d)
Alattar *et al*[11], 2020	Quatar	Retrospective	25	14	92/8	58	12 HTN	-/48	CKD 16/4	HCQ (100), AZITRO (96), lopinavir/ritonavir (96), ribavirin (88), and INF 1-α2a (60)	56 (invasive)	100/1
Alberici *et al* [12], 2020	Italy	Retrospective	6	4	-	-	-	-/-	-/-	Steroids, antivirals, HCQ	33 (16 worsened)	-/-
Capra *et al*[13], 2020	Italy	Retrospective (with ctr arm[1])	82 (*n* = 62 TCZ)	9	73/27	63	63 HTN	-/16	-/-	HCQ (100), lopinavir/ritonavir (100)	35.2 (27% worsened)	4.8/-
Colaneri *et al* [14], 2020	Italy	Retrospective with prop. score	112 (*n* = 21 TCZ)	7	90/10	62.3	47.6 HTN	0/9.5	19/4.7	HCQ, AZITRO, steroids (100)	-	14/-
Hassoun *et al* [15], 2020	United States	Retrospective	9	-	66/33	60	55 HTN	11/11	66/-	HCQ, AZITRO (100) steroids (33), antibiotics (66)	-	89/-
Klopfenstein *et al*[16], 2020	France	Case control	45 (*n* = 20 TCZ)	-	-	76.8	55 HTN/70 CVS disease	20/25	-/35	HCQ or lopinavir/ritonavir + antibiotics ± steroids (100)	-	0/-
Luo *et al*[17], 2020	China	Retrospctive	15	-	80/20	73	66 HTN	-/26.6	-/-	Steroids (53)	6.6 (33.3% worsened)	-/-
Quartuccio *et al* [18], 2020	Italy	Retrospective (with ctr arm[1])	111 (*n* = 42 TCZ)	17.8	78.6/21.4	62.4	47.6 HTN	-/-	-/-	Antivirals (100), HCq (92.9) steroids (40); antibiotics (28.6)	65 (invasive)	57/-
Sciascia *et al* [19], 2020	Italy	Prospective	63	-	89/11	62.6	45	4.7/9.5	-	Lopinavir/ritonavir (71), darunavir/cobicistat (29)	95	7.9/-
Toniati *et al*[20], 2020	Italy	Prospective	100	10	88/12	62	62	9/17	11/6	HCQ, lopinavir/ritonavir or remdesivir, antibiotic, steroids	69 (*n* = 23 worsened)	43/-
Xu *et al*[21], 2019	China	Retrospective	21	-	86/14	56.8	57.2	9.6/23.8	CKD 4.8/-	Lopinavir/ritonavir, IFN-α, ribavirin, steroids (100)	100	-/-
Ramaswamy *et al*[22], 2020	United States	Case control	86 (*n* = 21 TCZ)	-	61.9/38.1	63.2	14.3 HTN/heart disease, AF or stroke 19.1	28.6/14.3	-/0	HCQ (81), AZITRO (23.8), steroids (42.9)	-	47.6/-
Rimland *et al* [23], 2020	United States	Retrospective	11	17	82/18	59	73 HTN/18 CVS	27/36	Renal or liver 18/9	HCQ (36), AZITRO (64)	54 (10% worsened)	73/-

Sanchez-Montalva *et al* [24], 2020	Spain	Prospective	82	-	63/37	59.1	39 HTN/6.1 heart failure/12.2 AF	23.5/19.5	Liver 1.2/-	HCQ (98.9), lopinavir/ritonavir (76.8), AZITRO (96.3), darunavir/cobicistat (25)	53 (52% worsened)	2.9/-
Wadud *et al* [25], 2020	United States	Case control	94 (*n* = 44 TCZ)	-	-/-	55.5	-	-/-	-/-	-	-	-
Campochiaro *et al*[26], 2020	Italy	Retrospective	65 (*n* = 32 TCZ)	28	91/9	64	37 HTN/12 CAD	3/12	CKD 9/6	HCQ, AZITRO, lopinavir/ritonavir (100)	91	0/-
Morena *et al* [27], 2020	Italy	Prospective	51	30	78.4/21.6	60	29.4 HTN/49 CVS disease	9.8/11.8	5.9/5.9	HCQ (98), antibiotics (76), lopinavir/ritonavir (82), remdesivir (42)	66.6 (33% worsened)	11.8/-
Kimmig *et al* [28], 2020	United States	Retrospective (with ctr arm)	60 (*n* = 28 TCZ)	-	46.8/53.2	63.8	53.6 HTN/43 other	35.7/14.3	14/14.3	-	-	-
Roumier *et al* [29], 2020	France	Compassionate use	59 (*n* = 30 TCZ)	8	80/20	50	20 HTN/13 CVS	13/23	33/-	HCQ (6.6), steroids (6.6)	-	23.3/-
Ip *et al*[30], 2020	United States	Retrospective	547 (*n* = 134 TCZ)	30	78/22	62	71.6 HTN and coronary arthery disease	15/35	15/9	HCQ + AZITRO (92), steroids (66)	-	100/-
Perrone *et al* [31], 2020	Italy	Phase 2 and expansion cohort	1221 (*n* = 708 TCZ[3])	30	82/18	61% > 60	68 heart disease or HTN	-/15	-/-	HCQ (75), anti-retroviral (65), antibiotics (50), steroids (28)	-	16 invasive ventilation/-
Perez-Tanoira *et al*[32], 2020	Spain	Cohort study	562 (*n* = 36 TCZ)	-	-/-	-	-	-/-	-/-	-	-	-
Somers *et al* [33], 2020	United States	Observational	154 (*n* = 78 TCZ)	47	68/32	55	85 HTN or heart failure	54/13	CKD 35/-	HCQ (26), steroids (29), remdesivir (3)	56 (18 and worsened)	100/41 < 24 h, 36 > 48 h
Heili-Frades *et al*[34], 2020	Spain	Cohort study	4712 (*n* = 366 TCZ)[2]	-	-/-	-	-	-/-	-/-	-	-	40.7/-
Issa *et al*[35], 2020	France	Retrospective	10	-	100/0	66	60 HTN	-/30	-/-	HCQ (100), steroids (30)	50	70/7 d
Garcia *et al*[36], 2020	Spain	Retrospective	171 (*n* = 77 TCZ)	-	58.8/51.2	61.5	61 HTN or heart disease	10.3/15.6	-/-	Antivirals (100, steroids (50)	90	10.3/-
Ayerbe *et al* [37], 2020	United Kingdom	Retrospective	2075 (*n* = 421	8	-/-	-	-	-/-	-/-	-	-	-/-

			TCZ)									
Borku Uysal *et al*[38], 2020	Turkey	Retrospective	12	22	50/50	65.8	58 HTN	16/58	CKD 8/16	HCQ and antivirals (100), AZITRO (50), antibiotics (58)	82	17/-
Fernandez-Cruz *et al*[39], 2020	Spain	Retrospective	463 (*n* = 189 TCZ)	-	-/-	-	-	-/-	-/-	Steroids (100), other not available	-	-/-
Garibaldi *et al* [40], 2020	United States	Cohort study	832 (*n* = 39 TCZ)	-	-/-	-	-	-/-	-/-	-	-	-/-
Martínez-Sanz *et al*[41], 2020	Spain	Cohort study	1229 (*n* = 260 TCZ)	-	73/27	65	17 HTN, 8 CAD, 2 heart failure	18/15	CKD 4/-	-	-	19/6 d
Petrak *et al*[42], 2020	United States	Retrospective	145	-	64/36	58.1	-	-	-	Corticosteroids (60), HCQ + AZITRO (98.6)	-	-/-
Rossi *et al*[43], 2020	France	Case control	246 (*n* = 106 TCZ)	28	66/34	64	60 HTN, 23.6 CVS	16/45	-/5.7	Antibiotics (100), HCQ (83), steroids (40), lopinavir/ritonavir (0.9)	-	-/-

[1]Control arm consisted in patients treated with hydroxycloroquine + lopinavir/ritonavir before tocilizumab availability.
[2]Hospitalized cohort only.
[3]Modified intent to treat analysis. CVS: Cardiovascular disease; CAD: Coronary arthery disease; AF: Atrial fibrillation; HTN: Hypertension; CKD: Chronic kidney disease; pts: Patients; HCQ: Hydroxycloroquine; AZITRO: Azitromycin; -: Not availble; TCZ: Tocilizumab; ICU: Intensive care unit.

Outcome was improved with TCZ. In the primary analysis (n = 19 studies reporting data), mortality was reduced in patients treated with TCZ (OR = 0.64, 95%CI: 0.47-0.87; P < 0.01; Figure 2). In 9 studies where risk of death with TCZ use was controlled for other variables mortality was reduced by 57% (OR = 0.43, 95%CI: 0.27-0.7; P < 0.01). Intensive care need (mechanical ventilation) was also reduced (OR = 0.36, 95%CI: 0.14-0.89; P = 0.02). In all cases, a random effect model was used.

Egger's test indicated a significant publication bias (P = 0.01). Duvall and Tweedie's trim and fill procedure indicated 4 missing studies (see the funnel plot with imputed studies in Supplementary material). The adjusted effect size (after imputation of the missing studies) was 0.84 (95%CI: 0.63-1.14).

DISCUSSION

A large part of the ongoing research into COVID-19 infection is concentrated on finding an immunomodulatory therapy to down-regulate the cytokine storm, usually combining it with antiviral agents[6]. In fact, IL-6 binds either with transmembrane IL-6 receptors or soluble IL-6 receptors, and the resulting complex can combine with the

Table 2 Laboratory and radiological characteristics of patients treated with tocilizumab

Ref.	Fever (baseline) °C/%	O_2 sat. %	Cough %	Dyspnea %	Leucocytes 10^9/L	Lymphocites/Neutrophil 10^9/L	PLT 10^9/L	Hb g/dL	LDH	Liver tests IU/L	CRP mg/L	PCT ng/L	D-dimer	IL6 ng/L	Imaging %
Alattar *et al*[11], 2020	38/92	-	84	72	6.0	0.9/5.0	208	-	-	46/30	95.2	0.38	-	-	Infiltrates and ground glass opacities 100
Alberici *et al*[12], 2020	-/-	-	-	-	-	-/-	-	-	-	-	-	-	-	-	-
Capra *et al*[13], 2020	38/-	-	-	-	-	-/-	-	-	-	-	123	0.6	-	-	Bilateral pulmonary opacities 100
Colaneri *et al*[14], 2020	-/-	-	-	-	-	0.6/8.4	303	-	445	38/72	21.3	0.24	-	-	Interstitial lung disease 100
Hassoun *et al*[15], 2020	-/-	-	-	-	-	-/-	-	-	-	-/-	-	-	-	-	-
Klopfenstein *et al*[16], 2020	-/-	90	-	-	-	0.67/-	-	-	-	-/-	158	-	-	-	≥ 50% lung involvement 60
Luo *et al*[17], 2020	-/-	-	-	-	-	-/-	-	-	-	-/-	96	-	-	71	-
Quartuccio *et al*[18], 2020	-/-	-	-	-	5540	0.68/4.5	157	-	625	-/-	79.05	-	835	63.5	-
Sciascia *et al*[19], 2020	< 38/39.7	-	-	-	-	-	-	-	-	-	-	-	-	-	Bilateral pulmonary infiltrates
Toniati *et al*[20], 2020	> 37.5/85	-	55	73	6	0.78	177	13.6	413	55/39	97	-	525	41	Ground glass opacities and consolidation, bilateral pulmonary infiltration
Xu *et al*[21], 2019	-/100	-	66.7	-	6.3	0.97	170	-	370	31/29	75	0.33	0.8	153	Ground glass opacities and focal consolidation, peripheral and subpleural
Ramaswamy *et al*[22], 2020	-/-	-	-	-	-	1.1/6.7	200	-	-	60/43.5	15.9	2.2	2900	371	-
Rimland *et al*[23], 2020	-/-	-	-	-	8.5	-/0.8	230	-	1203	51/35	197.3	-	343.5	30.65	-
Sanchez-Montalva *et al*[24], 2020	37.7/91.5	94	86.6	65.9	9.2	0.86/	199	13.3	446	53/41	17.98	-	295	74.8	-
Wadud *et al*[25], 2020	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Campochiaro *et al*[26], 2020	37.6/-	-	-	-	-	-/-	-	-	469	-/-	156	-	-	-	-

Morena *et al*[27], 2020	74.5/-	-	62.7	54.9	9.1	0.8/7.3	230	-	470	48/39	189	-	1706	116	Bilateral pulmonary opacities 100
Kimmig *et al*[28], 2020	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Roumier *et al*[29], 2020	-	-	-	-	-	-	-	-	-	-	189	-	3712	-	-
Ip *et al*[30], 2020	80	-	78	80	-	-/-	-	-	-	-/-	-	-		-	-
Perrone *et al*[31], 2020	-/-	-	-	-	-	-/-	-	-	-	-/-	30	-	-	-	-
Perez-Tanoira *et al* [32], 2020	-/-	-	-	-	-	-/-	-	-	-	-/-	-	-	-	-	-
Somers *et al*[33], 2020	-/-	-	-	-	12.1	0.9/-	-	-	627	50/76	185	-	2400	-	-
Heili-Frades *et al* [34], 2020	-/-	-	-	-	-	-/-	-	-	-	-/-	-	-	-	-	-
Issa *et al*[35], 2020	-/100	-	-	-	-	-/-	-	-	-	-/-	246	-	1354	-	Ground glass opacities
Garcia *et al*[36], 2020	-/98.7	-	83	43	-	0.87/-	-	-	-	-/-	97	-	918	-	-
Ayerbe *et al*[37], 2020	-/-	-	-	-	-	-/-	-	-	-	-/-	-	-	-	-	-
Borku Uysal *et al* [38], 2020	-/92	92	100	67	6.1	1.09/4.3	180	13.8	259	33/39	54	-	599	-	Ground glass opacities
Fernandez-Cruz *et al* [39], 2020	-/-	-	-	-	-	-/-	-	-	-	-/-	-	-	-	-	-
Garibaldi *et al*[40], 2020	-/-	-	-	-	-	-/-	-	-	-	-/-	-	-	-	-	-
Martínez-Sanz *et al* [41], 2020	36.8/-	91	-	-	-	0.89/5.4	-	-	669	-/32	113	-	809	70	-
Petrak *et al*[42], 2020	-	-	-	-	-	-	-	-	538	-	53.3	-	1.3	-	-
Rossi *et al*[43], 2020	37.5/-	94	-	-	-	1.128/-	-	-	-	-	168	-	-	-	-

-: Not available; PLT: Platelets; Hb: Hemoglobin; CRP: C reactive protein; PCT: Procalcitonin C; IL-6: Interleukin-6; sat: Saturation; LDH: Lactate dehydrogenase.

signal-transducing component gp130 to activate the inflammatory response. In an emergent situation where no approved drugs are available and supportive measures are available only for critically ill patients, any new promising agent merits attention. A meta-analysis has correlated IL-6 concentration with COVID-19 severity. Those with severe cases show a 2.9-fold higher concentration than those without complications[7].

Table 3 outcome of patients treated with tocilizumab therapy

Ref.	N° TCZ administered (median doses)	Death %	Dismissed %	Median hospitalization (d)	TCZ AEs %	Comparison with other medications or no TCZ	NOS Scale	ROBIN risk
Alattar *et al*[11], 2020	1	12	36 (from ICU)	-	Anemia 64; ALT ↑ 44	HR for discharge from ICU 0.64 (0.37-1.11)	8	Low
Alberici *et al* [12], 2020	1	33	16	-	-	-	6	Moderate
Capra *et al*[13], 2020	1	8	92	12.5	-	OR for OS 0.036 (0.07-0.18)°	7	Low
Colaneri *et al* [14], 2020	2	23.8	85.7 (from ICU)	2	0	OR for OS 0.78 (0.06-9.34); OR for ICU 0.11 (0-3.38)	7	Low
Hassoun *et al* [15], 2020	1	22	55	13.5 (n = 7)	-	-	5	Low
Klopfenstein *et al*[16], 2020	1 or 2	25	55	13	-	OR for OS and ICU admission 0.36 (0.1-1.3) and 0.03 (0.002-0.56); OR for mechanical vent 0.05 (0.003-0.93)	5	Low
Luo *et al*[17], 2020	1	20	-	-	-	-	5	High
Quartuccio *et al* [18], 2020	1	9.5	28.5	-	-	OR for OS 14.5 (0.76-278.3); OR for ICU admission 220.9 (12.7-3826.1)	8	Moderate
Sciascia *et al* [19], 2020	1 (2 in 82.5%)	11	-	-	-	-	6	Moderate
Toniati *et al*[20], 2020	1 (2 in 87%)	20	15	-	Septic shock (n = 2), GI perforation (n = 1)	-	8	Low
Xu *et al*[21], 2019	1 (2 in 14.3%)	0	100	15.1	-	-	5	Moderate
Ramaswamy *et al*[22], 2020	1 (2 in 38%)	14.3	-	-	-	HR for OS 0.25 (0.07-0.9)	5	Moderate
Rimland *et al* [23], 2020	1	27	18	18	-	-	7	Low
Sanchez-Montalva *et al* [24], 2020	1	26.8	41.5	-	-	-	6	Low
Wadud *et al* [25], 2020	-	38.6	-	-	-	OR for OS 0.58 (0.25-1.32)	6	Moderate
Campochiaro *et al*[26], 2020	1 (2 in 28%)	15	63	13.5	SAEs (25)	OR for OS 0.38 (0.11-1.27); OR for ICU admission 0.33 (0.13-8.5)	8	Low
Morena *et al* [27], 2020	-	27	61	-	AST/ALT ↑ 29, PLT 14, neutropenia 6, rash 2	-	8	Low
Kimmig *et al* [28], 2020	1 (2 in 10.7%)	42.9	25	-	Infections 71.4	OR for OS 2.25 (0.75-2.24)	6	Moderate
Roumier *et al* [29], 2020	1	10	20	-	-	OR for OS 0.25 (0.05-1.03); OR for ICU 0.17 (0.06-0.48)	7	Low
Ip *et al*[30], 2020	1 (78%)	46	-	-	Bacteriemia (13), secondary pneumonia (9)	OR for OS 0.66 (045-0.99)	8	Low
Perrone *et al* [31], 2020	1 (59.8), 2 (54.5)	20	-	-	26.4 G3-5; 14.4 G1-2	OR for 30-d OS 0.7 (0.41-1.22) and 1.22 (0.86-1.92) in phase 2 and validation cohort	8	Low
Perez-Tanoira *et*	-	27.7	-	-	-	OR for OS 1.015 (0.47-2.18)	5	Moderate

al[32], 2020								
Somers *et al* [33], 2020	1	18	56	20.4	Superinfection (54)	OR 0.39 (0.18-0.82)	8	Low
Heili-Frades *et al*[34], 2020	-	22.4	-	-	-	-	6	Moderate
Issa *et al*[35], 2020	1	10	-	11 (ICU)	-	-	5	High
Moreno-Garcia *et al*[36], 2020	-	10.3	84.4	-	-	OR for ICU 0.3 (0.12-0.71) and OR for OS 0.52 (0.21-1.29)	5	Moderate
Ayerbe *et al* [37], 2020	-	21.1	-	-	-	OR for OS 1.9 (1.44-2.51)	5	High
Borku Uysal *et al*[38], 2020	2	0	100	-	-	-	6	Moderate
Fernandez-Cruz *et al*[39], 2020	-	-	-	-	-	OR for OS 0.69 (0.41-1.19)	5	High
Garibaldi *et al* [40], 2020	-	5	-	-	-	OR for OS 1.14 (0.46-2.81)	5	Moderate
Martínez-Sanz *et al*[41], 2020	1	23	-	13	-	OR for OS 2.19 (1.54-3.1)	5	Low
Petrak *et al*[42], 2020	1 (84.8), 2 (15.2)	28.3	48.3	-	-	-	5	Moderate
Rossi *et al*[43], 2020	1	28.9	-	-	-	HR for OS 0.29 (0.17-0.49)	8	Low

-: Not availble; NOS: Nottingham-ottawa-scale; ROBIN: Risk of bias of non-randomized studies; ALT: Alanine aminotransferase.

Siltuximab, a chimeric monoclonal antibody acting and blocking IL-6, is being tested in the SISCO study, including patients with acute respiratory distress syndrome related to COVID-19 infection (NCT04322188). Preliminary data from 21 patients showed a reduction in the C-reactive protein levels in 16 patients, a clinical improvement in 33% and disease stabilization in 43% of cases[8].

In this pooled analysis of 31 studies including 2898 patients treated with TCZ, we found a strong trend toward improved survival with the use of TCZ (a significant reduction in acute mortality risk by 36%). Tocilizumab administration was also independently associated with a 57% reduced risk of death in multivariable analysis. Tocilizumab reduced also the risk of mechanical ventilation and ICU admission by 64%. Overall mortality rate was 22%.

The limitations of these data are related to the observational nature of the studies, primarily monocentric and non-controlled. The population treated with TCZ was negatively selected for the worst clinical and inflammatory conditions. Also, due to the non-randomized design of all studies, final results might have been biased, and the added value of TCZ might not have been formally proven. However, despite a likely imbalance among clinical and laboratory baseline variables between the 2 groups, the effect of TCZ on clinical outcomes appears sustained. We finally recognize that some papers reported in the primary analysis were pre-printed in MedRxiv archive and not still finally reviewed and published in full.

At this time, 45 trials are underway to explore the contribution of TCZ when added to the standard of care for COVID-19. Four are in phase 3 trials: the COVACTA study (NCT04320615), in which TCZ is compared with placebo, the NCT04361552 study in which the control arm is represented by best practices, the COV-AID study (NCT04330638), a six-arm study including anakinra and the association of anakinra + TCZ, and the RECOVERY study (NCT04381936), also a six-arm study, including hydroxychloroquine, lopinavir/ritonavir, and low doses of steroids.

Recently, the use of hydroxycloroquine or chloroquine with or without a macrolide was associated with decreased survival and increased rate of ventricular arrhythmias in COVID-19 hospitalized patients[9]. Despite this alarming concern, article and data purity were subsequently questioned and article retracted. Similarly, results of a separate study with data attained from a different database, showed that hydroxycloroquine failed to reduce infection risk in people exposed to patients with confirmed COVID-19. Results indicated that the incidence of new illness compatible with

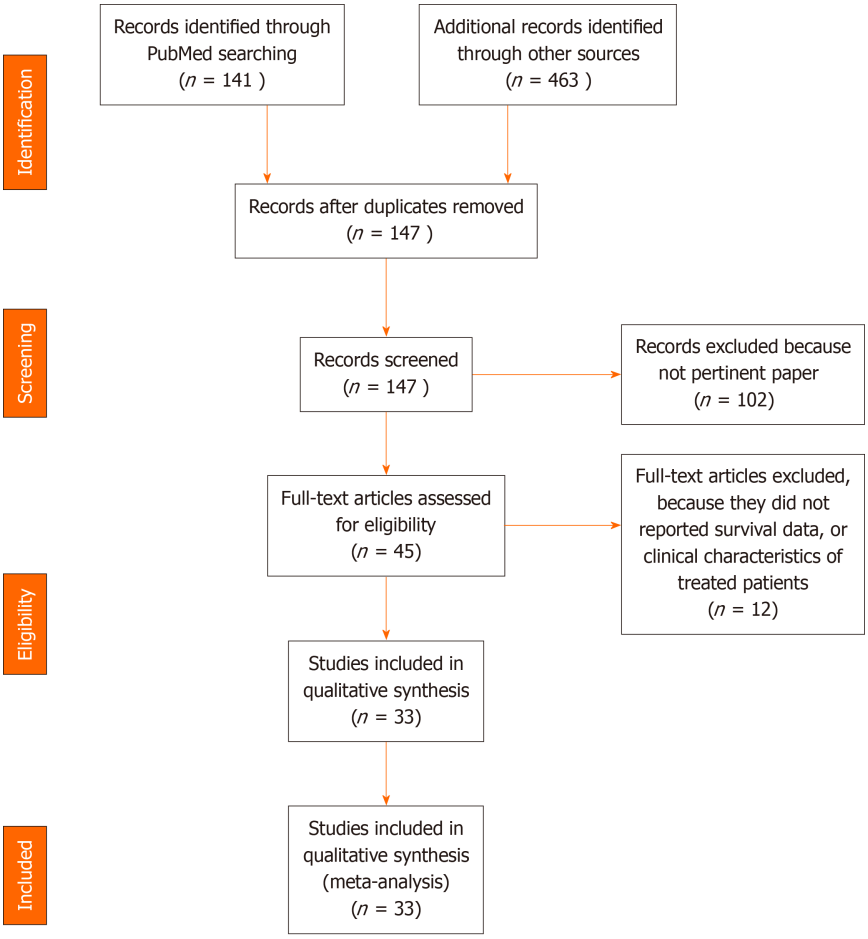

Figure 1 Thirty-three studies met inclusion criteria among 604 retrieved.

COVID-19 did not differ significantly between those who received hydroxycloroquine and those who received placebo[10]. Therefore, new combinations of potentially active drugs need to be tested, and efficacy confirmed in these patients[11-43].

CONCLUSION

In conclusion, we provide the first evidence that TCZ can improve the respiratory and clinical outcomes of patients with COVID-19 pneumonia in clinical practice, but its use merits further confirmatory trials.

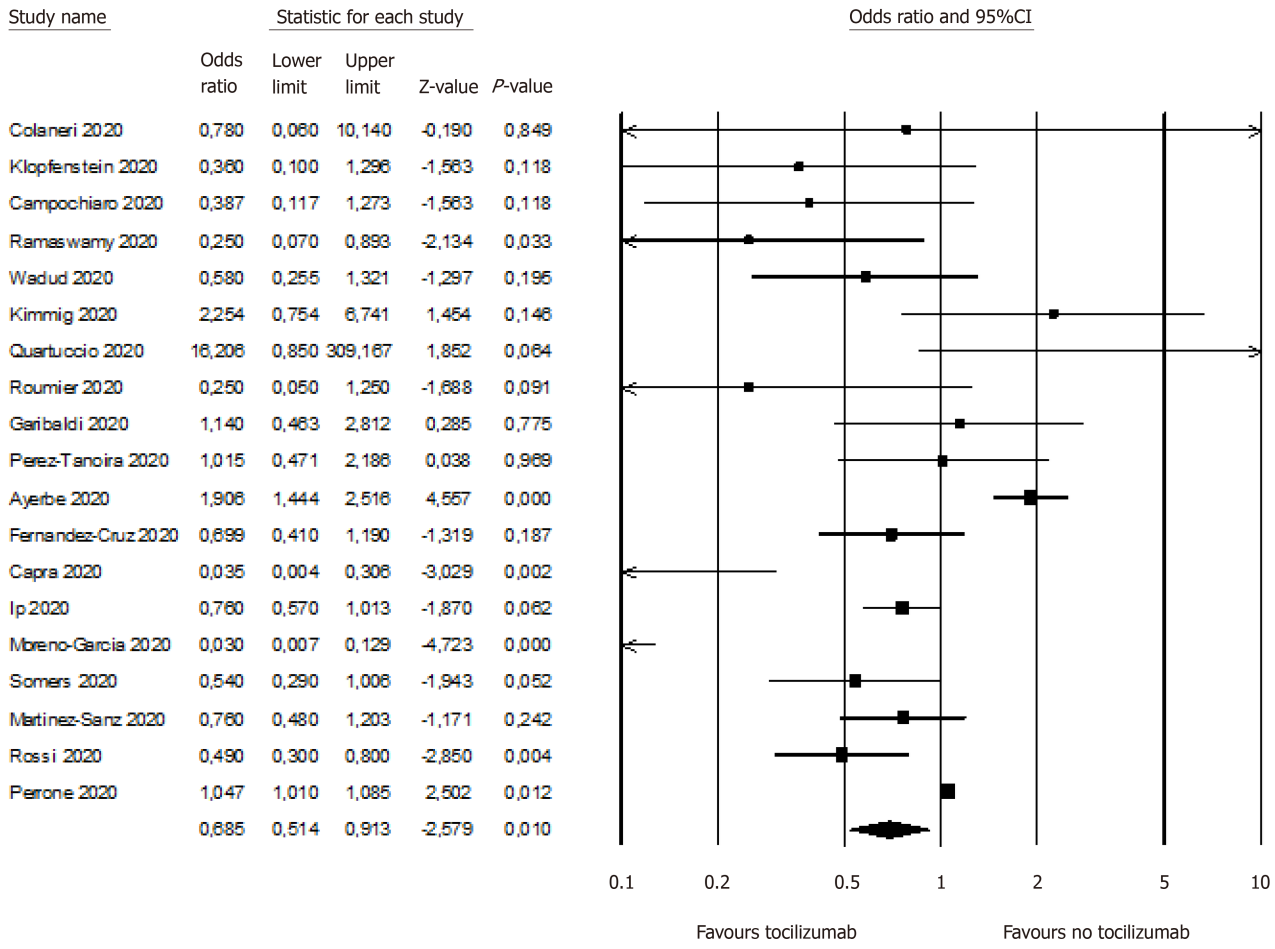

Figure 2 In the primary analysis, mortality was reduced in patients treated with tocilizumab.

ARTICLE HIGHLIGHTS

Research background

Coronavirus disease 2019 (COVID-19) infection is associated with a cytokine storm during acute phase.

Research motivation

Interleukin-6 is a key player in this systemic inflammation.

Research objectives

We evaluated the effect of tocilizumab (TCZ) on the outcomes of COVID-19 pneumonia.

Research methods

We performed a systematic review and pooled analysis of published literature.

Research results

Mortality was reduced in patients treated with TCZ (Odds ratio = 0.64, 95%CI: 0.47-0.87; $P < 0.01$).

Research conclusions

We conclude that TCZ may improve outcome of COVID-19 infected patients.

Research perspectives

Current use of tocilizumab in clinical practice has to be validated further through large randomized trials.

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ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2021 July 20; 11(4): 110-230

Published by Baishideng Publishing Group Inc

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RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Gerhard Litscher

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2222-0682/editorialboard.htm

PUBLICATION DATE
July 20, 2021

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World J Methodol 2021 July 20; 11(4): 110-115

DOI: 10.5662/wjm.v11.i4.110

ISSN 2222-0682 (online)

ACADEMIC ACTIVITY REPORT

Sacrum magnetic resonance imaging for low back and tail bone pain: A quality initiative to evaluate and improve imaging utility

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Author contributions: Chhabra A, Pezeshk P and Joodi R conceived of the project; Joodi R, Castillo S and Williams LE performed a retrospective chart review and data analysis under the guidance of Drs. Chhabra A and Pezeshk P. Chhabra A and Pezeshk P reviewed patient imaging; Castillo S and Pezeshk P prepared and performed clinician education; Castillo S, Pezeshk P and Chhabra A contributed to the final version of the manuscript.

Conflict-of-interest statement: Dr. Chhabra A receives payment as a consultant for Treace 3D Medical Inc. and Icon Medical and book royalties from Jaypee and Wolters. The remaining authors have no financial conflicts of interest to disclose.

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Abstract

As quality and cost effectiveness become essential in clinical practice, an evidence-based evaluation of the utility of imaging orders becomes an important consideration for radiology's value in patient care. We report an institutional quality improvement project including a retrospective review of utility of sacrum magnetic resonance (MR) imaging for low back pain at our institution over a four-year period and follow-up results after physician education intervention. Sacral MR imaging for low back pain and tailbone pain were only positive for major findings in 2/98 (2%) cases, and no major changes in patient management related to imaging findings occurred over this period, resulting in almost $500000 cost without significant patient benefit. We distributed these results to the Family Medicine department and clinics that frequently placed this order. An approximately 83% drop in ordering rate occurred over the ensuing 3 mo follow-up period. Sacrum MR imaging for low back pain and tail bone pain has not been a cost-effective diagnostic tool at our institution. Physician education was a useful tool in reducing overutilization of this study, with a remarkable drop in such studies after sharing these findings with primary care physicians at the institution. In conclusion, sacrum MR imaging rarely elucidates the cause of low back/tail pain diagnosed in a primary care setting and is even less likely to result in major changes in management. The practice can be adopted in other institutions for the benefit of their patients and improve cost efficiency.

Key Words: Sacrum magnetic resonance imaging; Low back pain; Tail bone pain; Musculoskeletal imaging; Quality improvement; Radiology

Manuscript source: Invited manuscript

Specialty type: Medical laboratory technology

Country/Territory of origin: United States

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Received: October 22, 2020
Peer-review started: October 22, 2020
First decision: November 25, 2020
Revised: December 20, 2020
Accepted: May 20, 2021
Article in press: May 20, 2021
Published online: July 20, 2021

P-Reviewer: Leung PC
S-Editor: Gao CC
L-Editor: A
P-Editor: Yuan YY

Core Tip: Sacrum magnetic resonance studies ordered for low back pain were suspected to lack clinical utility at our institution. A literature review demonstrated a lack of evidence based practice in ordering of this study for low back/tail bone pain. A quality project was then pursued to first assess the clinical usefulness of the study. Over four years these studies had no major impacts on management. An educational component of the quality project was then pursued with a rapid decrease in the number of studies ordered by referring providers.

Citation: Castillo S, Joodi R, Williams LE, Pezeshk P, Chhabra A. Sacrum magnetic resonance imaging for low back and tail bone pain: A quality initiative to evaluate and improve imaging utility. *World J Methodol* 2021; 11(4): 110-115
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/110.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.110

INTRODUCTION

Magnetic resonance imaging (MRI) of the sacrum ordered for vague low back pain and tail bone pain was seen with some frequency at our institution over the last decade and with little evidence on its clinical value as subjectively reported by our radiologists. The American College of Radiology (ACR) Appropriateness Criteria does not include sacrum imaging in its reviews for low back pain indication[1]. The ACR Appropriateness Criteria does however recommend sacral MR imaging in patients with chronic low back pain, symptoms of inflammatory arthropathy, and negative or equivocal radiographs[2]. Recommendations from the American College of Physicians and the American Pain Society also state that imaging should not be obtained in uncomplicated back pain without neurologic symptoms unless history or physical examination suggest a specific underlying etiology[3]. In fact, increases in imaging in low back pain have been associated with increases in procedures for low back pain without improvement in outcomes—a finding highly suggestive of overutilization[4,5]. Coccydynia (or tail bone pain) has many similar underlying etiologies to low back pain, including muscular, post traumatic, and degenerative etiologies[6,7]. Treatment options also bear similarity to those available in patients with low back pain, including surgical and local therapeutic options based on the underlying pathology[6,8]. However, conservative treatments including physical therapies are effective treatments and often the most appropriate therapy in patients affected by these conditions[6,8,9].

While adherence to guidelines recommending physical therapy in low back pain can decrease long term health care costs and unnecessary procedures, it has also been postulated that up to 5% of low back pain suffers could have some degree of inflammatory arthropathy[9,10]. The clinicians ordering these exams might want to exclude a serious and clinically meaningful diagnosis.

It is thus clear that a specific systematic review of these orders in the setting of low back pain would be of use to determine the true value of this imaging order and examination in the clinical practice. Literature on the usefulness of these orders in the absence of known recent trauma, infection or neoplastic etiologies is currently lacking, resulting in an interest in determining if these orders contribute to a meaningful alteration of the patient care. This project was a quality improvement and practice management project, which entailed retrospective imaging and electronic patient chart reviews, focused provider education and post-intervention assessment to evaluate the impact of such procedures.

METHODOLOGY

Problem assessment and pre-intervention analysis

Sacrum MRI orders and examinations at our institution from 2013-2017 were retrospectively reviewed. Sacrum MR orders were identified through the PACS (picture archiving and communications system, Mckesson, Alpharetta, GA, United States), identifying 322 unique patients with sacrum MRI orders over this time period.

Orders for already known diagnosis of inflammatory arthritis, concern for infection (osteomyelitis), known or suspected tumor, recent trauma (< 4 wk) and concern for occult fracture were excluded from further review, leaving 98 studies performed over this time period. All studies had an indication of low back pain and/or tail bone pain as described by the ordering physician/provider without indicating specific suspected etiology of pain.

Imaging, radiology reports, and patient charts were reviewed by a radiology resident and/or musculoskeletal imaging fellow for included patients to evaluate for major, minor, and incidental findings, as well as major or minor impact on the patient diagnosis and care. Major findings were defined as those that may have been a cause of patient's pain and could potentially be acted upon, particularly inflammatory spondyloarthropathy. Minor findings were findings felt to be unlikely the cause of patient's symptoms, routine degenerative changes without evidence of inflammation, or seen on concurrent exam. Incidental findings were felt not to be related to patient's symptoms and/or would be better evaluated by a different exam and/or modality of imaging. MRIs positive for major findings were reviewed by two expert subspecialty musculoskeletal radiologists with 4 and 10 years of post-fellowship attending experience.

Root cause analysis

The current process map for patients who present to their clinicians with low back pain and/or tail bone pain was evaluated with electronic chart reviews. Ordering providers were from multiple different clinics in the hospital system. Imaging evaluation usually started with lumbar spine, sacral or sacroiliac (SI) joint or pelvic X-rays. The interpreting radiologist qualitatively described degenerative findings of the spine, SI joints or hip joints and/or chronic pelvic enthesophytes of trochanters and ischial tuberosities, all common findings. Sacrum MRIs were then ordered for further evaluation for many reasons including chronic but increasing low back/tail bone symptoms, partially obscured sacrum on radiographs due to bowel shadows, psychological factors, no response to initial conservative treatment, imaging prior to caudal blocks, and lack of defined appropriateness criteria for tail bone pain. It was determined that the strategy that would most likely impact the patient care would involve presentation of results of the above retrospective imaging and chart reviews with focused teaching of the referring clinicians who ordered most of these examinations.

Interventions

The results were tabulated with frequencies of findings identified from the imaging and chart reviews, completed in June 2018. Impact on patient care was inferred from the electronic chart reviews, treatments patients received and their follow-up procedures. Powerpoint (Microsoft, Redwood, Seattle) presentations were generated on preliminary findings and presented at the department meeting of family medicine in April 2018. The presentations were also distributed to the clinicians at the care centers ordering most of these examinations as continuing education.

Post-intervention analysis

Finally, sacrum MRIs were again identified through the same methods over a 3-mo period after the distribution of the teaching materials, and the results were tabulated.

Statistical analysis

The frequencies were tabulated as percentages. No *P* values were generated due to clearly successful intervention.

RESULTS

Frequency and distribution of MRI findings

There were 98 MRIs of the sacrum that met the inclusion criteria. The included patients were 65% female, with a mean 48.1 years of age. All included studies were non-contrast examinations. Majority of cases (85/98) had incidental findings including lumbar spine degeneration (often seen on concurrently performed lumbar spine MRIs, which were performed in 65 of the 98 studies) and incidental visceral findings in the pelvis such as incidental uterine fibroids and partially visualized renal lesions.

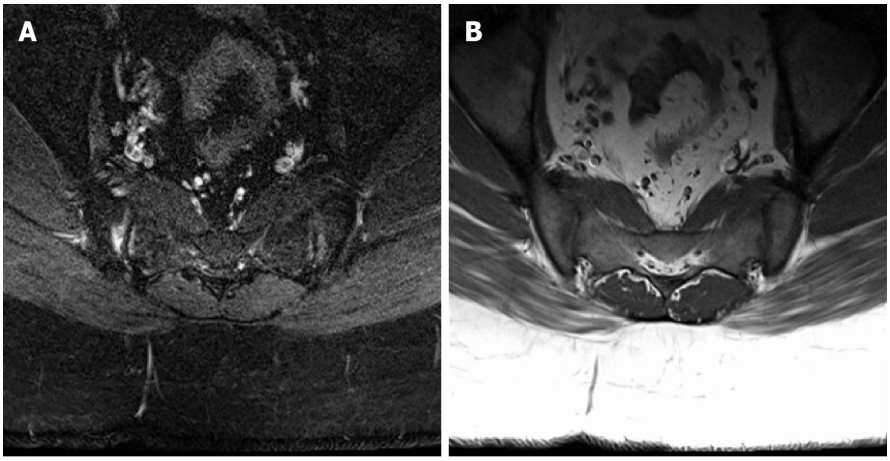

Figure 1 Axial T1 and STIR images demonstrate bilateral sacroiliac joint edema and irregularity consistent with sacroiliitis, considered a major change in diagnosis. These inflammatory changes resulted in no changes to management in this patient who eventually underwent microdiscectomy for disc extrusion seen on concurrent lumbar spine magnetic resonance. A: Axial T1 image; B: STIR image.

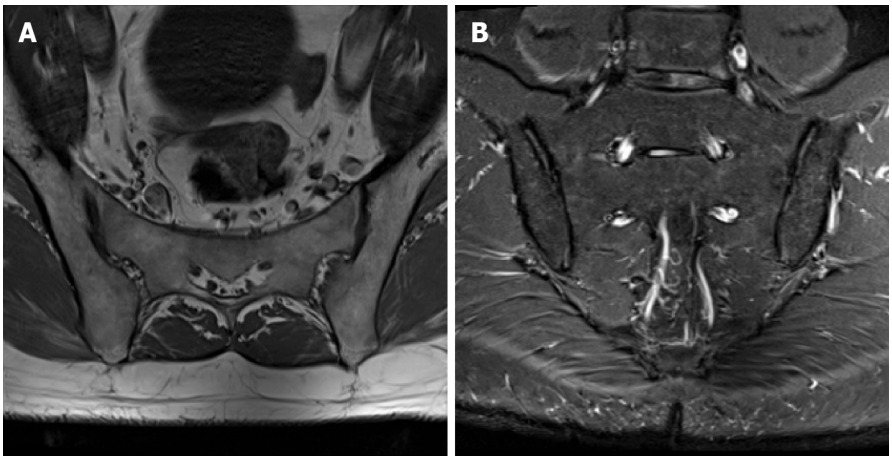

Figure 2 Axial T1 and coronal STIR images demonstrate mild bilateral sacroiliac joint degeneration. This patient was also noted to have incidentally noted lower lumbar spine degeneration, for which he subsequently underwent a dedicated lumbar spine magnetic resonance. A: Axial T1 image; B: Coronal STIR image.

Frequency of change in diagnosis and management

Two patients had a major change in diagnosis based on Sacral MR findings, one with acute SI inflammation and one with chronic inflammation. Forty-three patients had minor findings such as SI degenerative disease, Tarlov cysts, or old post-traumatic changes of the sacrum/coccyx. Of these patients with changes in diagnosis, 12 patients had minor changes in management, which included surgical referrals for degenerative disease and further imaging of incidental or minor findings. No major changes in management occurred as a result these examinations (Figure 1-3).

Cost of sacrum MRIs

At the above rate, based on current charges for sacrum MRI examination at our institution ($4900 per MRI sacrum without contrast), patients were charged a total of $480200 for two major findings, no major changes in management, and only 12 minor changes in management. This doesn't include costs of their travel times, parking, time away from work, increased clinic visits for management of minor or incidental findings, patient anxiety and discomfort from MRIs, *etc.*

Post-intervention results

Post-intervention, during 3-mo follow-up, only 1 study meeting the inclusion criteria had been ordered and this was also negative for major or minor findings or any changes in management. This is less than the expected 2 exams per month average over the duration of the pre-intervention period, suggesting a positive impact on

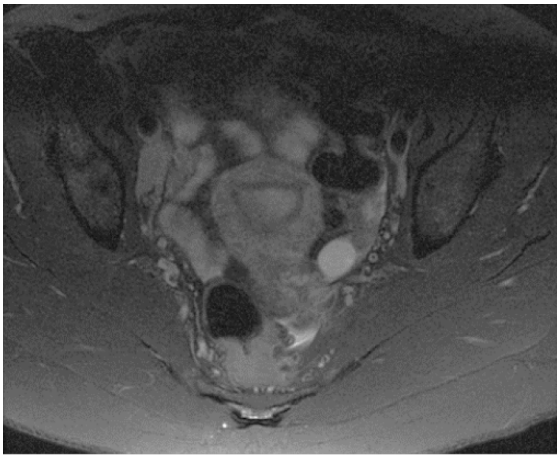

Figure 3 Axial STIR image demonstrates an incidentally noted small left ovarian cyst and borderline enlarged right external iliac lymph nodes in this reproductive age patient with an underlying systemic illness. No musculoskeletal abnormalities were present on her exam.

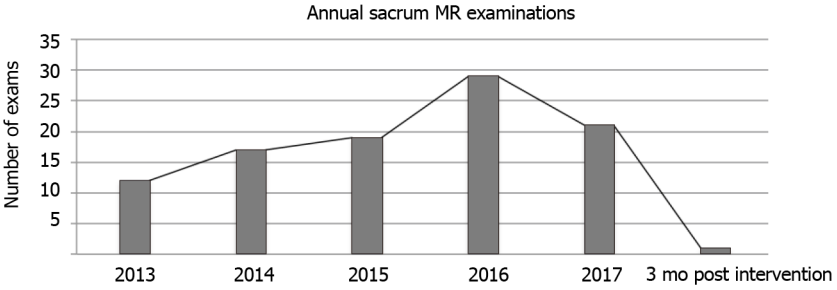

Figure 4 Number of sacral magnetic resonance examinations performed per year during the retrospective review followed by 3 mo post intervention. MR: Magnetic resonance.

clinician ordering patterns. We hope to add this in the decision support software used by the ordering physicians at our institute with best practice alerts if there is no history of known tumor, recent trauma, infection, or spondyloarthritis (Figure 4).

DISCUSSION

Sacrum MRI at our institution was overall found to be an inefficient method of diagnosis of low back or tailbone pain, not lending itself to cost effectiveness or significant impact on patient care. This further strengthens the argument that advanced imaging should be avoided in favor of conservative therapies in patients with vague low back pain without neurologic symptoms or other clinical red flags (such as recent trauma or malignancy)[7,8]. Physician education led to at least a short-term decrease in frequency of these orders by approximately 83%, effectively saving $24500 over a 3-mo time period. In future, similar studies at other institutions may be helpful to confirm minimal, if any utility of sacral MR imaging in low back and/or tail bone pain.

CONCLUSION

Applications

Our findings add to the literature supporting clinician decision making when deciding to pursue conservative therapies in patients with low back or tail bone pain prior to imaging, as well as supporting literature which indicates this reduces health care cost. Additionally, our project supports the utility of clinician education and communication to improve hospital system quality of care and resource utilization.

Limitations

Due to the nature of this retrospective review, we couldn't evaluate the referring physician or patient factors driving the need for sacrum MRI examinations during their management, or the satisfaction they obtained from the positive or negative results. The negative predictive value of such examinations may also play a role in the alleviation of patient concerns of something unknown or significant that may be causing their symptoms. Eliminating redundant procedures and unnecessary diagnostic services, such as costly advanced imaging examinations is however in line with the strategy of best practice management while simultaneously decreasing health care costs and increasing patient care quality and medical efficiency.

Further research

Future research is needed to confirm similar lack of changes in clinical management based on sacral MRI in additional institutions. Furthermore, long term analysis of clinician response to education is needed as this may have a dampened effect over time.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 July 20; 11(4): 116-129

DOI: 10.5662/wjm.v11.i4.116

ISSN 2222-0682 (online)

OPINION REVIEW

Delphi methodology in healthcare research: How to decide its appropriateness

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Author contributions: Nasa P, Jain R, and Juneja D contributed equally to this work; Nasa P and Jain R designed the idea of the study; Nasa P, Jain R, and Juneja D performed the systematic search of the literature; Nasa P contributed the figures; Jain R and Nasa P analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

Conflict-of-interest statement: Prashant Nasa declared to be on the advisory board of Edwards life sciences. Other authors do not declare any conflict of interest.

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Abstract

The Delphi technique is a systematic process of forecasting using the collective opinion of panel members. The structured method of developing consensus among panel members using Delphi methodology has gained acceptance in diverse fields of medicine. The Delphi methods assumed a pivotal role in the last few decades to develop best practice guidance using collective intelligence where research is limited, ethically/logistically difficult or evidence is conflicting. However, the attempts to assess the quality standard of Delphi studies have reported significant variance, and details of the process followed are usually unclear. We recommend systematic quality tools for evaluation of Delphi methodology; identification of problem area of research, selection of panel, anonymity of panelists, controlled feedback, iterative Delphi rounds, consensus criteria, analysis of consensus, closing criteria, and stability of the results. Based on these nine qualitative evaluation points, we assessed the quality of Delphi studies in the medical field related to coronavirus disease 2019. There was inconsistency in reporting vital elements of Delphi methods such as identification of panel members, defining consensus, closing criteria for rounds, and presenting the results. We propose our evaluation points for researchers, medical journal editorial boards, and reviewers to evaluate the quality of the Delphi methods in healthcare research.

Key Words: Delphi studies; Quality tools for methodology; Research methods; Delphi technique; Consensus; Expert panel; Coronavirus disease 2019; SARS-CoV-2

Manuscript source: Invited manuscript

Specialty type: Methodology

Country/Territory of origin: United Arab Emirates

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 12, 2021
Peer-review started: January 12, 2021
First decision: February 14, 2021
Revised: February 26, 2021
Accepted: May 19, 2021
Article in press: May 19, 2021
Published online: July 20, 2021

P-Reviewer: Boos J
S-Editor: Wang JL
L-Editor: Filipodia
P-Editor: Yuan YY

Core Tip: There are no standard quality parameters to evaluate Delphi methods in healthcare research. Delphi methods' vital elements include anonymity, iteration, controlled feedback, and statistical stability of consensus. Published studies have used modified versions of Delphi, and details on methods like expert panel selection, defining consensus, or closing criteria for Delphi rounds are not explicit. We suggest quality assessment tools for readers and researchers for a systematic assessment of Delphi studies.

Citation: Nasa P, Jain R, Juneja D. Delphi methodology in healthcare research: How to decide its appropriateness. *World J Methodol* 2021; 11(4): 116-129
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/116.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.116

INTRODUCTION

This review used the "Delphi study" for the published studies that used Delphi methodology. "Delphi rounds" is used for the survey questionnaire rounds to develop iterative discussion among panel members. "Delphi process" is used for the steps of Delphi methods in research.

The term "Delphi" originated from ancient Greek mythology and was believed to be the precinct of Pythia (a major oracle), where prophecies were made to dictate and direct vital state affairs. In its literal sense, Delphi methods can be defined as a structured technique to modulate a group communication process effectively in allowing a group of individuals, as a whole, to deal with a complex problem[1]. The Delphi method was initially developed for business forecasting using an expert panel's interactive discussion, assuming collective judgments are more valuable than individuals.

Possibly the first application of Delphi methodology was during the cold war in the 1950s by the United States army. They used it for their military project RAND to develop consensus among experts using repeated rounds of anonymous feedback, forecasting future enemy attacks[2]. After this first application, it has been used in many other academic domains like finance, economics, development planning, and healthcare, where group forecasting makes sense in the absence of accurate tested data. In modern times, this forecasting tool has evolved into a statistical methodology to collate individual opinions and converge them into statistically generated consensus with collective intelligence. A constant theme is observed across all domains with vital elements like anonymity, iteration, controlled feedback, and group response (or consensus)[3].

The anonymity of individual members in a Delphi study removes the inherent bias like dominance and group conformity (defined as groupthink) observed with face-to-face group meetings. The primary purpose of the Delphi technique is to generate a reliable consensus opinion of a group of experts by an iterative process of questionnaire interspersed with controlled feedback[2]. After initial slow acceptance in healthcare, it is now a widely used method to generate group consensus, develop qualitative practice points, or identify future research areas. In healthcare, the Delphi process had been used in diverse areas: (1) Evaluate current knowledge; (2) Resolving controversy in management[4]; (3) Formulating theoretical or methodological guidelines[5,6]; (4) Developing assessment tools and indicators[7,8]; and (5) Formulating recommendations for action and prioritizing measures[9].

The Delphi methods from its inception have undergone modifications to structure effective and faster consensus. The modified Delphi does not have a standard criterion, but in principle, a steering group facilitates the group communication process effectively. There are no set standards for reporting Delphi studies in healthcare research, unlike other qualitative and quantitative clinical research tools. There are also no validated quality parameters to evaluate Delphi studies. In a recent meta-analysis of Delphi studies in healthcare research, many studies were found to be of questionable quality[10]. The protocol design, the definition of consensus, and closing criteria were not set *a priori* and vary widely in Delphi studies. There have been attempts to identify quality parameters to conduct and evaluate Delphi studies[10-12]. The guidance on conducting and reporting of Delphi studies (CREDES) is a popular

tool, developed for Delphi studies on palliative care. The authors acknowledged significant variation in the reporting and methodology of Delphi studies and proposed CREDES standards for reporting and conducting such studies[12]. However, these tools are neither been validated in other fields of medicine nor universally accepted for the conduct of Delphi studies. The discrepancy in conduct and transparency of reporting may overshadow the consensus recommendations generated by Delphi studies. There is an urgent need of simple tools for systematic assessment of the quality of Delphi studies. Like other statistical research studies, readers must consider if the methodology has been followed appropriately for the key elements of Delphi technique. This article recommends critical appraisal of a Delphi study in healthcare sequentially by nine qualitative evaluation points in a four-step methodological process (Figure 1).

PROBLEM AREA

The Delphi study is practical in problematic areas where either statistical model-based evidence is not available, knowledge is uncertain and incomplete, and human expert judgment is better than individual opinion[1]. The emerging disease or conditions in healthcare often simulates such areas, where either standard research pathways cannot be adopted or become impractical. Various approaches can identify these problem areas: (1) Extensive systematic literature search; (2) Group discussion among a defined steering group; and (3) Open-ended discussion rounds among panel members.

The process of identifying problem areas and its communication among all participating panel members should be explicit and must be done before the final survey rounds to achieve consensus.

Evaluation point

The criteria used to identify the problem area and process followed should be documented. The systematic search of the literature must mention period, keywords, and database included in the search.

PANEL MEMBERS

The members who participate in the anonymous voting process of the Delphi survey are called panelists. The panel member selection is undoubtedly the most crucial aspect of Delphi research studies[13]. The methods used for the identification and selection of panel members are discrepant in published Delphi studies. There are no standard criteria used for the definition of panel members[10]. The readers should consider the following issues while evaluating the Delphi study: Homogeneity of panel, labelling panel members as an 'expert', and size of the panel.

Homogeneity of the panel

A diverse panel helps to achieve a broader perspective and generalization of consensus. The homogenous group, on the other hand, may be more reliable in a particular study objective. The homogenous panel is suitable when resolving unsettled issues of a focused problem like management of acute respiratory distress syndrome, while the heterogeneous panel is appropriate in a broader situation like when studying the impact of mental illness. The methodology should represent the process followed for achieving homogeneity in the study.

Expert panel

The labelling of panel members as 'experts' is most contentious. The expert can be defined as someone with knowledge and experience on a particular subject matter; however, it is practically difficult to measure experience quantitatively. Despite its controversy, the experts are commonly used in the Delphi studies for panel members without a uniform selection criterion. The common goal behind using experts is to increase the qualitative strength of recommendations or consensus. The readers must evaluate the criteria for expert panel selection. Panel selection should adhere to a predefined criteria[4,6,14].

Size

There is no standard size of the panel members and varies from 10 to 1000 (typically

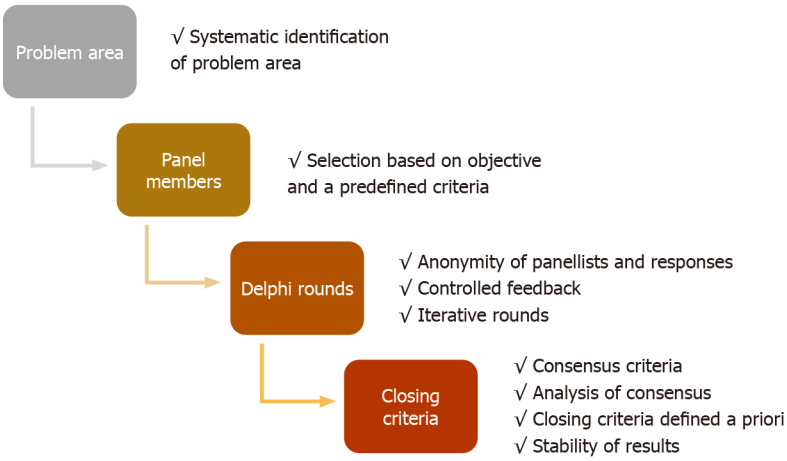

Figure 1 Stepwise quality assessment of Delphi studies.

between 10-100) in published studies. However, due to data management difficulties and logistic issues (rounds of the survey), a panel with three-digit sample size is unusual[10,15]. Generally, a double-digit number close to 30-50 is considered optimum in concluding rounds for a homogenous Delphi[4,14]. Appropriate size depends on the complexity of the problem, homogeneity (or heterogeneity) of the panel, and availability of the resources. Apart from panel members with knowledge, some studies recruit members from diverse academic and practice backgrounds or involve end-users in the process[16].

Generalizability of Delphi results requires an appropriate panel size, diverse representation of members from different specialties, and geographical distribution.

The electronic Delphi survey (also called e-Delphi) helps in the global representation of panel members, saves time, and fastens the survey rounds using technology without physical voting. This process involves selecting experts after research for eligibility on the world wide web; further email invitations to participate in the project can be sent. The acceptance rate among experts can be low, and researchers usually consider this higher attrition rate during the invitation process.

Evaluation point

The selection of a panel or voting members in a Delphi study should be based on objective and predefined criteria and related to the problem under study.

DELPHI ROUNDS

The strength of Delphi process is anonymity of panelist in the survey rounds, controlled feedback and iterative discussions. Anonymous survey rounds have advantages over face-to-face or group encounters in reducing dominance and group conformity. Participants feel more comfortable in providing anonymous opinions on uncertain, unsettled issues. The interpretation of items may sometimes become a critical issue in anonymous Delphi rounds and may affect the consensus process.

The "controlled feedback" is another classic characteristic of the Delphi study. It is termed as "controlled" because moderator decides about feedback provisions based on responses to the items and open comments. After each of the survey rounds, obtained data are analyzed and presented in an easily interpretable format to all the panel experts. It can include simple charts and statistics showing the stability of responses. Statistics usually include the measurement of central tendencies with dispersion, percentage, and frequency of distribution[17]. Even anonymous comments can be incorporated as a part of the feedback. Sometimes individual feedback along with group responses are also provided. Controlled feedback gives insight to the individual member about the trend and one can change its response if needed. Panel members should clear their position if they have an extreme choice of response in a particular situation.

Analysis of successive iterative rounds provides an opportunity to evaluate data for consensus and interspersed stability among the two successive rounds. The repetitive and interactive survey rounds are useful for gathering qualitative information,

improving framing of the statements for panel members, and achieving consensus.

Evaluation point

The Delphi survey should be assessed for iterative discussions and controlled feedback while maintaining a strict anonymity of the panel members and their responses.

CLOSING CRITERIA

As Delphi is a method to generate consensus of individual panel member opinion on unsettled critical issues, the consensus and closing criteria vary widely among the studies[10,12,15]. The definition of consensus used in published Delphi studies is discrepant.

Consensus

Traditionally, a consensus is considered as the primary outcome of the Delphi study. However, its understanding is quite confusing among various studies. Consensus can mean a group opinion, solidarity towards a sentiment, or sometimes absolute alignment of the opinion of experts[18]. Hence, various measures have been used to define consensus. A meta-analysis[10] to evaluate quality of published Delphi studies found 73% of the studies reported a consensus method, and only 68% did so in an advanced declared protocol. It was even observed that some studies declare achieving consensus but do not provide the process to reach the consensus and its definition[10,12,15]. The definition of consensus used in published Delphi studies is discrepant[12,19]. The consensus definition used commonly is the percentage of agreement based on a predefined cut-off, central tendency, or a combination of both. However, percentage agreement varies widely from 50%-97% and is selected arbitrarily[10,12].

Closing criteria

The conventional design of the Delphi study had at least four rounds. However, the essence of good Delphi surveys is an iterative process and controlled feedback to generate consensus. The closing criteria in most of the Delphi studies include consensus achieved after a prefixed (usually two) rounds[10,12]. The stability of the responses or consensus cannot be checked with two rounds of Delphi. Any change in the items or controlled feedback may alter the response of panelists. However, these responses may not be stable and hence a fixed number of rounds without assessment of the stability of the results is a compromise on statistical robustness. The invention of "modified Delphi" arbitrarily uses two-three rounds of survey decided *a priori* as a closing criterion. The "modified" term in Delphi studies is, however, discrepant and without any universal accepted criterion. The only common thing in modified Delphi methodology is the active effort of the steering group in generating consensus. The steering group performed a systematic search of the literature in the problem area and, instead of open-end, initial Delphi rounds are focused on achieving consensus among panelists. The group also review the results after each round and items that reached consensus are dropped for the next rounds, but the items that are consistently not achieving consensus despite controlled feedback can also be dropped[20]. However, this active participation of the steering group can cause bias through opinion of members.

Stability

Understanding the stability of responses is even more confusing than consensus, and the stability of the consensus is rarely used in Delphi studies as a closing criterion. Classically, consensus or a pre-fixed round of surveys served as a closing criterion. It comes with an inherent risk that a significant change in responses occurred in the last round, affecting the stability of the results or consensus. Hence some authors believed that achieving a consensus is meaningless with unstable responses[1,21-23]. The stability of the results is thus considered the necessary criterion. Stability is defined as the consistency of responses between successive rounds of a study[21]. The researchers believe that specific results of two separate rounds for a particular question can occur by chance, which can be decreased by obtaining statistically significant stability (or variance) of the responses[10]. In other words, consensus can be there in unstable responses, and stability can be there without consensus, and hence achieving response stability should be an appropriate closing criterion. However, every effort to achieve consensus should be made[21,23]. Therefore, a hierarchical stopping criterion should be adopted as a closing criterion for Delphi (Figure 2).

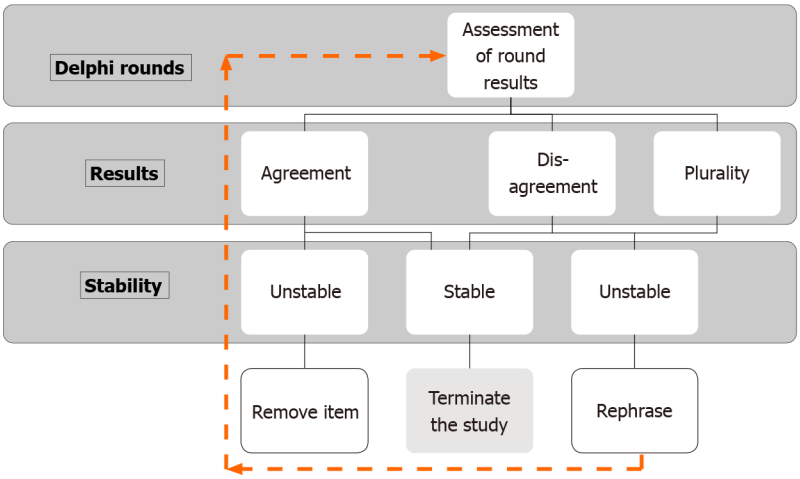

Figure 2 Stability assessment for Delphi rounds.

Evaluation point

The criteria for stopping the Delphi rounds based on consensus or stability should be identified *a priori*. The alternative plans and method to drop items should be defined if consensus is used as a stopping criterion of Delphi rounds. Stability of the responses is important for statistical stability of the consensus.

EVALUATION OF RECENT DELPHI STUDIES

We used our nine qualitative evaluation points to assess the quality of recent Delphi studies on coronavirus disease 2019 (COVID-19).

Search strategy and selection criteria

A systematic search of the literature was conducted from PubMed and MEDLINE databases between January 1, 2020 and December 31, 2020. We used a combination of keywords, "Delphi technique" OR "Delphi study" OR "Delphi" AND "COVID-19" OR "SARS-CoV-2". We excluded search results that have non-human study subjects, non-English literature, and alternative medicine.

Included studies

Fifty-two Delphi studies were assessed as *per* the inclusion criteria, and 34 (67.3%) studies[24-57], were finally analyzed using nine evaluation points (Table 1). The data on medical specialty, geographical location, the purpose of the study, conclusion format, number of experts, and Delphi rounds were collected for each study (Table 2). The study methods were scrutinized using nine qualitative evaluation points on a 3-point scale, "yes", "no", and "not clear" (Table 2).

Summary of Delphi studies assessment

COVID-19 is a new disease coined by World Health Organization in February 2020. The exponential growth of the COVID-19 pandemic disrupted public health, healthcare, and the global economy in an unprecedented manner. The absence of quality evidence on pathophysiology, infection transmission or control, and management of COVID-19 made researchers deploying Delphi methodology for consensus recommendations in various medicinal fields affected by COVID-19. We used our evaluation points for the quality assessment of Delphi technique in 34 selected studies that met the inclusion criteria. The studies from various fields of medicine were included in this analysis. Most of the studies (60%) were done in Europe or North America. The median of 20 (interquartile range-41) experts participated in two (interquartile range-1) Delphi rounds (Table 1).

No single study met all nine evaluation points for quality assessment (Table 1). The systematic identification of the problem area was explicitly declared in 28 (79.41%) studies. The anonymity of panelist was missing in nine (26.47%) studies and not disclosed clearly in another 13 (38.24%) articles. The confidentiality in the identity of panelists was breached in few studies either for video/audio conference or in the final

Table 1 Evaluation of Delphi studies on coronavirus disease 2019 that were published in 2020 on nine qualitative evaluation points

No.	Ref.	Medicine field	Geographical location (Country/Continent)	Aim or purpose	Guidance format
1	Vitacca *et al*[24]	Rehabilitation	Italy, Europe	Consensus on pulmonary rehabilitation in patients with COVID-19 after discharge from acute care.	Recommendations from experts' panel.
2	Mikuls *et al*[25]	Rheumatology	USA, North America	Guidance to rheumatology providers on the management of adult rheumatic diseases during COVID-19 pandemic.	77 initial guidance statements converted to 25 final guidance statements.
3	Greenhalgh *et al* [26]	Primary health	UK, Europe	To develop early warning score for patients with suspected COVID-19 who need escalation to next level of care.	Development of software for early warning score in COVID-19 patients.
4	Lamb *et al*[27]	Respiratory medicine and critical care medicine	USA, NA	Guidance to physicians on the preparation, timing, and technique of tracheostomy in COVID-19 patients.	Eight recommendations.
5	Welsh Surgical Research Initiative (WSRI) Collaborative[28]	General surgery	Global	Identify the needs of the global OR workforce during COVID-19.	Statements, predominantly standardization of OR pathways, OR staffing, and preoperative screening or diagnosis.
6	Eibensteiner *et al* [29]	Nephrology	Europe	To gather expert knowledge and experience to guide the care of children with chronic kidney disease during the COVID-19 pandemic.	Qualitative expert statements and answers.
7	Bhandari *et al*[30]	Gastroenterology	Global	Guidance on how to resume endoscopy services during COVID-19.	Best practice recommendations to aid the safe resumption of endoscopy services globally in the era of COVID-19.
8	Guckenberger *et al* [31]	Radiotherapy	NA and Europe	To develop practice recommendations pertaining to safe radiotherapy for lung cancer patients during COVID-19 pandemic.	Consensus recommendations in common clinical scenarios of radiotherapy for lung cancer.
9	Aj *et al*[32]	General surgery	NA, Europe and Australia	Validation of international COVID-19 surgical guidance during COVID-19 pandemic.	Area of consensus and contentious areas from previous guidelines.
10	Gelfand *et al*[33]	Dermatology	NA	Guidance on the management of psoriatic disease during the COVID-19 pandemic.	22 guidance statements.
11	Allan *et al*[34]	Surgery	Global	Guidance on surgery and OR practices during COVID-19 pandemic.	Development of research priorities in discipline of surgery related to COVID-19.
12	Shanbehzadeh *et al* [35]	Medical informatics and public health	Iran, Middle east	Development of minimum data set for COVID-19 surveillance system.	Conceptual COVID-19 surveillance model.
13	Bergman *et al*[36]	Long-term nursing care	NA	Consensus guidance statements focusing on essential family caregivers and visitors in nursing homes during COVID-19 pandemic.	Recommendations for visitors in long term nursing homes.
14	Daigle *et al*[37]	Ophthalmology	Canada	Risk stratifying for oculofacial plastic and orbital surgeries in context of transmission of SARS-CoV-2.	Risk based algorithm for oculoplastic surgeries and recommendations for appropriate PPE.
15	Sorbello *et al*[38]	Anaesthesia	Europe	Review of available evidence and scientific publications about barrier-enclosure systems for airway management in suspected/confirmed COVID-19 patients.	Recommendation on enclosure barrier systems.
16	Jheon *et al*[39]	Cardiovascular and thoracic surgery	Asia	Thoracic cancer surgery during COVID-19 pandemic.	Recommendations on timing, approach, type of surgery, and postoperative requirements.
17	Olmos-Gómez *et al* [40]	Behavioural sciences	Spain, Europe	To know the impact of learning environments and psychological factors.	Future research priorities.
18	Sawhney *et al*[41]	Gastroenterology	Global	Study to emphasize patient-important	Recommendations on procedural

				outcomes while considering procedural timing.	timing for common indications for advanced endoscopy during COVID-19.
19	Sciubba *et al*[42]	Neurosurgery	USA	Study to device scoring system to help with triaging surgical patients during the COVID-19 pandemic.	Scoring system to triage spinal surgery cases during COVID-19 pandemic.
20	Errett *et al*[43]	Environmental health science	USA	Study to develop an Environmental Health Sciences COVID-19 research agenda.	To validate, find limitations, and identify future research priorities.
21	Arezzo *et al*[44]	Minimal access surgery	Global	To study and provide recommendations for recovery plan in minimally invasive surgery amid COVID-19 pandemic.	Framework for resumption of surgery with focus on minimally invasive surgeries following COVID-19 pandemic.
22	Dashash *et al*[45]	Healthcare education	Syria	To identify essential competencies required for approaching patients with COVID-19.	Core competency points for health care professionals to prepare them for COVID-19 pandemic.
23	Ramalho *et al*[46]	Psychiatry	Global	To create a practical and clinically useful protocol for mental health care to be applied in the pandemic.	Consensus protocol for use of telemedicine in psychiatry consults during COVID-19 pandemic.
24	Saldarriaga Rivera *et al*[47]	Rheumatology	Columbia, SA	To produce recommendations for patients with rheumatological diseases receiving immunomodulatory and immunosuppressive therapies.	Recommendations for pharmacological management of patients with rheumatic diseases during COVID-19 pandemic.
25	Tchouaket Nguemeleu *et al* [48]	Public health	Canada, NA	Study for development and validation of a time and motion guide to assess the costs of prevention and control interventions for nosocomial infections.	Development and validation of a new instrument for systematic assessment of costs relating to the human and material resources used in nosocomial infection prevention and control.
26	Santana *et al*[49]	Nursing	Brazil, SA	To develop an adaptable acceptable nursing protocol during the pandemic.	Protocol for nurse managers to cope with pandemic.
27	Tang *et al*[50]	Oncology	China	To develop a risk model based on the experience of recently resumed activities in many cancer hospitals in China to reduce nosocomial transmission of SARS-CoV-2.	Risk model development on the basis of experience from recently resumed cancer hospital.
28	Jiménez-Rodríguez *et al*[51]	Public health	Spain	Develop recommendations for telemedicine in video consultations during COVID-19.	Consensus recommendations for healthcare professionals for proper management of video consultation.
29	Reina Ortiz *et al* [52]	Public health	Ecuador	Development of bio-safety measures to reduce cross-transmission of SARS-CoV-2.	Biosafety-at-home flyer for high-risk group and health care workers to reduce the risk of cross-transmission.
30	Douillet *et al*[53]	Internal medicine	France and Belgium	Identify reliable criteria for hospitalization or outpatient management in mild cases of COVID-19.	Development of toolkit "HOME-CoV rule", a decision-making support mechanism for clinicians to target patients with suspected or confirmed COVID-19 requiring hospitalization.
31	Richez *et al*[54]	Rheumatology	France	Management of anti-inflammatory agents and disease-modifying-anti-rheumatic-drugs for rheumatological patients during COVID-19.	Recommendations to rheumatologists on management.
32	Yalçınkaya *et al*[55]	Physiotherapy and rehabilitation medicine	Turkey	Recommendations for the management of spasticity in Cerebral palsy children during COVID-19 pandemic.	Consensus recommendations for spasticity management in cerebral palsy children.
33	Tanasijevic *et al*[56]	Haemato-oncology	USA	To identify minimum hemoglobin for safe transfusion in myelodysplastic syndrome during COVID-19 pandemic.	Recommendations for lowest value of hemoglobin for which transfusions can safely forgo.
34	Alarcón *et al*[57]	Dentistry	Latin America	Education and practice in implant Dentistry during COVID-19 pandemic.	Consensus recommendations.

COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; OR: Operating room.

round to generate consensus on the items[25,27,29]. The consensus based on the percentage of agreement and consensus analysis was mentioned in 27 (79.41%)

Table 2 Basic information of the Delphi studies included for evaluation

No.	Ref.	Identification of problem area	Selection of panel members	Anonymity of panellist	Controlled feedback	Iterative rounds	Consensus Criteria	Analysis of consensus	Closing criteria	Group stability	Number of rounds	Number of experts
1	Vitacca *et al* [24]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	2	20
2	Mikuls *et al* [25]	Yes	Yes	Not clear	Yes	Yes	Yes	Yes	Yes	No	2	14
3	Greenhalgh *et al*[26]	Yes	Not clear	Yes	Yes	Yes	Not clear	Yes	Yes	No	4	72
4	Lamb *et al*[27]	Yes	Yes	No	No	No	Yes	Yes	Yes	No	1	13
5	Welsh Surgical Research Initiative (WSRI) Collaborative [28]	Yes	No	Yes	No	No	Yes	Yes	Yes	No	1	339
6	Eibensteiner *et al*[29]	Yes	Yes	Not clear	Yes	Yes	No	No	No	No	4	13
z	Bhandari *et al* [30]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	2	34
8	Guckenberger *et al*[31]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	3	32
9	Aj *et al*[32]	Yes	No	Yes	Yes	No	Yes	Yes	Yes	No	1	339
10	Gelfand *et al* [33]	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No	2	18
11	Allan *et al*[34]	No	No	Yes	No	Yes	Yes	Yes	No	No	3	213
12	Shanbehzadeh *et al*[35]	Yes	Yes	No	No	Yes	Yes	Yes	Yes	No	2	40
13	Bergman *et al* [36]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	2	21
14	Daigle *et al* [37]	Yes	Yes	Not clear	Yes	Yes	Yes	Yes	Yes	No	2	18
15	Sorbello *et al* [38]	Yes	Not clear	Not clear	Not clear	Not clear	Not clear	Not clear	Not clear	Not clear	-	0
16	Jheon *et al*[39]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	2	26
17	Olmos-Gómez *et al*[40]	Yes	Yes	Not clear	Not clear	Yes	Yes	Yes	Yes	Yes	3	441
18	Sawhney *et al* [41]	Not clear	Not clear	Not clear	Yes	Yes	Yes	Yes	No	Not clear	3	14
19	Sciubba *et al* [42]	Not clear	Not clear	No	Not clear	Yes	No	Yes	No	Not clear	3	16
20	Errett *et al*[43]	Not clear	Not clear	Yes	Not clear	Yes	Yes	Yes	Yes	No	3	28
21	Arezzo *et al* [44]	Yes	Not clear	Yes	Yes	No	Yes	Yes	Yes	No	2	55
22	Dashash *et al* [45]	Yes	Not clear	Not clear	Yes	No	Yes	Yes	No	No	3	20
23	Ramalho *et al* [46]	Yes	Not clear	Not clear	Yes	Yes	Yes	Yes	Not clear	No	2	16
24	Saldarriaga Rivera *et al* [47]	Yes	No	Not clear	Yes	Yes	Yes	Yes	Not clear	No	3	11
25	Tchouaket Nguemeleu *et al*[48]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	2	18

26	Santana *et al* [49]	No	Not clear	No	Yes	Yes	Yes	Yes	No	Not clear	4	6
27	Tang *et al*[50]	Yes	Yes	Not clear	No	Not clear	No	No	No	No	1	83
28	Jiménez-Rodríguez *et al*[51]	No	Yes	Not clear	Yes	Yes	Yes	Yes	Yes	No	3	16
29	Reina Ortiz *et al*[52]	No	Yes	Not clear	Yes	Yes	No	No	Not clear	No	2	12
30	Douillet *et al* [53]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Not clear	4	51
31	Richez *et al* [54]	Yes	Yes	No	Yes	No	No	No	Not clear	No	2	10
32	Yılmaz Yalçınkaya *et al*[55]	Yes	Yes	Not clear	No	No	Yes	Yes	No	No	1	60
33	Tanasijevic *et al*[56]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	3	13
34	Alarcón *et al* [57]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	3	197

studies. The assessment for stability of the results or consensus was missing in many of the studies with only two studies mentioning this in their methodology.

This systematic evaluation of Delphi studies in medical fields highlights the variations in the research methodology used. There was no single study that could score in all the nine evaluation points.

STRENGTH AND LIMITATIONS OF THE ASSESSMENT

We assessed our evaluation points in a wide variety of Delphi studies across various medical fields. These evaluation points are a focused qualitative tool set to assess any Delphi study on a 3-point scale. The evaluation points can be used by the readers, journal editors, and reviewers to assess the quality of the Delphi methodology.

The limitations of this assessment are the inclusion of only English language published studies in the medical field. The evaluation points were qualitative and did not assess the reporting method of the results.

Despite the limitations mentioned above, the nine evaluation points can rapidly assess the quality of Delphi studies and, thus, the creditability of scientific research presented through them.

CONCLUSION

There are no standard quality parameters to evaluate Delphi methods in healthcare research. The vital elements of Delphi methodology include anonymity, iteration, controlled feedback, and statistical stability of consensus. The published studies have used modified Delphi, and details on methods like expert panel, consensus, or closing criteria are not explicit. We suggest tools for readers and researchers for a systematic assessment of the quality of the Delphi studies.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 July 20; 11(4): 130-143

DOI: 10.5662/wjm.v11.i4.130

ISSN 2222-0682 (online)

REVIEW

Looking into key bacterial proteins involved in gut dysbiosis

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Author contributions: Li M conceived and initiated this review; Zeng XY did literature search, filter, and organization; Li M and Zeng XY wrote the paper and approved the final version of this manuscript.

Supported by National Natural Science Foundation of China, No. 81700457.

Conflict-of-interest statement: There is no conflict of interest associated with any of authors who contributed their efforts in this manuscript.

Manuscript source: Invited manuscript

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Abstract

The gastrointestinal microbiota plays a pivotal role in health and has been linked to many diseases. With the rapid accumulation of pyrosequencing data of the bacterial composition, the causal-effect relationship between specific dysbiosis features and diseases is now being explored. The aim of this review is to describe the key functional bacterial proteins and antigens in the context of dysbiosis related-diseases. We subjectively classify the key functional proteins into two categories: Primary key proteins and secondary key proteins. The primary key proteins mainly act by themselves and include biofilm inhibitors, toxin degraders, oncogene degraders, adipose metabolism modulators, anti-inflammatory peptides, bacteriocins, host cell regulators, adhesion and invasion molecules, and intestinal barrier regulators. The secondary key proteins mainly act by eliciting host immune responses and include flagellin, outer membrane proteins, and other autoantibody-related antigens. Knowledge of key bacterial proteins is limited compared to the rich microbiome data. Understanding and focusing on these key proteins will pave the way for future mechanistic level cause-effect studies of gut dysbiosis and diseases.

Key Words: Gut microbiota; Pyrosequencing; Bacteria; Protein; Immune; Dysbiosis

Core Tip: Revealing the causal-effect relationship between specific dysbiosis features and diseases requires understanding the roles of key bacterial proteins that are involved in dysbiosis. Some bacterial proteins may affect the microbiome by their inherent

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: China

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 29, 2021
Peer-review started: January 29, 2021
First decision: May 6, 2021
Revised: May 11, 2021
Accepted: June 16, 2021
Article in press: June 16, 2021
Published online: July 20, 2021

P-Reviewer: Biondi A
S-Editor: Ma YJ
L-Editor: Wang TQ
P-Editor: Zhang YL

functions. Others shape the microbiome mainly by eliciting host immune responses. These key proteins warrant attention in future bioinformatic analyses and mechanistic studies.

Citation: Zeng XY, Li M. Looking into key bacterial proteins involved in gut dysbiosis. *World J Methodol* 2021; 11(4): 130-143
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/130.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.130

INTRODUCTION

The gastrointestinal microbiota is linked to numerous diseases, including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), colorectal cancer, cirrhosis, and many others. Thanks to the rapid decrease in the cost of pyrosequencing, the gut microbiota, often represented by the fecal bacteria composition, is now easy to profile by 16S rDNA sequencing and shotgun metagenomic sequencing. With the accumulation of known microbiome-disease correlations in many descriptive studies, the mechanisms of known dysbiosis features in the pathogenesis of related diseases have become a new frontier to be explored. Understanding these mechanisms is a prerequisite to developing the precise intervention methods targeting the gut microbiome. Thus, it is necessary to review the key microbial proteins involved in gut dysbiosis.

The gut microbiome produces numerous products for itself and the host. The collection of small molecules produced by the gut microbiota, termed the metabolome, represents promising targets for investigation and translation. The methodology and findings of studies of the gut metabolome have been reviewed elsewhere[1,2]. In addition, the gut microbiota produces exosomes, which have been reviewed by other excellent reviews[3]. The virome[4,5], parasitome[6], helminths, and protozoa-omics[6] are also recognized by omic-approaches but with less well documented mechanisms. In this review, we will focus on the key peptides, proteins, and antigens produced by bacteria and fungi in the context of dysbiosis and diseases.

To organize the review, we categorize the bacterial proteins into two groups: (1) The primary key proteins, whose action mainly depends on their inherent properties (Table 1); and (2) The secondary key proteins, whose action mainly depends on the host response to them (Figure 1, Table 2). This classification mainly depends on the current knowledge and is relative. Often, the bacteria-host interaction is bilateral. Thus, this classification is subjective and only helps navigate the mechanisms. For each group, we organize the key proteins according to their functions to assist in navigating this field rapidly.

PRIMARY KEY PROTEINS

Biofilm inhibitors

Biofilm formation is a process of extracellular synthesis by bacteria, and it has adverse effects on the immune response of the host[7], resulting in dysbiosis[8]. Bacteria are found in the intestinal mucosa of humans and clinical observations have revealed bacterial biofilms associated with mucosal colonization in patients with IBD[7]. Many infections also involve pathogens forming biofilms, including enterohemorrhagic *Escherichia coli* (EHEC)[9]. Probiotics have been documented to produce enzymes degrading biofilms of other species. *Escherichia coli* (*E. coli*) Nissle 1917 (EcN), a probiotic capable of alleviating inflammation, can produce its own biofilm and outcompete that of other intestinal pathogens[10]. Fang *et al*[11] found that DegP, a bifunctional (protease and chaperone) periplasmic protein secreted by EcN, contributes to the inhibition of EHEC biofilms by directly interacting with the EHEC cell surface while not affecting its own biofilm. Another probiotic, *Lactobacillus rhamnosus GG* (LGG), could also disrupt the biofilm formation of pathogenic *E. coli* and *Salmonella*[12]. This effect is mediated by its lectin like proteins, termed Llp1 (lectin-like protein 1) and Llp2[12]. Llp2, which is more active than Llp1, showed inhibitory

Table 1 Summary of primary key proteins

Classification	Name	Function	Ref.
Enzyme	DegP	Inhibiting EHEC biofilms.	[11]
Enzyme	Llp1, Llp2	Inhibiting biofilm formation of pathogen.	[12]
Enzyme	Protease of *S. boulardii*	Digesting both *C. difficile* toxin A and its receptor binding sites.	[15]
Enzyme	Lon protease	Degrading the oncogene c-MYC.	[19]
Secreted protein	P9	Inducing the secretion of GLP-1. Inducing the secretion of IL-6 in macrophages.	[21, 22]
Secreted protein	Peptide B7	Reducing CCR2 expression on all APCs from health people.	[25]
Secreted protein	MAM	Inhibiting the NF-κB pathway and several cell immune responses. Inducing expression of TGF β.	[26-28]
Surface layer protein	MIMP	Inducing the secretion of anti- inflammatory cytokines and inhibiting inflammatory cytokines. Enhancing the intestinal barrier.	[29]
Enzyme	OGA	Hydrolysing O-GlcNAcylated NF-κB-p65 and IKKβ to inhibit NF-κB signaling.	[30]
Bacteriocins	PediocinPA-1/AcHnisin Z	Reducing colonization of VRE *in vivo*.	[38]
	Microcin	limiting the expansion of pathogens.	[39, 40]
Bacteriocins	Enterocins	Inhibiting a wide spectrum of Gram-positive bacteria. Inhibiting the growth of cancer cells.	[42, 43]
Bacteriocins	Bacteriocin A, B	Degrading pathogenic biofilm and having antibacterial potential.	[36]
Bacteriocins	Nisin A	Changing the integrity of the cancer cell membrane.	[44]
Secreted protein	P8	Inducing host cell growth arrest at the G2 phase.	[46, 47]
Ribosomal proteins	HPRP-A1; HPRP-A2	Resisting infection. Arresting the cancer cells cycle at the G0/G1 phase and G2/M phase.	[48-53]
Innermembrane protein	Pre-FadA	Binding host epithelial cells.	[54]
Secreted protein	m-FadA	Inducing the invasion of host cells.	[54]
Outer membrane protein	Fap2	Leading to colonization of Fn. Facilitating tumor immunity evasion. Binding to and activating TIGIT. Inducing host lymphocyte apoptosis.	[55-57]
Secreted proteins	OMVs of Fn	Inducing the colonization of host epithelial cells.	[58-60]
Cell envelope-associated multiprotein systems	Sus-like systems	Inducing the colonization of host epithelial cells.	[64]
Pili	SpaCBA	Inducing the adhesion of mucus.	[66, 67]
Secreted proteins	EVs	Inducing the expression of the TJ protein-encoding genes and regulating the intestinal barrier. Inducing the expression of *PPARα* and *PPARγ* genes and *ANGPTL4* gene. Inhibiting blood lipase lipoproteins in the bloodstream.	[71, 72]
Secreted proteins	TcpC OMVs of EcN	Enhancing epithelial barrier.	[77-81]

EcN: *Escherichia coli* Nissle 1917; EVs: Extracellular vesicles; OMVs: Outer membrane vesicles.

activity against biofilm formation by various pathogens, including clinical *Salmonella* species and uropathogenic *E. coli* (UPEC) [12]. Thus, biofilm production and inhibition might represent key bacterial events in microbiome evolution, as well as promising targets to manage dysbiosis.

Toxin degraders

Probiotics may degrade pathogenic toxins and thus contribute to the homeostasis of gut microbiota. *Clostridium difficile* (*C. difficile*) mediates intestinal inflammation and mucosal damage by releasing two potent exotoxins, toxin A and toxin B[13], while the fungal probiotic *Saccharomyces boulardii* (*S. boulardii*) is known as the most efficient

Table 2 Summary of secondary key proteins

Classification	Name	Function	Ref.
Flagellin		Inducing the secretion of proinflammatory cytokines.	[87]
Flagellin		Recuiting flagellin specific CD4+ T-cells.	[85]
Flagellin		Inducing the secretion of flagellin antibodies.	[88,89]
Flagellin		Inducing the secretion of AMPs.	[90]
Flagellin		Inducing the secretion of human β-defensin 2.	[91]
Flagellin		Inducing the expression of lncRNA (HIF1A-AS2) and suppressing NF-kB signaling pathway activation.	[92]
Outer membrane protein	OmpC, OmpW	Adhesion and invasion of the CD-associated *Escherichia coli* in intestinal epithelial cells. Cross-reactive bacterial proteins.	[95-97]
Outer membrane protein	FomA	Inducing upregulation of CD86, MHC II, and primary B cells. Inducing secretion of antigen-specific antibody IgA and IgG.	[99,100]
Bacterial division protein	FtsZ	Cross-reacting with TBB-5 and mediating the secretion of p-ANCA.	[102, 103]
Bacterial heat shock protein	GroEL	Cross-reacting with Hsp60 and inducing antibodies.	[110, 111]

p-ANCA: Perinuclear antineutrophil cytoplasmic antibody.

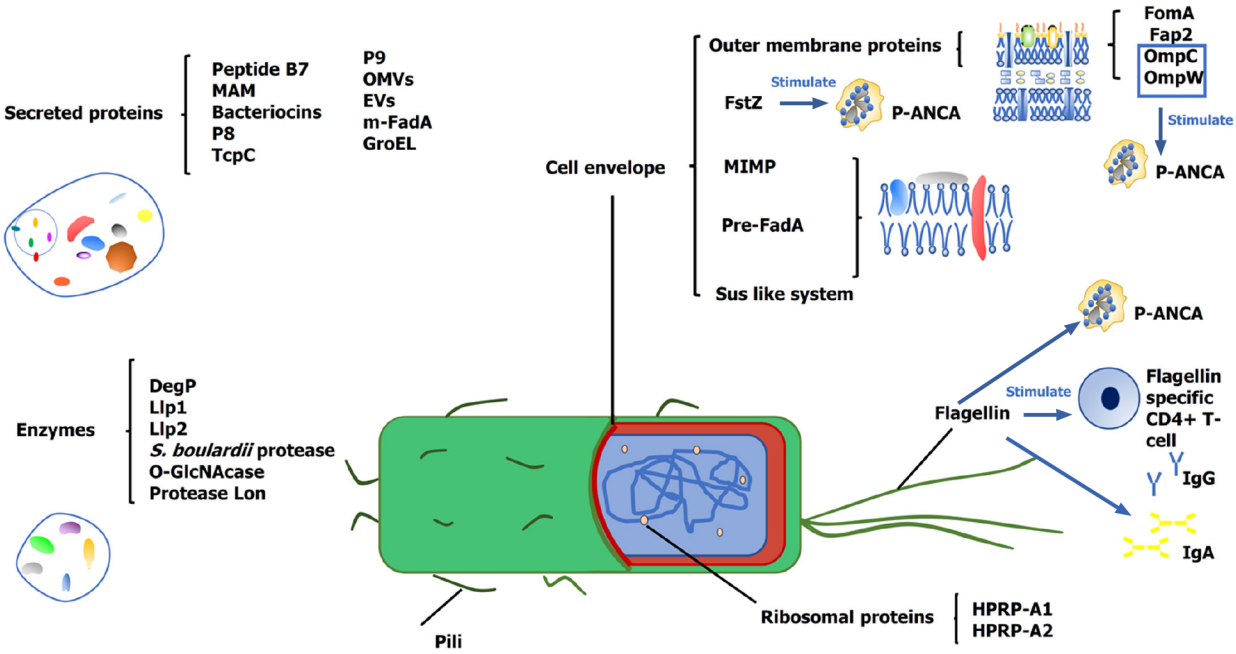

Figure 1 Summary of the location or form of key bio-active microbiota proteins. FtsZ and outer membrane proteins OmpC and OmpW were testified to stimulate perinuclear antineutrophil cytoplasmic antibody (p-ANCA). Flagellin was proved to stimulate p-ANCA, flagellin specific CD4+ T-cells, and flagellin associated IgG and IgA.

probiotic to prevent intestinal inflammation and mucosal damage associated with *C. difficile* infection[14]. The protective effect of *S. boulardii* is dependent on a 54 kDa protease, which digests both toxin A and its receptor binding sites[15]. Several human studies demonstrated that treatment with *S. boulardii* CNCM I-745 in dysbiosis leads to faster reestablishment of a healthy microbiome[16].

Oncogene degraders

Oncogene c-MYC is associated with oncogenic transcription in malignant tumor driven by chronic bacterial infections[17], and the up-regulated c-MYC also indicates a poor prognosis in some human cancers[18]. The Lon protease from UPEC shows potential for therapeutic targeting of c-MYC in cancers, the degradation of c-MYC is

dependent on both direct Lon protease cleavage and Hly-dependent activation of CK1 α1, and UPEC represses transcriptional MYC regulators to inhibit c-MYC expression [19]. In mice, the recombinant Lon (rLon) protease without major toxicity delayed tumor development and increased survival in MYC-dependent bladder and colon cancer models[19]. These results indicate that probiotics may block tumor proliferation by degrading the oncogene.

Adipose metabolism modulators

Akkermansia muciniphila (A. muciniphila), one of the gut microbiota, is connected with metabolic disorders, and it reduces the energy absorption under cold conditions in the intestine epithelium[20]. P9 is an 84 kDa protein, which is secreted by *A. muciniphila*. P9 increases the glucagon-like peptide-1 (GLP-1) secretion in a calcium-dependent manner and specifically promotes interscapular brown adipose tissue (iBAT) non-shivering thermogenesis in the gut hormone-releasing L cells and HFD mice[21]. The ligand–receptor capture (LRC)-TriCEPS technology shows that the P9 interacts with intercellular adhesion molecule 2 (ICAM-2), and ICAM-2 reduces the secretion of the P9-induced GLP-1 in a dose-dependent manner[21]. Moreover, P9 induced the secretion of interleukin-6 (IL-6) in macrophages[21], and IL-6 can stimulate GLP-1 secretion by intestinal L cells[22].

Anti-inflammatory peptides

The mucosal immune response plays an important role in IBD pathogenesis, and perturbations of the gut microbiota are a key element[23]. Probiotics can modulate the intestinal cytokine milieu to treat IBD[24] and other diseases. Peptide B7 from the probiotic *Bifidobacterium longum* decreases CCR2 expression on all antigen presenting cells from healthy controls but not from active IBD patients[25]. Although this bioactive peptide is useless for the treatment of active IBD patients, we cannot ignore its potential to prevent inflammation flares in the quiescent phase[25]. Another probiotic, *Faecalibacterium prausnitzii* (*F. prausnitzii*), one of the most abundant species in the human gut microbiota, possesses a 15 kDa protein with anti-inflammatory properties, termed a microbial anti-inflammatory molecule (MAM)[26]. The inflammatory suppressive role of MAMs from *F. prausnitzii* may be related to their effects on the inhibition of the NF-κB pathway, several cell immune responses such as Th1, Th2, and Th17 cells, and the expression of TGF-β[27,28]. The micro integral membrane protein (MIMP) identified from *Lactobacillus plantarum* was found to decrease proinflammatory cytokines (IFN-γ, IL-17 and IL-23), increase anti-inflammatory cytokines (IL-4 and IL-10), and fortify the intestinal barrier in a dextran sulphate sodium induced colitis model[29]. Probiotics have been documented to produce enzymes hydrolyzing key proteins in the NF-κB pathway[30]. O-GlcNAcase (OGA) is rich in *Bacteroidetes* and *Firmicutes*, the major probiotics distributed in the human gut, and reduced expression of bacterial *OGA* genes has been found in ulcerative colitis (UC)[30]. Bacterial OGAs are an advanced therapeutic strategy in UC that act by hydrolyzing O-GlcNAcylated NF-κB-p65 and IKKβ to inhibit NF-κB signaling in both immune cells and intestinal epithelial cells[30].

Bacteriocins

Bacteriocins are ribosomally synthesized bactericidal or bacteriostatic peptides[31,32]. Bacteriocins from probiotics maintain the microbial population-level and community-level dynamics and inhibit other strains[33]. Bacteriocins are mainly divided into two classes: Posttranslationally modified class I and unmodified class II[34,35]. In a previous study, pediocin, enterocin-A, and enterocin-B were regarded as class II bacteriocins[31], and nisin belonged to class I bacteriocins[36,37]. Pediocin PA-1/AcH secreted by *Pediococcus acidilactici* (*P. acidilactici*) MM33 and nisin Z secreted by *Lactococcus lactis* (*L. lactis)* MM19, have been proven to reduce colonization of vancomycin-resistant enterococci (VRE) *in vivo*[38]. Microcin-producing EcN limits the expansion of competing *Enterobacteriaceae*, including commensal *E. coli*, adherent-invasive *E. coli*, and *Salmonella enterica* in the inflamed gut[39] by utilizing catecholate siderophores[40]. *Enterococcus faecium* produces two synergistic bacteriocins, enterocin-A (a pediocin-like bacteriocin) and enterocin-B. Although the inhibitory spectra of enterocins A and B have small differences, both enterocins from *Enterococcus faecium* TI36 inhibit a wide spectrum of Gram-positive bacteria but not Gram-negative bacteria [41]. With a similar inhibitory spectrum, enterocin A has lower minimum inhibitory concentration (MIC) values than enterocin B[41].

Furthermore, the bactericidal effect is drastically increased when a mixture of the two bacteriocins is used[41]. The findings of a previous study suggested that the

heterodimer of bacteriocin A and B from *Enterococcus faecium* por1 had antibacterial, pathogenic biofilm degradation potential but did not result in haemolysis of human red blood cells[42]. A cancer cell growth inhibitory potential of enterocins has been demonstrated, and apoptotic makers were observed in enterocin treated cancer cells including HeLa, HT-29, and AGS cells[42]. The mechanism of their effects on cancer is that cancer cells have more microvilli on their surface, which allows the membrane of cancer cells to bind large quantities of bacteriocins[43]; thus, Nisin A from *L. lactis* changes the integrity of the cancer cell membrane and obstructs the rearrangement of phospholipids, resulting in increased ion permeability[44]. These bacteriocins enable probiotics to treat enterobacterial infections in the gut and even some cancers.

Host cell circle regulators

Some oral bacteria disseminate into the colon and alter the composition of the microbiota in the colon, resulting in intestinal dysbiosis and possibly leading to colorectal cancer (CRC)[8]. FadA from *Fusobacterium nucleatum* drives CRC proliferation through E-cadherin and increases the expression of transcription factors and inflammatory genes *via* activation of β-catenin signaling[45]. Some bacterial proteins provide new strategies to treat cancer. An 8 kDa protein called p8 was isolated from *Lactobacillus rhamnosus* (LR) KCTC 12202BP, which regulates the p53-p21-Cyclin B1/Cdk1 signaling pathway and causes cell growth arrest at the G2 phase in a dose-dependent manner[46]. Bacterial drug delivery systems are being applied to treat CRC. The p8 protein from *Pediococcus pentosaceus* SL4 (PP-p8) showed antiproliferative activity in a mouse CRC model[47]. Moreover, endogenous p8 expression was much more effective than exogenous recombinant- p8 expression. This makes gene therapy possible[47].

HPRP-A1 and its enantiomer HPRP-A2 are derived from ribosomal protein L1 (RpL1) of *Helicobacter pylori*[48]. These proteins can resist infection including fungi, bacteria, and parasites[49,50]. Moreover, they have anticancer potential, and both peptides lead to apoptosis *via* caspase-3-, caspase-8-, and caspase-9-dependent pathways and inhibit cancer cell growth by arresting the cell cycle at the G0/G1 phase and G2/M phase. HPRP-A1 and its enantiomer HPRP-A2 play an important role in the inhibition of gastrointestinal cancer[51-53].

Adhesion and invasion molecules

Fusobacterium nucleatum (Fn) is associated with CRC and promotes tumor formation. Fn is able to adhere to and invade intestinal endothelial cells by binding to adhesin FadA, a virulence factor from Fn[54]. FadA from *E. coli* enhances the connection between host epithelial cells and bacteria. FadA has two forms, anchored form (pre-FadA) and secreted form (mature FadA), thus the pre FadA-mFadA complex is regarded as a unique adhesin/invasin[54]. Fusobacterial lectin (Fap2) might mediate the binding of Fn to the host factor Gal-GalNAc in CRC, and Gal-GalNAc is highly expressed in human colorectal adenocarcinoma and metastases[55]. Other findings support that Fap2 of Fn not only leads to colonization but also facilitates tumor immunity evasion[55]. Fap2 directly binds to and activates TIGIT (an inhibitory receptor on human natural killer cells and different T cells), and the interaction between these two molecules inhibits the cytotoxicity of NK cells and the activities of cytotoxic T lymphocytes and T helper cells, increasing the immune evasion of tumor cells[56]. Fap2, as an apoptosis-inducing protein, also induces host lymphocyte apoptosis and destroys the host immune response, facilitating Fn survival[57]. Liu *et al* [58] identified outer membrane vesicles (OMVs) in Fn by LC/MS/MS analysis and identified several pathogenic proteins in OMVs, including FadA, Fap2, MORN2, YadA (Yersinia adhesin)-like protein, and autotransporter proteins[58]. The MORN2 domains of Fn may contribute to adhesion and active invasion[59]. Two YadA-like proteins exist in OMVs and outer membrane fractions, which reveal great adhesion ability[58]; therefore, YadA-like proteins are involved in resisting host immune defenses dependent on resisting serum killing activity and phagocytosis[60]. OMVs provide new insight into the research and development of vaccines against Fn[58].

Bacteroidetes is one of the most numerous Gram-negative bacteria in the mammalian gastrointestinal tract[61]. Cell envelope-associated multiprotein systems, namely, Sus (starch utilization system)-like systems[62], are abundant in *Bacteroides*. Polysaccharide utilization loci (PULs) in Sus-like systems are not only used to bind to and degrade dietary sugar[63], but they also encode a unique pathway, the *ccfA–E* genes, called commensal colonization factors (CCF systems) for species-specific saturable niche colonization[64]. Moreover, the CCF system is medicated by *B. fragilis* colonization during infection with *Citrobacter rodentium* and antibiotic treatment[64].

LGG has a very good mucus adhesive capacity compared to another *Lactobacillus strains*[65].The LGG-specific SpaCBA pili are long and thin proteinaceous protrusions on bacterial surface, which involved in three pilin monomers: SpaA , SpaB, and SpaC [66]. The SpaCBA pili mediate adhesive capacity to mucus and contribute to biofilm formation[67]. Moreover, the SpaCBA pili may also regular immune response. The spaCBA knockout LGG had twofold increased IL-8 and some pro-inflammatory markers in Caco-2 cells compared to wild-type[67].

Intestinal barrier regulators

Under dysbiosis, increased permeability of the intestinal epithelium leads to low-grade inflammation and metabolic dysfunctions[68]. However, according to the leaky gut hypothesis, if only the *F. prausnitzii* is present as a probiotic, it will not beneficial to the intestine health and dysbiosis-induced diseases but enter the bloodstream by passing though the gut barrier and may cause systemic consequences because of obesity and a high-fat diet (HFD)[69,70]. Moosavi *et al*[71] show that *F. prausnitzii* –derived extracellular vesicles (EVs) contain different proteins with a molecular weight of 11 to 245 kDa. Compared with *F. prausnitzii*, its EVs in the Caco-2 cell line significantly regulate the intestinal barrier permeability due to increasing the expression of the tight junction (TJ) protein encoding genes *ZO1* and *OCLN*, as well as *PPARα and PPARγ* genes and their targeted gene *ANGPTL4* at the mRNA level[71]. TJ proteins connect the adjacent epithelial cells and block the paracellular space in order to obstruct pathogens[72]. ANGPTL4 inhibits blood lipase lipoproteins in the bloodstream, which reduces the intake of free fatty acids and cholesterol into the tissues[73-76].

TcpC from EcN enhanced the intestinal barrier function by increasing the expression of the TJ proteins ZO-1, ZO-2, and claudin-14[77-79]. Moreover, the positive strains ECOR63 and ECOR57 increased the transepithelial electrical resistance (TER) in T-84 monolayers to strengthen the intestinal barrier[80]. In addition, OMVs and other soluble factors from these probiotic bacteria increase the upregulation of ZO-1 and claudin-14, but downregulation of claudin-2[81]. Raising claudin-2 levels lead to increased barrier permeability[82] and result in CD and UC[83,84]. OMVs and soluble factors, rather than TcpC, are able to strengthen the intestinal barrier[81].

SECONDARY KEY PROTEINS

Flagellin

Flagellin is a common conserved component of bacteria, and it induces both innate and specific immunity, showing a close relationship between dysbiosis and IBD[85], but flagellin of some probiotics has anti-inflammatory effects[86]. Flagellin is regarded as the major antigen in pathogenic bacteria. Flagellin binds with the pattern-recognition receptor Toll-like receptor 5 (TLR5), inducing the secretion of proinflammatory cytokines[87]. Compared with healthy controls, both Crohn's disease (CD) and UC patients have a relative increase in the proportion of flagellin specific CD4+ T-cells. Cook *et al*[85] found a positive correlation between the relative abundance of bacteria [*Escherichia*/*Shigella* and (*Ruminococcus*) *gnavus* group] in IBD patients and high concentrations of flagellin antibodies, including anti-Fla2 IgG and anti-Fla2 IgA[88]. Specifically, CBir1 flagellin has been associated with complicated CD, and enzyme-linked immunosorbent assays proved that anti-CBir1 IgG is independently associated with CD[89]. Flagellin may provide a clinically novel approach to prevent pathogen infections, including vancomycin-resistant Enterococcus (VRE). Intestinal epithelial cells and Paneth cells secrete the antimicrobial protein (AMP) RegIIIγ to kill microorganisms and directly respond to flagellin *via* the Toll-like receptor (TLR)–myeloid differentiation factor 88–mediated pathway[90]. Flagellin of EcN stimulates intestinal epithelial cells to produce human β-defensin 2 *via* three main MAP kinase pathways, including ERK1/2, JNK, and p38[91]. Bacterial flagellin also induces negative regulation of inflammation. *Roseburia intestinalis* (*R. intestinalis*), a dominant symbiotic microbiota in the intestine, suppresses inflammation by inducing Treg cells and upregulating anti-inflammatory cytokines. However, *R. intestinalis* is significantly reduced in CD patients[86]. Flagellin in *R. intestinalis* induces the expression of lncRNA (HIF1A-AS2) in a dose- and time-dependent manner *via* p38 STAT1 activation, and HIF1A-AS2 inhibits the expression of inflammatory genes by suppressing NF-kB signaling pathway activation[92].

Outer membrane proteins

Some evidence linking intestinal dysbiosis with autoimmune diseases has shown that they are both associated with increased inflammation[93,94]. Bacterial outer membrane proteins are more likely to trigger an immune response, and the perinuclear antineutrophil cytoplasmic antibody (p-ANCA) present in many autoimmune diseases cross-reacts with outer membrane proteins. P-ANCA autoantibody is associated with UC. A p-ANCA monoclonal antibody detects outer membrane porins OmpC and OmpW expressed by colonic bacteria *Bacteroides caccae* and *E. coli*[95,96]. A structural relationship of the cross-reactive bacterial proteins and the p-ANCA autoantigen has been observed in IBD[95,96]. OmpC also enhances the adhesion and invasion of the CD-associated *E. coli* strain LF82 in intestinal epithelial cells through the sigma (E) regulatory pathway[97]. *Fusobacterium nucleatum* (Fn) has been found to be increased in the microbiota of diarrhea dominant IBS. The FomA protein is a major outer membrane protein of Fn[98]. The FomA of Fn is an immune adjuvant, which is a Toll-like receptor 2 (TLR2) agonist that induces upregulation of CD86 and MHC II in mice and primary B cells *in vitro* and antigen-specific antibody IgA and IgG secretion *in vivo* [99]. These characteristics enhance inflammation in the small intestine epithelium in both cell and mouse experiments[99]. Fn causes microbial dysbiosis, exacerbates visceral hypersensitivity in a colonization-independent manner, and induces the specific IgA agonist FomA[100]. Moreover, FomA has been proven to be an antigen that stimulates the secretion of symptom-associated antibodies[100].

Other autoantibody-related antigens

Primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH) are frequently associated with chronic IBD, including UC and CD[101]. The immune reaction in PSCs is mediated by autoantibodies, including pANCA, that recognize both β-tubulin isotype 5 (TBB-5) and the bacterial antigen cell division protein FtsZ[102]. Human TBB-5 and FtsZ share a high degree of structural homology in evolutionarily conserved epitopes[103]. Moreover, B cells respond directly to microbial constituents in PSCs and AIH[104].

Helicobacter hepaticus (Hh) can induce intestinal inflammation in DC-LMP1/CD40 mice[105]. These immunodeficient mice lost intestinal CD103+ DCs and IL-10+ Helios−induced Tregs (iTregs) but had increased IL17+ IFNγ+ Th17/Th1 cells and pathogenic IFNγ+ Th1 cells[106,107]. They developed fetal colitis similar to human IBD, because CD40-CD40L interactions are connected with the pathogenesis of IBD [108,109].A 60 kDa Hh-protein, GroEL, as the main antigen recognized by antibodies in an iTreg-free setting, triggers fatal colitis[110]. The bacterial GroEL and human heat shock protein 60 (Hsp60) share a high similarity and molecular mimicry[111,112], hence the antibodies cross-react with Hsp60 and GroEL, which contribute to IBD and autoimmune diseases[110].

CONCLUSION

Understanding the key bacterial proteins is significant to both the diagnosis and management of dysbiosis related diseases. For the incendiary proteins involved in autoimmune diseases and tumors, the presence of the specific marker in the microbiota or its specific antibodies might indicate the prognosis of diseases. The therapeutic value of targeting these markers would also be tempting. Knowing the key elements of microbiota could provide much more specific target than generally modulating the microbiota, which is super-high dimensional in taxonomy.

The current mainstream microbiome manipulation approaches are intensively investigated, including supplement of probiotics and prebiotics, and fecal microbiota transplantation (FMT). However, the probiotics should be strain-defined to gain standardized safety, dose, and effect; the adverse events associated with FMT have been found recently. The transplanted probiotics met indigenous microbiome-mediated mucosal colonization resistance in mice and even a specific colonization resistance in a person-, strain-, and region- dependent manner in humans[113]. Our recent mathematical model studies also suggested intriguing behavior of microbiome in response to probiotic supplement[114]. For FMT, the risk of unknown infections is still inevitable even after rigorous tests on the donors. The specific microbial proteins are easier to be cloned, purified, tested, optimized, and standardized, which is crucial for the pharmacology. Furthermore, the natural beneficial bacterial proteins can be artificially engineered and optimized to maximum their mechanism. This review summarizes the pathogenic and therapeutic mechanisms of some bioactive microbial

proteins. This field is cutting edge, and there is a need for further studies to explore the role of the key gut microbial proteins in dysbiosis associated diseases.

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World Journal of Methodology

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World J Methodol 2021 July 20; 11(4): 144-159

DOI: 10.5662/wjm.v11.i4.144

ISSN 2222-0682 (online)

REVIEW

Connecting inorganic mercury and lead measurements in blood to dietary sources of exposure that may impact child development

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Author contributions: Dufault RJ wrote all but one section of the manuscript and created the figure, and supplementary material; Wolle MM wrote the analytical methods section and created the table; Kingston HMS, Gilbert SG, and Murray JA contributed to the format design and revision of the manuscript.

Conflict-of-interest statement: The authors declare no conflict of interests.

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Abstract

Pre-natal and post-natal chemical exposures and co-exposures from a variety of sources including contaminated air, water, soil, and food are common and associated with poorer birth and child health outcomes. Poor diet is a contributing factor in the development of child behavioral disorders. Child behavior and learning can be adversely impacted when gene expression is altered by dietary transcription factors such as zinc insufficiency or deficiency or by exposure to toxic substances permitted in our food supply such as mercury, lead, or organophosphate pesticide residue. Children with autism spectrum disorder and attention deficit hyperactivity disorders exhibit decreased or impaired *PON1* gene activity which is needed by the body to metabolize and excrete neurotoxic organophosphate pesticides. In this current review we present an updated macroepigenetic model that explains how dietary inorganic mercury and lead exposures from unhealthy diet may lead to elevated blood mercury and/or lead levels and the development of symptoms associated with the autism and attention deficit-hyperactivity disorders. *PON1* gene activity may be suppressed by inadequate dietary calcium, selenium, and fatty acid intake or exposures to lead or mercury. The model may assist clinicians in diagnosing and treating the symptoms associated with these childhood neurodevelopmental disorders. Recommendations for future research are provided based on the updated model and review of recently published literature.

Manuscript source: Invited manuscript

Specialty type: Public, environmental and occupational health

Country/Territory of origin: United States

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 28, 2021
Peer-review started: January 28, 2021
First decision: May 14, 2021
Revised: May 27, 2021
Accepted: June 16, 2021
Article in press: June 16, 2021
Published online: July 20, 2021

P-Reviewer: Yen TH
S-Editor: Ma YJ
L-Editor: A
P-Editor: Yuan YY

Key Words: Autism; Paraoxonase 1; Selenium; Inorganic mercury; Lead; Attention deficit hyperactivity disorder

Core Tip: Connecting inorganic mercury and lead measurements in blood to dietary sources of exposure that may impact child development is a challenge. Autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) diagnoses and treatment efficacy may include the collection of the biomarker measurements of selenium, mercury, and lead levels in red blood cells and behavioral checklist data before and after healthy dietary interventions. We discuss the analytical measurement methods for determining mercury and lead levels in blood and how these biomarkers have been used in ASD and ADHD studies with and without dietary intervention.

Citation: Dufault RJ, Wolle MM, Kingston HMS, Gilbert SG, Murray JA. Connecting inorganic mercury and lead measurements in blood to dietary sources of exposure that may impact child development. *World J Methodol* 2021; 11(4): 144-159
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/144.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.144

INTRODUCTION

Autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) are neurodevelopmental disorders that are diagnosed according to behavior descriptions outlined in the Diagnostic and Statistical Manual of Mental Disorders (DSM) published by the American Psychiatric Association (APA). The conditions are similar in that children with either diagnosis have difficulty functioning in a social environment[1]. While a child with ASD may find it difficult to verbally hold a reciprocal conversation with a peer, a child with ADHD may talk excessively or interrupt his classmate during a discussion[1]. Children with either diagnosis often have difficulty in school settings where there are established rules for acceptable behaviors in and out of the classroom. While a child with ASD may become distressed when it is time to transition to another school activity, a child with ADHD may have difficulty standing still in the recess lines[1]. Children with either diagnosis often fail to develop and maintain appropriate peer relationships and have difficulty learning in the general education school environment. In the United States (U.S.), children with ASD or ADHD are eligible to receive special education and related services under federal law to help them achieve their learning goals[2].

Although the causes of ASD and ADHD remain unknown, there is strong evidence to suggest mercury and lead exposures are significant factors in their etiology. Regarding ADHD, He *et al*[3] found seven case-control studies in a literature review of the effects of blood lead levels in children with ADHD symptoms. The case groups showed significant increases in ADHD symptoms with blood lead measurements and, in some cases, at levels < 3 μg/dL[3]. This finding is alarming given the fact the United States Centers for Disease Control and Prevention (CDC) only uses a 5 μg/dL reference value for blood lead levels to identify children who have been exposed to lead[4]. In a separate review of seventeen studies published in the last five years, Donzelli *et al*[5] determined there is a positive association between lead exposure and ADHD and even low levels of blood lead are of concern. Regarding ASD, Saghazadeh and Rezaei[6] identified 48 studies in a review of the literature for use in assessing the role of heavy metals in the etiology of ASD. They found children with ASD had higher erythrocyte levels of lead and mercury and significantly higher blood lead levels compared to controls[6]. Jafari *et al*[7] focused their review and analysis exclusively on mercury measurements reported in 44 studies. They found mercury levels in whole blood and red blood cells were significantly higher in patients with autism compared to healthy subjects[7]. Jafari *et al*[7] concluded that their results revealed mercury to be an important causal factor in ASD with its accumulation in blood tissue likely due to impaired mercury detoxification and excretory mechanisms. The United States CDC

currently has no reference value for blood mercury levels to identify children at risk of autism.

A mercury toxicity model introduced previously in 2009 by Dufault *et al*[8] provides a macroepigenetic explanation of how child neurodevelopment can be adversely impacted when dietary transcription factors such as zinc deficiency or exposures to heavy metals interfere with the expression of the zinc dependent metallothionein (MT) gene. The MT gene produces the metal binding protein metallothionein that makes it possible for the body to excrete the neurotoxicants mercury and lead[9]. The mercury toxicity model was revised and re-introduced by Dufault *et al*[10] in 2012 to show how lead levels may accumulate in the body with the consumption of high fructose corn syrup (HFCS) and the accompanying calcium losses that may occur when dietary magnesium intake is low.

United States Department of Agriculture (USDA) scientists warned long ago that when dietary magnesium intake is low, HFCS consumption may adversely affect micromineral homeostasis in humans by lowering calcium, zinc, and phosphorus balances[11]. Mahaffey *et al*[12] previously reported a significant and independent inverse relationship exists between blood lead levels and dietary calcium (Ca) intake. In addition to these homeostatic relationships, the revised model published in 2012 shows that with inadequate dietary Ca intake or Ca losses, PON1 gene activity may decrease[10]. The *PON1* gene is responsible for producing the Ca dependent paraoxonase-1 enzyme that breaks down and detoxifies the organophosphate (OP) pesticides[13] used widely by United States farmers[14]. United States CDC researchers found diet to be the primary source of OP exposure in children[15]. They warned children have significantly higher OP exposures compared to adults and are at greater risk of neurotoxic harm[15]. In addition to fructose[16], mercury and lead are potent inhibitors of PON1 gene expression[17]. When PON1 gene expression is inhibited, the body is unable to detoxify OP pesticide residues. This triggers the mechanism of oxidative stress in the brain which impacts a child's ability to learn[8,10].

Mercury (Hg) and lead (Pb) accumulation in the body may also create these same conditions of oxidative stress[18] which impact child behavior and learning especially when MT is unable to perform its antioxidant role[19] due to dietary zinc (Zn) deficiency or homeostatic mechanisms that lead to Zn losses and copper (Cu) gains. Figure 1 below show the updated macroepigenetic model for Hg and Pb toxicity resulting from this review of the literature. The new model shows selenium (Se) deficit resulting from unhealthy diet is a key factor in decreasing PON1 activity levels.

The literature indicates unhealthy diet remains a significant factor in the development of symptoms associated with ASD[20] and ADHD[21-23]. Evidence suggests ultra-processed food consumption may be a source of heavy metal exposure not often considered, especially in the case of inorganic mercury and lead[24-28]. Both elements are neurotoxic. In a recent review, Dórea[29] reported low-level exposures to lead concurrent with other neurotoxic substances such as mercury and arsenic, show a measurable impact on child neurodevelopment. In addition, children with ASD and ADHD continue to show elevated levels of Hg and/or Pb in their blood[30-39]. Hg and Pb exposures can be identified through blood analyses and should be determined by the clinician when a patient presents with symptoms of ASD or ADHD. If blood testing shows elevated Hg or Pb levels, the physician could refer the patient for dietary assessment and healthy diet instruction.

The following section provides a review of the availability of Pb and Hg analyses and current practices in the laboratory and clinical settings.

ANALYTICAL METHODS FOR BLOOD HG AND PB MEASUREMENT IN CLINICAL STUDIES

Despite the difference in toxicity related to Hg species, total Hg is often measured for exposure studies. As can be seen from Table 1, cold vapor atomic absorption spectrometry (CV-AAS) is commonly used to evaluate the association between blood Hg levels and autistic symptoms[34,40-42]. Other studies couple CV-AAS to atomic fluorescence spectrometry (CV-AFS) for better sensitivity and selectivity[31,43]. Current methodologies mainly rely on inductively coupled plasma mass spectrometry (ICP-MS) due to its unique advantages of high sensitivity, wide dynamic range, and multi-isotopic analysis capabilities. ICP-MS can be used to analyze blood with less sample preparation mainly involving matrix solubilization with acidic or alkaline solutions, which can be accelerated by microwave irradiation[44]. Table 1 Lists a few studies on measurement of Hg in blood by ICP-MS[30,32,34,45]. The United States

Table 1 Summary of methods for the determination of blood Hg and Pb measurements

Analyte	Sample and sample size	Method	Limit of detection	Ref.
Hg	Blood (20-30 mL)	CV-AAS	NA	[34]
	Red blood cells[1]		NA	[40]
	Blood (3-5 mL)		NA	[41]
	Red blood cells (0.4 mL), plasma (0.5 mL)		0.09 ng/mL	[42]
Hg	Blood[1]	CV-AFS	0.09 μg/L[2]	[31]
	Blood (0.5 g)		0.001 μg/L	[43]
Hg	Blood (20 mL)	DMA	0.01 μg/L	[47,48]
Pb	Blood (3-5 mL)	AAS	NA	[35]
	Blood (3 mL)		25.01 μg/L	[38]
	Blood (3-5 mL)		NA	[41]
	Blood (3-5 mL)		0.03 μg/dL	[55]
Pb	Blood[1]	GF-AAS	2.85 μg/dL[2]	[31]
	Blood (20-30 mL)		NA	[34]
	Blood (50 μL)		0.042 μg/dL	[36]
	Blood (20 mL)		0.01 μg/dL	[47]
	Blood[1], hair (1 g)		NA	[56]
	Blood (0.5-2 mL)		25 μg/L	[57]
Pb	Bone[1]	X-ray fluorescence	2 μg/g	[38]
Hg, Pb	Blood (1 mL), hair (5-10 mg)	ICP-MS	NA	[30]
	Blood (2-3 mL)		0.25 μg/L (Hg)	[32]
	Blood (2-3 mL)		0.25 μg/dL (Pb)	[32]
	Blood (20-30 mL)		0.1 μg/L (Hg)	[34]
	Blood (20-30 mL)		0.002 μg/dL (Pb)	[34]
	Blood[1]		1.3 μg/dL (Pb)	[37]
	Blood[1]		0.24 μg/L (Hg)	[45]
	Blood (20 mL)		0.3 μg/dL (Pb)	[59]
Hg, Pb	Blood (46 μL)	DBS with ICP-MS	0.65 μg/L (Hg)	[60]
	Blood (46 μL)		0.27 μg/dL (Pb)	[60]
	Blood (30 μL)		NA	[61]
	Blood (50 μL)		0.13 ppb[2] (Hg)	[62]
	Blood (50 μL)		2.38 ppb[2] (Pb)	[62]
	Blood (6.2 μL)		0.7 μg/L (Hg)	[63]
Methylmercury	Blood (0.5 g)	GC-CV-AFS	0.0001 μg/L	[43]
	Dental amalgam[1]		NA	[50]
	Blood (0.5 mL)		0.1 pg/L	[51]
I-Hg, methylmercury	Blood (0.5 g)	GC-ICP-MS with SIDMS	NA	[26]
I-Hg, methylmercury	Blood (35-50 μL)	DBS with GC-CV-AFS	0.3 μg/L (methylmercury)	[64]
	Blood (40-60 μL)		1.9 μg/L (I-Hg)	[65]

	Blood (40-60 μL)	[65]

[1]Sample size unspecified.
[2]Lowest detected concentration reported in the study (limit of detection not available).
NA: Limit of detection and lowest detected concentration not available; CV-AAS: Cold vapor atomic absorption spectrometry; CV-AFS: CV-AAS to atomic fluorescence spectrometry; DMA: Direct mercury analysis; AAS: Atomic absorption spectrometry; GF-AAS: Graphite furnace AAS; ICP-MS: Inductively coupled plasma mass spectrometry; SIDMS: Speciated isotope dilution mass spectrometry; DBS: Dried blood spot.

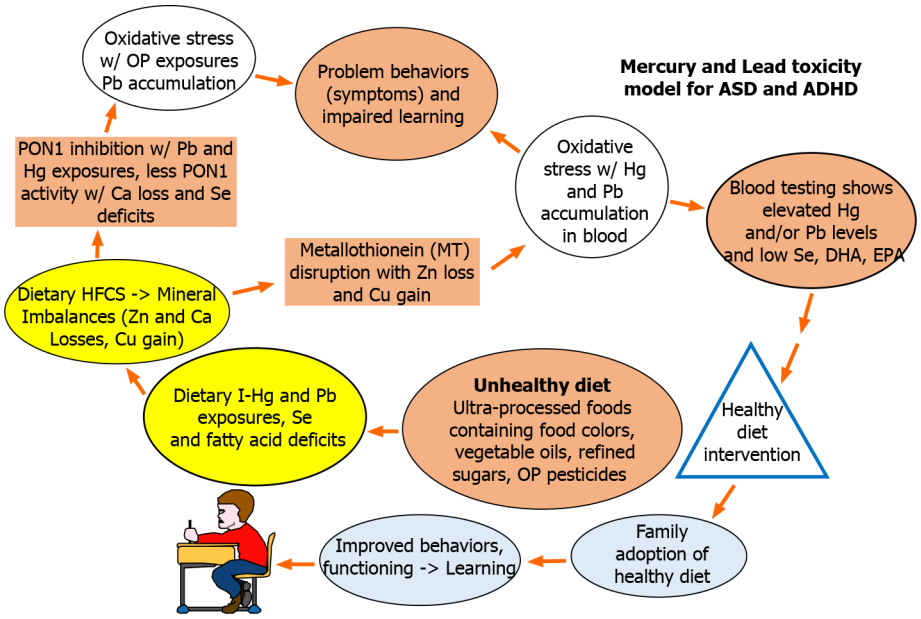

Figure 1 Macroepigenetic model for role of unhealthy diet in mercury and lead toxicity. ASD: Autism spectrum disorder; ADHD: Attention deficit hyperactivity disorder.

CDC uses this technique to measure blood Hg after solubilizing the matrix with a solution of tetramethylammonium hydroxide[46].

Direct mercury analysis (DMA) enables direct Hg measurement without any sample preparation. This high throughput and cost-effective method minimize analytical errors associated with sample preparation and reduces hazardous waste generation. Chinese researchers used DMA to measure blood Hg levels to evaluate the association between Hg exposure and child neurobehavioral development and the influence of sex and dietary intake on these relationships[47,48]. DMA has the constraint that its detection limit is not comparable with those of CV-AAS, CV-AFS and ICP-MS.

Although exposure studies often focus on total Hg determination, the health effects of Hg depend on its chemical forms. Hence, there are now analytical methods to determine its distinct species. The CDC used a non-chromatographic method to speciate blood Hg based on selective determination of inorganic and total Hg by CV-AAS with their difference accounting for organic Hg[49]. Halbach and Welzl[42] predicted the levels of inorganic and organic Hg from total blood Hg based on the difference in partition of the two forms of Hg between erythrocytes and plasma. Advanced methods of Hg speciation analysis use a chromatographic system coupled to element specific and sensitive detector such as CV-AFS and ICP-MS. Several clinical studies used gas chromatography (GC) coupled to CV-AFS to determine Hg species in blood[43,50,51]. Liquid chromatography (LC) can be used to separate Hg species without derivatization, thus reducing potential risk of contamination or loss of unstable analytes. Its main setback is higher detection limit which can be improved by using micro- or nano-HPLC systems or preconcentration of analytes.

Despite the advances in instrumentation and methodology, speciation analysis is complicated by potential transformation of analytes during sample collection, storage, preparation, and analysis that may lead to erroneous results. Hg species may undergo alkylation, dealkylation, oxidation and/or reduction depending on the matrix composition and analytical processes[52]. Such species transformations cannot be identified using conventional methods involving external calibration or standard additions. Speciated isotope dilution mass spectrometry (SIDMS), EPA Method 6800

[53], is uniquely capable to track and correct for in-situ transformation of species, thereby enabling accurate and precise determination of analytes. The U.S. CDC applies this methodology to determine Hg species in blood by GC-ICP-MS after preconcentration on a solid phase microextraction fiber[54]. Dufault *et al*[26] used this method to study the association between blood inorganic mercury (I-Hg) with glucose levels in the human population and its link to processed food intake.

Several methods are available for blood Pb measurement. Studies evaluated the association between Pb exposure and autistic behaviors by measuring Pb in blood using AAS[35,38,41,55]. Graphite furnace AAS (GF-AAS) enables analysis of small volume of sample with minimum preparation[31,34,36,47,56,57]. The introduction of ICP-MS has made it possible for laboratories to achieve lower detection limits and make accurate and precise blood Pb measurement. A method by the U.S. CDC measures Pb in whole blood using ICP-MS after simple dilution[58]. The use of ICP-MS has also been widely reported to evaluate the relationship between blood Pb levels and ASD, see Table 1[30,32,34,37,45,59]. Portable and easy-to-operate devices using anodic stripping voltammetry technology are also available to measure Pb at point of care on a small amount of blood.

Clinical studies are emerging on measurement of Hg and Pb on dried blood spot (DBS). Here, a small volume of blood is blotted and dried on a filter encased in a paper card *via* a simple prick. DBS allows easy and less invasive blood collection, small volume sampling as well as simple transport and storage. The use of DBS is growing through the introduction of advanced analytical techniques, that have expanded testing options and improved throughput. A pilot study in 2008 used DBS for measurement of Hg and Pb where samples from newborns were analyzed by ICP-MS[60]. Subsequent studies using DBS evaluated children's exposures to heavy metals including Hg and Pb by ICP-MS[61-63]. Recent studies investigated the feasibility of DBS for the measurement of Hg species by GC-CV-AFS[64,65].

It was difficult to summarize the lowest and highest concentrations of analytes found in the studies discussed in this section because the data presentation lacks uniformity; some studies reported mean or median concentrations while others compared the levels of the elements before and after treatment. Therefore, the detection limits of the techniques are summarized in Table 1. In cases where detection limits were not available, the lowest reported concentrations were provided in Table 1.

LACK OF UNIFORMITY AMONG MEASUREMENT RESULTS AND IMPACT ON SYMPTOM-BASED DIAGNOSIS

The analytical method used to determine Hg or Pb levels in blood during clinical trials yields varied results lacking in uniformity; this measurement problem continues to be a barrier in identifying toxic exposures. An exposure to Pb or Hg becomes toxic when the child exhibits symptoms of behavioral duress. Children presenting symptoms of ADHD or ASD can be diagnosed using the appropriate behavioral checklist in conjunction with blood testing. The amount of blood sample required to measure mercury or lead using any of the methods listed in Table 1 vary depending on the analyst or laboratory conducting the blood test. Clinical laboratories should be consulted before a phlebotomy is performed to obtain the blood sample. The sample size needed by the laboratory will be based on the instrument to be used, its limit of detection for the analyte to be measured and the method of analysis.

In choosing an appropriate behavior checklist, the Conners rating scales or symptom checklists are used widely by physicians to diagnose ADHD when children present with problem behaviors[66]. In a brief review of the diagnostic accuracy of tests used to diagnose ADHD in children, Gaba *et al*[67] recommend using the Conners Abbreviated Symptom Questionnaire because of its brevity and high diagnostic accuracy. In the case of ASD diagnosis, the most widely used behavior rating scale is the childhood autism rating scale, otherwise known as CARS[68]. Whether diagnosing a case of ADHD or ASD, appropriate behavioral checklists are available for use and can be used in conjunction with blood testing. Several studies show these diagnostic tools to be effective when used together.

In a study of five hundred seventy-eight children with ADHD, Huang *et al*[37] measured blood Pb levels using ICP-MS and found children with low exposure (≤ 5 μg/dL) exhibited hyperactive and impulsive behaviors but not inattentiveness. In conjunction with the blood testing, Huang *et al*[37] administered the Conners' Rating Scale-Revised (CRS-R) checklist to the mothers to assess their children's behavior. In a study of one thousand seven hundred and seventy-eight children with no current

ADHD diagnosis, Ha *et al*[41] measured blood Pb levels using AAS in conjunction with administering a Conner's rating ADHD scale to the parents to determine symptoms and trouble behaviors in their children. The blood Pb levels were positively associated ($P < 0.0001$) with the Conners' ADHD score[41]. As blood lead levels increased so did the children's symptoms associated with ADHD[41].

Alabdali *et al*[69] measured both Pb and I-Hg in the red blood cells of fifty-two male children diagnosed with ASD and thirty age and sex matched healthy controls. Measurement of the severity of symptoms associated with ASD was accomplished using the CARS questionnaire[69]. Compared to the control group of children, the ASD group had significantly higher I-Hg ($P < 0.001$) and Pb ($P < 0.001$) levels in their red blood cells (RBCs)[69]. A flameless AAS method developed by Magos[70] was used to measure mercury species and the GF-AAS method was used to measure lead [69] in the RBCs. Hassan *et al*[71] measured several biomarkers (*e.g.*, serum cholesterol, *Helicobacter pylori* in stool, heavy metals in blood) in a cohort of one hundred and forty-six children comprised of seventy-three males with ASD and seventy-three healthy age- and sex-matched controls. Behavioral assessments were conducted on the children with ASD using the CARS questionnaire[71]. The biomarker study showed Hg, Pb and aluminum levels in whole blood were significantly higher ($P < 0.5$) among the children with ASD *vs* the control group[71]. The blood samples were collected and preserved in vials of EDTA and the metals were measured using AAS[71]. The CARS questionnaire data did not reveal any differences in autism severity associated with the different metals[71]. Mostafa *et al*[72] measured blood Hg levels using CV-AAS in eighty-four children with ASD and eighty-four healthy-matched controls. The blood samples were collected in a heparinized syringe and analyzed promptly[72]. The Hg levels were significantly higher ($P < 0.001$) in the children with ASD compared to the controls[72]. The CARS questionnaire was used to evaluate the severity of symptoms in the children with ASD; the data showed a significant ($P < 0.0001$) and positive linear relationship between blood Hg levels and CARS autism behavior severity scores[72].

From this review, it appears the behaviors seen in children diagnosed with ADHD and autism are influenced by the Hg and Pb levels found in their blood. Regardless of why these bioaccumulations are occurring in these children, a key goal in their care should be to reduce their exposures to Hg and Pb. Figure 1 show unhealthy diet may be a significant source of I-Hg and Pb exposure in these afflicted children.

UNHEALTHY WESTERN DIET IS A SOURCE OF I-HG AND PB EXPOSURE

Unhealthy dietary factors that may link to the development of symptoms associated with ASD and ADHD include exposures to I-Hg, Pb and/or pesticide residues[8,10,73] found in highly processed food[74-76]. In the United States, certified food color consumption in processed foods increased five-fold between 1950 and 2012 from 12 mg/capita per day to 68 mg/capita per day[76]. These food colors may contain residues of I-Hg, Pb, and arsenic[75]. Allowable heavy metal residues in certified food colors are regulated by the United States Food and Drug Administration through the batch certification process[77]. Stevens *et al*[78] studied the amounts of certified food colors found in the United States processed food supply (*i.e.*, beverages, food, and sweets) and concluded that many children are consuming far more food colors than previously thought. Stevens *et al*[78] recommended that parents avoid serving food or beverages containing certified food colors and limit their children's consumption of added sugars to improve child behavior and health outcomes.

The most common added sugars found in processed food are the corn sweeteners which are at risk of Hg contamination due to their manufacturing process. I-Hg mercury can enter the corn sweetener product line in one of two ways: (1) With the use of Hg cell chlor-alkali chemicals in the manufacturing process[79] and (2) With the intentional addition of mercuric chloride to the corn starch mix at the front end of the manufacturing process to inhibit naturally occurring degrading enzymes produced by bacteria[80]. Rideout *et al*[81] and Wallinga *et al*[82] both reported finding low levels of mercury in samples of corn syrup or products containing corn syrup or high fructose corn syrup.

In addition to food colors and added sugars, numerous other food ingredients found in the typical western diet may contain mercury or lead residues[83]. The western diet is characterized by the high intake of ultra-processed food products containing numerous ingredients including refined added sugars, fats, vegetable oils and grains with allowable organophosphate (OP) pesticide residues and the inadequate intake of whole foods, especially pesticide-free fruits, vegetables, nuts, healthy

fats (*e.g.*, cold pressed) and organically grown grains[83]. In the cases of ADHD and ASD, several recent diet studies show children afflicted with these neurodevelopmental disorders are typically eating unhealthy diets as shown in Figure 1.

RECENT STUDIES SHOW CHILDREN DIAGNOSED WITH ADHD AND ASD ARE EATING UNHEALTHY DIETS

In a review of the literature to determine the role of diet in reducing symptoms in children diagnosed with ADHD, Millichat *et al*[21] found diets to reduce symptoms include those that restrict the intake of sugars or eliminate additives including preservatives. Recommendations in the use of diet as a treatment option for ADHD were made in cases of parental or patient preference, iron deficiency, medication failure or when a change from a western diet to an ADHD-free healthy diet was warranted[21]. Millichat *et al*[21] concluded that greater attention needs to be paid to the education of parents and their children on what constitutes a healthy dietary pattern with a focus on removing ingredients shown to exacerbate symptoms. Shareghfarid *et al*[22] conducted a pooled meta-analysis of dietary pattern studies that included eight thousand and sixteen children diagnosed with ADHD and found a healthy dietary pattern decreased the risk of ADHD while a western dietary pattern increased symptoms. In their conclusion, Shareghfarid *et al*[22] determined a "healthy" dietary pattern consisting of vegetables, fruits, legumes, and fish decreased the odds of ADHD while a "Western" dietary pattern consisting of red meat, refined grains, processed meats and hydrogenated fats and oils increased ADHD. In another recent literature review, Farsad-Naeimi *et al*[23] conducted a pooled meta-analysis of fourteen studies that included twenty-five thousand nine hundred and forty-five subjects to determine whether there is a relationship between the consumption of sugar and sugar sweetened beverages and symptoms of ADHD. The results of the pooled data analyses indicate a strong positive relationship (P = 0.01) exists between sugar and sugar-sweetened beverage consumption and symptoms of ADHD[23].

Sugar-sweetened beverages often contain food color ingredients (*e.g.*, yellow #5/tartrazine/E-102, red #40/allura red/E-129, and yellow #6/sunset yellow/E110) that require certification in the United States[77] to determine if heavy metal levels exceed allowable concentrations. Products containing these same food color ingredients must carry the following warning label in the European Union and United Kingdom: *"May have an adverse effect on activity and attention in children"*[84]. The warning label requirement is a result of the findings of a study commissioned by the United Kingdom government[84]. The study was led by McCann and involved two cohorts including one comprised of one hundred fifty-three 3-year-old children and another comprised of one hundred forty-four 8/9-year-old children from the general population[85]. Children in both age groups were divided into three different groups [85]. One group received a placebo juice containing no food colors, another group received a juice mix (Mix A) containing yellow #5, yellow #6, and sodium benzoate and the final group received a juice mix (Mix B) containing yellow #6, sodium benzoate, and red #40[85]. Sodium benzoate is a common preservative with allowable levels of lead up to 2 ppm[86]. Behavior checklists were administered to parents and teachers during the study[85]. The children in the 3-year-old group that received Mix A exhibited a significant adverse effect in behavior (P = 0.044) compared to the children in the placebo and Mix B groups[85]. The children in the 8/9-year-old group exhibited a significant adverse effect in behavior in both the Mix A (P = 0.023) and Mix B (P = 0.001) groups compared to the placebo group[85]. From their data analyses, McCann *et al*[85] determined the food colors or sodium benzoate or both exposures result in increased hyperactivity in the general population of children falling into the 3-year and 8/9-year-old age groups.

In a review of dietary impacts on children diagnosed with ASD, Peretti *et al*[20] found diet is a key factor in the worsening of symptoms and is a modifiable risk factor in the treatment of ASD. Many parents of children with ASD have placed their children on a gluten and casein free diet without realizing the gluten free foods are highly processed and may lead to the accumulation of Hg, Pb, and cadmium in their children's blood over time. Raehsler *et al*[87] analyzed data collected by the United States CDC from the National Health and Nutrition Examination Survey to determine whether a gluten free diet was associated with increases in heavy metal accumulation. The data was collected from adult participants 18 years of age and older between 2009 and 2012[87]. The United States CDC analyzed participant whole blood samples for heavy metals using the ICP-MS method[87]. Raehsler *et al*[87] used univariate statis-

tical analysis to compare the dietary intake of participants following a gluten free diet with those not following a gluten free diet. After controlling for age, smoking, sex, race, and fish and shellfish consumption, blood Hg levels were significantly higher (P = 0.04) in people following a gluten free diet compared to those who did not[87]. Raehsler *et al*[87] found blood Pb levels were statistically significantly higher (P = 0.001) in women following a gluten free diet but not in men.

In a review of studies conducted to determine the effectiveness of placing children with autism on a gluten free and casein free (GFCF) diet, Piwowarczyk *et al*[88] found there is little evidence that such a diet is beneficial in reducing symptoms. Parents of children with autism that suffer from intestinal abnormalities need more information as to whether gluten intolerance or sensitivity is the cause of their child's symptoms and should thus consult a physician before placing their child on a GFCF diet. Organophosphate pesticide (OP) exposures from grain consumption may also create gastrointestinal disturbance and other symptoms observed with gluten intolerance[83]. Since children with autism lack the bioavailability and catalytic activity of the PON1 gene [89] and PON1 activity is significantly decreased in children with ADHD[90,91], they are more susceptible to the toxicity and resulting symptoms associated with organophosphate pesticide exposures. More research is needed to determine if the consumption of gluten-free food products contribute to heavy metal or OP pesticide exposures in children with autism and ADHD and if their consumption may be safely included in a healthy diet.

HEALTHY DIET INCLUDES WHOLE FOODS AND MAY INCLUDE SUPPLEMENTS WHEN WARRANTED

One recent promising study led by Adams incorporated aspects of a (GFCF) diet in a supplement intervention with promising results[92]. In a randomized, controlled trial design, thirty-seven children and adults with ASD diagnoses participated in the treatment group which received the intervention, thirty children and adults with ASD served in the non-treatment group, while 50 neurotypical people of similar age and gender served as controls[92]. The treatment intervention involved a 12-mo regime which included supplementation with a variety of vitamins and minerals beginning on Day 0, with additional supplements added on Day 30, Day 90, and Day 180 until finally on Day 210 the Healthy Gluten Free Casein Free (HGFCF) diet was introduced to the protocol[92]. The researchers administered a variety of behavior checklists prior to beginning, and after completing, the 12-month treatment intervention[92]. The most significant reductions in symptoms of ASD (P = 0.0002) seemed to occur in the children with pervasive developmental delay (PDD). Biomarkers were also analyzed before and after the intervention period[92], presumably before Day 0 and at Day 365. The most significant biomarker findings for supplementation involved vitamin B2 (riboflavin), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), in which there were large and significant increases in the treatment group compared to the non-treatment group with corresponding P values: P = 0.00000001, P = 0.000000001, P = 0.000000001)[92]. The fish oil supplements introduced on Day 30 of the intervention could explain the significant increases in DHA and EPA levels. The healthy diet component of the intervention included adequate intake of leafy greens, whole fruit, and protein while eliminating food colors and preservatives and reducing junk foods [92]. The diet also included the adoption of gluten free, casein-free, and soy-free foods [92]. Heavy metal biomarkers were not collected and analyzed by the research team [92]. Selenium (Se) supplementation was introduced on Day 0 of the treatment protocol; there was a significant increase in Se levels (P = 0.001) in the treatment group compared to the non-treatment group[92] while other mineral supplements made no difference. Se binds to Hg in a 1:1 ratio[8]. The supplementation in the treatment group could have provided enough excess Se to mitigate any Hg exposures occurring from the consumption of highly processed gluten-free, casein-free foods. After analyzing their data, Adams *et al*[92] concluded a comprehensive nutritional and dietary intervention is effective in reducing symptoms in most individuals with ASD. Although the results of the Adams *et al*[92] study are encouraging, the impact of eating a whole food healthy diet and/or supplements on reducing symptoms of autism remains unclear.

Supplementation in diet should be done cautiously because supplements have the potential to be toxic and are poorly regulated with respect to their efficacy and composition[93]. Fish oil supplementation may be warranted in children who refuse to eat salmon or other low mercury fish and shellfish. DHA and EPA are important for

healthy brain development and function. The 2015-2020 Dietary Guidelines for Americans recommend eating low Hg fish as part of a healthy dietary pattern due to their DHA and EPA content[94]. In addition, fish provide more vitamin B-12 and vitamin D than many other foods[94]. Se and Hg tend to bind in fish tissue. When Se is present in excess of Hg in fish, there is no public health concern about Hg exposure from eating fish[95,96].

Se is a key mineral in reducing oxidative stress in children with ASD and ADHD who may have elevated blood Hg levels. Se supplementation could be avoided by the consumption of Brazil nuts in moderation by eating one Brazil nut each day or, if children are amenable, drinking a daily fruit/vegetable smoothie that contains one Brazil nut per child. Brazil nuts are known to be the richest source of Se and provide magnesium and Zn which are important micronutrients in preventing the adverse neurodevelopmental outcomes presented in Figure 1 that result from eating the western diet[8,10]. Cardoso *et al*[97] conducted a review of clinical trials using Brazil nuts as an alternative to selenium supplementation. Although Cardoso *et al*[97] did not focus their review specifically on children with developmental disorders, they found many positive outcomes such as improvements in lipid serum profiles, anti-inflammatory response, and cognitive impairment in the various trial participants. Se measurements in blood should be a part of any protocol used in future healthy diet intervention studies conducted to reduce symptoms of ASD or ADHD.

FUTURE HEALTHY DIET INTERVENTION STUDIES

The results of this review indicate there is a need for future intervention studies to conclusively connect I-Hg and Pb measurements in blood to dietary sources of exposure in children with ASD and ADHD. Khan *et al*[98] confirmed dietary heavy metal intake correlates significantly with the heavy metal levels in the blood of children and adults. Dietary exposures to Hg and Pb can be measured through blood analyses using the methods discussed in the previous sections with careful consideration as to which component of blood (*e.g.*, RBCs, Plasma, Whole Blood) is best for measuring elements of interest in children diagnosed with ASD or ADHD. In children with ASD, Alibadi *et al*[69] measured levels of I-Hg and Pb in the RBCs. Adams *et al*[92] measured Se levels in the RBCs of the children with ASD during their diet intervention study. In the case of autism, RBCs may be the best biomarker for measuring Hg, Pb and Se levels. In the case of ADHD, Pb levels are generally measured in whole blood rather than plasma or serum. However, because Pb is found within the blood cells, consideration should be given to testing Pb, along with Se, levels in the RBCs of children diagnosed with ADHD. In addition to mitigating Hg exposure, blood Se levels are important to measure in both ASD and ADHD because PON1 gene expression may be regulated by dietary Se status[99]. As can be seen in Figure 1, increasing PON1 gene activity may be key to reducing the oxidative stress in the brain that impacts a child's ability to learn.

In addition to collecting and measuring biomarker levels in future diet intervention studies, it is important to collect information on processed food consumption so that the link between Hg/Pb exposures and diet can be established definitively. Processed food consumption data can be gathered using a diet survey that focuses on pre- and post-intervention eating patterns. Dufault *et al*[26] developed such a survey for use in determining the link between processed food consumption and blood I-Hg levels. Supplementary Tables 2 accompanying this manuscript provides a list of the survey questions in checklist format along with scoring instructions. The questions were also used during a clinical trial to educate parents of children with ASD and ADHD on the benefits of healthy diet[100]. In administering the survey pre and post-intervention, diet scores can be compared to determine changes in processed food consumption patterns.

HEALTHY DIET CONSIDERATIONS FOR CHILDREN WITH AUTISM AND ADHD

In devising a healthy diet for children with autism and ADHD it is important to consider elements needed to boost PON1 gene activity. Xotlanihua-Gervacio *et al*[101] determined the dietary factors that promote increased PON1 gene activity include selenium, cholesterol, and overall lipid intake. The majority of PON1 activity occurs on

the high-density lipoprotein (HDL) in humans[102]. HDL is known as "good cholesterol" and its promotion can be accomplished by the adoption of a healthy Mediterranean type of diet which is characterized by the consumption of fruits, vegetables, whole grains, fish and shellfish, nuts, olive oil, and poultry. In the case of children with autism or ADHD, this literature review indicates a healthy type organic (pesticide free) Mediterranean diet includes leafy greens (*e.g.*, spinach, kale, collards), nuts (*e.g.*, Brazil), seeds, legumes, whole grains, fish (*e.g.*, salmon) and shellfish, and excludes the consumption of conventionally grown vegetables and fruits with allowable pesticide residues, products containing ingredients with allowable mercury and lead residues (*e.g.*, food colors, preservatives), hydrogenated fats, sugar sweetened beverages (*e.g.*, corn sweeteners), most vegetable oils, processed meats, and refined grains.

CONCLUSION

This review has resulted in the novel finding of the role dietary selenium may play in supporting PON1 activity in children with ASD or ADHD. Unhealthy diet resulting in the bioaccumulation of Hg or Pb may jeopardize the body's ability to regulate the expression of PON1 resulting in decreased or impaired PON1 activity. An updated Mercury and Lead Toxicity Model for ASD and ADHD is presented to assist clinicians in diagnosing and treating the symptoms associated with ASD and ADHD. The model can also be used as a guide in the design of future intervention studies to determine the role of dietary factors in creating conditions for the development of autism and ADHD.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 July 20; 11(4): 160-186

DOI: 10.5662/wjm.v11.i4.160

ISSN 2222-0682 (online)

REVIEW

Fascinating history of groin hernias: Comprehensive recognition of anatomy, classic considerations for herniorrhaphy, and current controversies in hernioplasty

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Author contributions: Hori T and Yasukawa D wrote this review; historical turning points was investigated by Hori T; Yasukawa D originally drew all illustrations and schemas; Hori T and Yasukawa D assessed important papers; Hori T supervised this review; Hori T and Yasukawa D contributed equally to this work.

Conflict-of-interest statement: None of the authors has a potential conflict of interest.

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Abstract

Groin hernias include indirect inguinal, direct inguinal, femoral, obturator, and supravesical hernias. Here, we summarize historical turning points, anatomical recognition and surgical repairs. Groin hernias have a fascinating history in the fields of anatomy and surgery. The concept of tension-free repair is generally accepted among clinicians. Surgical repair with mesh is categorized as hernioplasty, while classic repair without mesh is considered herniorrhaphy. Although various surgical approaches have been developed, the surgical technique should be carefully chosen for each patient. Regarding as interesting history, crucial anatomy and important surgeries in the field of groin hernia, we here summarized them in detail, respectively. Points of debate are also reviewed; important points are shown using illustrations and schemas. We hope this systematic review is surgical guide for general surgeons including residents. Both a skillful technique and anatomical knowledge are indispensable for successful hernia surgery in the groin.

Key Words: Inguinal hernia; Groin; History; Anatomy; Hernioplasty; Herniorrhaphy

Core Tip: Groin hernias include indirect inguinal, direct inguinal, femoral, obturator, and supravesical hernias. Groin hernias have a fascinating history in the fields of anatomy and surgery, and the concept of tension-free repair is generally accepted

Manuscript source: Unsolicited manuscript

Specialty type: Surgery

Country/Territory of origin: Japan

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 13, 2021
Peer-review started: March 13, 2021
First decision: March 31, 2021
Revised: April 2, 2021
Accepted: May 15, 2021
Article in press: May 15, 2021
Published online: July 20, 2021

P-Reviewer: Eaupanitcharoen S, Mannelli L
S-Editor: Gong ZM
L-Editor: A
P-Editor: Yuan YY

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Citation: Hori T, Yasukawa D. Fascinating history of groin hernias: Comprehensive recognition of anatomy, classic considerations for herniorrhaphy, and current controversies in hernioplasty. *World J Methodol* 2021; 11(4): 160-186
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/160.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.160

INTRODUCTION

The etymology of the term "hernia" originates from the Latin word for "prolapse," and the earliest evidence of an inguinal hernia was recorded in approximately 1552 BC in ancient Egypt[1,2]. In the early 1950s, the term "groin hernia" was first used by Henri Fruchaud (1894–1960)[3]. Hernias in the groin include indirect inguinal, direct inguinal, femoral, obturator, and supravesical hernias[4]. Herniorrhaphy has been performed to treat inguinal and femoral hernias since the 18th century. Edoardo Bassini (1844–1924) established a modern herniorrhaphy technique[5]; thereafter, groin herniorrhaphy became the most common technique performed in the field of general surgery[4]. Recent studies have shown that approximately 750000 patients undergo this procedure yearly in the United States[4,6]; the direct annual cost is 2.5 billion dollars[4].

In 1804, Astley Cooper (1768–1841) stated, "No disease of the human body, belonging to the province of the surgeon, requires in its treatment a greater combination of accurate anatomical knowledge with surgical skill than hernia in all its varieties."[7] Notably, herniorrhaphy treatment of pediatric patients is useful for accurate evaluation of the skills of general surgeons and residents[8]. Sir William Heneage Ogilvie (1887–1971) once stated, "I know more than a hundred surgeons whom I would cheerfully allow to remove my gallbladder but only one to whom I should like to expose my inguinal canal."[9] Notably, both technical skill and anatomical recognition are crucial for safe and reliable surgery. Irving L. Lichtenstein (1920–2000) established the concept of tension-free repair (TFR) in 1986[10,11]. Various meshes, including biological mesh, are currently available for groin surgery[12]. Surgical repair with mesh is categorized as hernioplasty, while classic repairs without mesh are termed herniorrhaphy. Many physicians focus on the preperitoneal (posterior) space (PPS)[3,7,13-21], topographic nerves[22-31], and regional vessels[4,32-34].

This review discusses existing knowledge regarding groin hernia. It summarizes historical turning points in the anatomy and surgery of groin hernias, described the current status of anatomical recognition and surgical repair, examines points of contention, and considers future perspectives. Despite the current global pandemic due to Chinese Wuhan pneumonia (so-called COVID 2019), general surgeons including residents thrive. We hope that this review including milestones of history, anatomy, and surgery will be informative for surgeons involved in the treatment of groin hernias.

ETIOLOGY

Inguinal hernias constitute 75% of abdominal wall hernias and have the lifetime risks of inguinal hernias are 27% in men and boys and 3% in women and girls[35]. Indirect (external or lateral) inguinal hernias (IIHs) outnumber direct (internal or inner) inguinal hernias (DIHs) by a ratio of approximately 2:1[35]. Suspected congenital causes for persistent patency of processus vaginalis include cryptorchidism, lack of carbachol response, absence of cholinergic receptors, absence of myofibroblasts, absence of apoptotic nuclei, failed apoptosis of smooth muscle, catecholaminergic

activity related to luteinizing and gonadotropin-releasing hormones, and deficiencies involving epithelial–mesenchymal transition[36-42]. Possible risk factors for groin hernias include elevated abdominal pressure (*e.g.*, by constipation, coughing, and obesity)[6], diabetes[6], smoking[6], collagen distribution[43,44], peritoneal dialysis [45], radical prostatectomy[46], and family history[47].

SYMPTOMS AND EVALUATION

Groin hernias are extremely common and can often be diagnosed by simple anamnesis collection and a physical examination[48]. Surgical repair is elective unless incarceration or strangulation is present[48]. Palpation of both testes in the scrotal sac should be performed during the clinical examination to rule out cryptorchid testis[49]. Local pain in the scrotal sac, which may indicate testicular torsion, should never be overlooked. From an immunological perspective, testicular torsion requires urgent surgery[50]. An undescended testicle harbors an increased risk of infertility and malignancy; thus, it requires orchiopexy in early childhood[48]. The development of autoimmune antisperm antibody induced by testicular torsion influences testicular function and subsequently causes male sterility[50].

The inguinal canal (IC) is traversed by the spermatic cord (SC) in men and boys, and by the round ligament (RL) of the uterus in women and girls. The RL is attached to the uterus and is accompanied by a pouch of parietal peritoneum in the IC, known as the canal of Nuck[51]. A hydrocele of the canal of Nuck is a differential diagnosis for groin hernia[51]. Hydroceles of the SC and testis are also differential diagnoses for groin hernia; deliberate surgery is required for SC hydroceles[52]. Hydroceles in infancy may resolve without surgery[48].

The bladder may be involved in groin hernias[53,54]; thus, injury to the urinary tract should be avoided. Notably, immature infants at birth may easily develop bladder prolapse[55]; therefore, the body weight at birth should be checked before surgery to avoid serious iatrogenic injuries[56].

In 1559, Caspar Stromayr (1530–1567) classified inguinal hernias as either IIH or DIH[57]. Inguinal hernias are currently classified as indirect or direct and primary or recurrent[6]. Some classifications are based on anatomical findings in relation to the development of the hernia (*e.g.*, posterior floor integrity, enlarged inguinal rings, and hernia size)[58,59]. The severity of the groin hernia may be difficult to determine prior to surgery; size alone may not be associated with severity in some patients[6]. However, the size of the groin hernia affects the choice of the surgical approach because surgeries performed under local or conduction anesthesia are contraindicated for huge hernias[59]. The European Hernia Society has proposes a simple classification (based on Aachen's classification[60]), which is used worldwide[61,62]. Briefly, this classification is based on orifice size and anatomical localization[60,61]. The size of the hernia orifice is recorded as 1 (≤ 1 finger), 2 (1-2 fingers), or 3 (≥ 3 fingers)[60]. Thus, a hernia orifice of 2.5 cm is considered a size 2 hernia[60]. For anatomical localization, the criteria are lateral (L), medial (M), and femoral (F)[60,61]. In addition, hernias are regarded as primary (P) or recurrent (R)[60,61].

HISTORY

Groin hernias have an interesting anatomical and surgical history[1,11,63-65] (Table 1). Pierre Franco (1500–1561) and Ambroise Paré (1510–1590) used conservative treatments with a strong bandage[1,66]. Specific anatomical structures in the IC were clarified in the 18th century[1]; the first report of successful transabdominal repair was described in 1716 by Demetrius Cantemir (1673–1723)[1]. Lorenz Heister (1683–1758) first reported successful bowel resection *via* laparotomy for a strangulated hernia[67].

Franz Kaspar Hesselbach (1759–1816) was the first to describe various anatomical structures [*e.g.*, cribriform fascia (Hesselbach's fascia) and interfoveolar ligament (Hesselbach's ligament)]. Hesselbach also defined the inguinal triangle (*i.e.*, "Hesselbach's triangle"), which is superolaterally bounded by the inguinal ligament (IL), the exterior border of the abdominal rectus muscle, and the inferior epigastric vessels[68,69].

Henry O. Marcy (1837–1924) stated that failure to close the internal (deep) inguinal ring (IIR), or low ligation of the hernia sac, could result in recurrence; he described an accurate reconstruction technique in 1871[70]. Marcy's repair was the first implementation of high ligation of the sac and closure of the IIR[70].

Table 1 History of anatomy and surgery for groin hernia

Name	Year	Remarks
G. Falloppio (1523-1562)		Importance of the IL (Etiology)
F. Poupart (1661- 1709)		Poupart's ligament (*i.e.*, the inguinal ligament)
P. Camper (1722-1789)		Camper's fascia (Anatomy)
A. Scarpa (1752-1832)		Scarpa's fascia (Anatomy)
D. Cantemir (1673-1723)	1716	Successful surgery (*via* a laparotomy)
L. Heister (1683-1758)		Bowel resection for strangulated hernia
P. Roland Arnaud de Ronsil	1724	Obturator hernia
C. Amyand (1660-1749)	1735	Amyand's hernia
AG. Richter (1742-1812)	1777	Strangulated hernia
AP. Cooper (1768-1841)		Cooper's ligament (Anatomy)
HO. Marcy (1837-1924)	1806	Marcy's repair (Anterior approach)
FK. Hesselbach (1759-1816)	1871	Hesselbach's triangle (Anatomy)
WJ. Mitchell Banks (1842–1904)	1882	Simple high ligation in infants and children
E. Basssini (1844-1924)	1887	Bassini's repair (Anterior approach)
WS. Halsted (1852-1922)		Modified Bassini's repair
EW. Andrews (1824-1904)		Modified Bassini's repair
L. Tait (1845-1899)	1891	Transabdominal approach
J. Lucas Championniere (1843-1913)	1892	Simple high ligation in infants and children
G. La Roque (1876-1934)	1919	Transabdominal approach
GL. Cheatle (1865-1951)	1920	TEP approach
RH. Russel (1860-1933)	1925	Sac removal in infants and children
A. Henry (1886-1962)	1936	Transabdominal approach
CB. McVay (1911–1987)	1939	McVay's repair (Anterior approach)
BJ. Anson (1894-1874)		Importance of the TF
WJ. Potts (1895-1968)	1945	Potts' method in infants and children
EE. Shouldice (1981-1965)	1953	Shouldice's repair (Anterior approach)
H. Fruchaud (1894-1960)	1956	The PPS (Anatomy)
CE. Koop (1916-2013)	1957	Koop's fixation
FC. Usher (1908-1980)	1958	Monofilament polypropylene mesh (Anterior approach)
LM. Nyhus (1923-2008)	1959	IPT repair (Preperitoneal approach)
J. Rives (1873-1985)	1965	Mesh placement in the PPS (Preperitoneal approach)
RE. Stoppa (1921-2006)	1969	Prosthetic reinforcement in the PPS (Preperitoneal approach)
P. Fletcher	1979	Laparoscope use (Laparoscopic approach)
R. Gel	1982	Laparoscopic repair (Laparoscopic approach)
IL. Lichtenstein (1920-2000)	1986	Mesh plug (Anterior approach)
		The concept of TFR
S. Bogojavalensky	1989	Laparoscopic repair with mesh plug (Laparoscopic approach)
L. Schultz	1990	The first series of laparoscopic repair (Laparoscopic approach)
JL. Dulucq	1991	Mesh placement in the PPS (Endoscopic approach)
FK. Toy and RT. Smoot, Jr.	1991	Intraperitoneal onlay mesh repair (Laparoscopic approach)
RJ. Fitzgibbons, Jr.	1991	Intraperitoneal onlay mesh repair (Laparoscopic approach)

AT. Spaw and LP. Spaw	1991	Triangle of doom (Anatomy)
AI. Gilbert	1992	Sutureless technique (Anterior approach)
ME. Arregui	1992	TAPP repair (Laparoscopic approach)
GS. Ferzli	1992	TEP repair (Endoscopic approach)
JM. Himpens	1992	TEP repair (Endoscopic approach)
JB. McKernan and HL. Laws	1993	TEP repair (Endoscopic approach)
EH. Phillips	1993	TEP repair (Endoscopic approach)
R. Annibali, TH. Quinn and RJ. Fitzgibbons Jr.	1993	Triangle of pain (Anatomy)

IL: Inguinal ligament; IPT: Iliopubic tract; PPS: Preperitoneal (posterior) space; TAPP: Transabdominal preperitoneal; TEP: Totally extraperitoneal; TF: Transversalis fascia; TFR: Tension-free repair.

Bassini[5] elucidated the anatomy of the anterior IC in 1884, then ushered safe and effective surgery into the modern era by describing Bassini's repair in 1887[5,71]. Notably, Bassini advocated the importance of Marcy's theory and emphasized floor reconstruction involving approximation of the internal abdominal oblique muscle (IAOM) and transverse abdominal muscle with the transversalis fascia (TF), combined with a shelving edge of the IL[5,71].

William Stewart Halsted (1852–1922) and Edmund W. Andrews (1824–1904) modified Bassini's repair[72-74]. The antiseptic concept was accelerated in the 19th century after Joseph Lister (1827–1912) introduced antisepsis[72]; additionally, Halsted was the first to use surgical gloves for aseptic technique during surgery[75]. Modified Bassini's repair (*i.e.*, "North American Bassini" repair) was implemented worldwide, although its use was associated with a higher recurrence rate[76]. Subsequently, Arthur Keith (1866–1955) described a shutter mechanism in 1923[13].

Chester Bidwell McVay (1911–1987) first employed Cooper's ligament for repair in 1939[77]; McVay's method involved repair of a femoral hernia through the posterior wall of the IC[78]. McVay and Barry J. Anson (1894–1874) focused on the importance of the TF[79]. Initially, the arch of the transverse abdominal muscle was approximated to Cooper's ligament, the iliopubic tract (IPT), and the IL. Subsequently, a relaxing incision was placed in the anterior rectus sheath.

In 1919, George La Roque (1876–1934) utilized abdominal and cutaneous incisions, then ligated the retracted hernia sac from the abdominal cavity[1]. In 1936, Arnold Henry (1886–1962) devised an analogous approach by means of a lower abdominal midline incision. In 1920, a totally extraperitoneal approach was first executed by George Lenthal Cheatle (1865–1951) as a radical curative operation for both inguinal and femoral hernias using a lower mid-abdominal preperitoneal approach[80].

Lloyd M Nyhus (1923–2008) introduced IPT repair in 1959[15], in accordance with the concept of the preperitoneal (posterior) approach[81]. The anterior rectus sheath was divided, and the abdominal rectus muscle was retracted medially; the TF was then exposed and the PPS was accessed. The IIH sac was ligated, and the defect was closed by approximating the conjoint tendon to the IPT and IL. Thereafter, based on the concept of the preperitoneal approach, prosthetic reinforcement in the PPS was first described by Jean Rives (1873–1985) in 1965 for unilateral hernias[16] and by René Stoppa (1921–2006) in 1969 for bilateral inguinal hernias[82].

Edward Earle Shouldice (1890–1965) introduced a multiple layer repair of the posterior inguinal wall in 1953[14]. The TF was divided from the IIR to the pubic tubercle and lifted from the peritoneum. Shouldice's repair proposed an imbrication of the TF and strengthening of the posterior wall of the IC by using four layers of the fascia and aponeuroses of the IAOM. Henri Fruchaud clarified the preperitoneal anatomy in the early 1950s[3]; preperitoneal and laparoscopic approaches were thereafter realized based on his work.

Jean Rives placed mesh into the PPS in 1965[16], and René Stoppa used a large Dacron prosthesis to reinforce the TF[83]. In 1986, Irving L. Lichtenstein first introduced a mesh plug made by rolling a piece of flat polypropylene into the shape of a cigarette to fill a femoral defect[10,11]. The mesh was fixated with interrupted sutures; this "cigarette plug" was used to repair inguinal, femoral, and recurrent hernias. Hence, Lichtenstein established a TFR technique that ushered a new surgical era[10,11]. Classic hernia repairs used sutures under tension, which led to a high recurrence rate; TFR dramatically improved the rates of recurrence and infection[84]. Prosthetic

mesh was used to reinforce the TF; specifically, polypropylene mesh was superiorly fixed to the IL, lateral edge of the rectus sheath, and conjoint tendon. Francis Usher (1908–1980) introduced the use of monofilament polypropylene mesh in 1958[85,86] and parietalized the SC[87]. Thereafter, Arthur I Gilbert (1913–2001) improved TFR with a sutureless technique[88,89]. Lichtenstein was first to coin the term "tension-free hernioplasty"[90]; the concept of TFR revolutionized hernia surgery[11,63]. The advantageous simplicity of "mesh-plug hernioplasty" was first described in 1993[91]; thereafter, this procedure became preferable and spread worldwide[92,93].

Although Lichtenstein's repair produced excellent results, George E Wantz (1923–2000) warned that polypropylene mesh did not cover the entire myopectineal orifice (MO) and that Lichtenstein's repair was therefore inadequate to prevent a femoral hernia[94]. Notably, he also stated that incomplete coverage of the MO by the mesh could predispose patients to subsequent DIH[94].

WALL LAYERS

Petrus Camper (1722–1789), Antonio Scarpa (1752–1832), and Franz Kaspar Hesselbach provided detailed descriptions of inguinal structures, particularly of important ligaments[1]. Following elaborate anatomical studies, François Poupart (1661–1709) in 1695 recognized the importance in hernia pathology of the IL[1], which had been described previously by Gabriele Falloppio (1523–1562)[1]. Astley P. Cooper published original anatomical views regarding the IC in 1804 and 1807[1]. Part of the aponeurosis of the external abdominal oblique muscle composes the IL, although the IAOM is observed under the aponeurosis of the external abdominal oblique muscle at the groin. The IL is also known as the Fallopian ligament or Poupart's ligament.

The abdominal wall at the groin is classically considered to have nine layers[95]: skin, subcutaneous fat, superficial fasciae (Camper's and Scarpa's fasciae), innominate (unnamed or untitled) fascia, IL, IAOM, TF, PPS [superficial parietal layer (SPL) and deeper visceral layer (DVL)], and peritoneum. This classification involves the innominate (unnamed or untitled) fascia, which is a thin membrane on the IL, and does not indicate division of the PPS into the SPL (*i.e.*, anterior subperitoneal fascia) and DVL (*i.e.*, posterior subperitoneal fascia) (Figure 1). The PPS comprises preperitoneal fat.

The IC is a passage in the anterior abdominal wall that conveys the SC in men and boys, whereas it conveys the RL in women and girls. The IL is bordered by the aponeurosis of the external abdominal oblique muscle anteriorly, the IAOM and transverse abdominal muscle superiorly, the IL and lacunar ligaments inferiorly, and the TF posteriorly.

PREPERITONEAL (POSTERIOR) SPACE AND MYOPECTINEAL ORIFICE

In the early 1950s, Henri Fruchaud reported that all hernias at the groin result from a defect of the TF and pass through the myopectineal orifice (MO) (all three triangles of the groin)[3]. The oval-shaped MO is the origin of all groin hernias[4] (Figure 2). In 1965, Jean Rives proposed reinforcement of the TF with a prosthesis placed in the preperitoneal (posterior) space (PPS), if local structures are weak[16]. Although an "on-lay patch," (placed on the anterior side of the TF) was historically used, the importance of an "under-lay (in-lay) patch" placed between the TF and peritoneum is now widely accepted. Hence, general surgeons commonly recognize the concept of optimal repair at the PPS. The PPS is observed between the TF and peritoneum; adequate creation of an extended PPS is important for optimal surgery[64,96]. Several physicians [Astley P. Cooper in 1807[7], William James Lytle (1896–1986) in 1945[13], Edward Earle Shouldice in 1953[14], Henri Fruchaud in 1956[3], Lloyd M. Nyhus in 1959[15], Jean Rives in 1965[16], R. Fowler Jr. in 1975[17], René Stoppa in 1977[18,19], Raymond C. Read in 1992[20], and Maurice E. Arregui in 1997[21]] historically made mention of this important space[97] (Table 2). In particular, anatomical recognition of the SPL and DVL was a milestone for further developments of surgical repair techniques[97]. The anterior subperitoneal fascia is recognized as the SPL, while the posterior subperitoneal fascia is recognized as the DVL[29] (Figure 3).

Anatomical recognition of the MO is also crucial for reliable treatment[98,99], and laparoscopic exploration easily reveals this orifice[4]. Full coverage of the MO is considered optimal surgery[98-100]. This orifice should be fully reinforced in a TFR manner to prevent IIHs, DIHs, femoral hernias, and obturator hernias[98-102]. Mesh implantation into an extended cavity of the PPS is currently considered optimal

Table 2 Preperitoneal (posterior) space and myopectineal orifice

Name	Year	SPL (Posterior sub-peritoneal fascia)	DVL (Anterior sub-peritoneal fascia)
AP. Cooper	1807	TF-inner portion	-
WJ. Lytle	1945	Preperitoneal fascia	-
EE. Shouldice	1953	Preperitoneal fascia	-
H. Fruchaud	1956	PPS at the MO	-
LM. Nyhus	1959	PPS	-
J. Rives	1965	Inguinal PPS	-
R. Fowler	1975	Preperitoneal fascia-membranous layer	Preperitoneal fascia-areolar layer
RE. Stoppa	1977	Urogenital fascia	Urogenital fascia
		Umbilico-prevesical fascia	Umbilico-prevesical fascia
		Spermatic sheath	Spermatic sheath
RC. Read	1992	TF-posterior lamina	-
ME. Arregui	1997	Attenuated rectus sheath	Umbilical prevesicular fascia
		TF-posterior lamina	Preperitoneal fascia

DVL: Deeper visceral layer; MO: Myopectineal orifice; PPS: Preperitoneal (posterior) space; SPL: Superficial parietal layer; TF: Transversalis fascia.

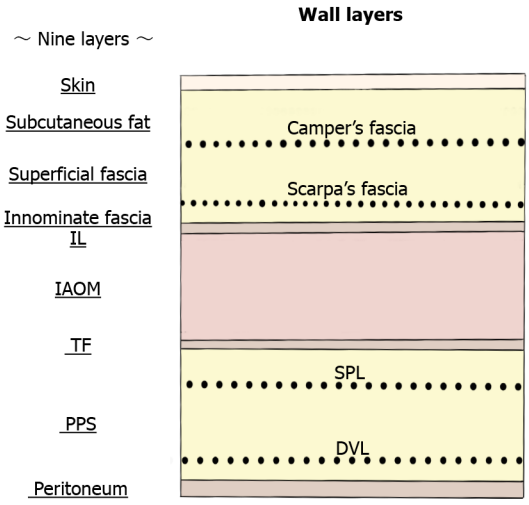

Figure 1 Wall layers. The abdominal wall at the groin contains the following components: skin, subcutaneous fat, superficial fasciae (Camper's and Scarpa's fasciae), innominate (unnamed or untitled) fascia, IL, IAOM, TF, PPS [SPL (anterior subperitoneal fascia) and DVL (posterior subperitoneal fascia)], and peritoneum. DVL: Deeper visceral layer; IAOM: Internal abdominal oblique muscle; IL: Inguinal ligament; PPS: Preperitoneal space; SPL: Superficial parietal layer; TF: Transversalis fascia.

surgery[98-102].

Some physicians have performed detailed investigations of the layer in which the bladder exists[19,21,103] (Figure 4). The terms "spermatic sheath" and "urogenital fascia" established by René Stoppa[19] and "umbilical prevesical fascia" established by Maurice E. Arregui[21] have become widespread.

PEDIATRIC HERNIORRHAPHY

Many pediatric surgeons focus on herniorrhaphy in infants and children[104-107]. Notably, a contralateral hernia develops after unilateral surgery in 8% to 11% of patients[108,109]. The low incidence of contralateral hernia after unilateral herniorrhaphy does not justify routine contralateral groin exploration[108,109].

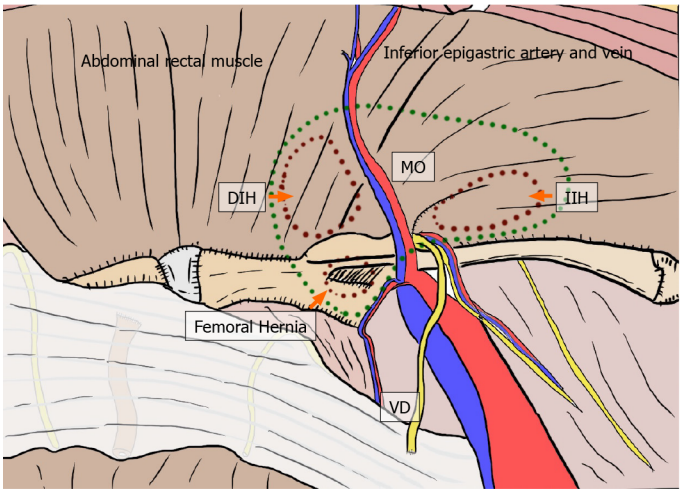

Figure 2 Myopectineal orifice. The oval-shaped myopectineal orifice (green dotted circle) is the origin of all groin hernias (brown dotted circles). DIH: Direct inguinal hernia; IIH: Indirect inguinal hernia; MO: Myopectineal orifice; VD: Vas deferens.

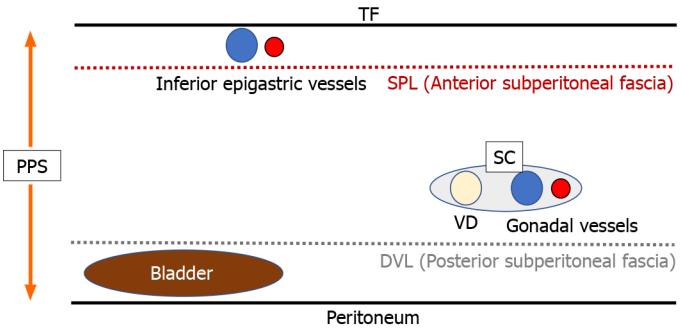

Figure 3 Preperitoneal space. DVL: Deeper visceral layer; PPS: Preperitoneal space; SC: Spermatic cord; SPL: Superficial parietal layer; TF: Transversalis fascia; VD: Vas deferens.

Just Lucas-Championnière (1843–1913) described a simple high ligation technique in 1892[110]. William Mitchell Banks (1842–1904) in 1882[111] and Willis J. Potts (1895–1968) in 1950[104,107] described high ligation by means of a partial incision of the IC without opening the external (superficial) inguinal ring. Alexander Hug Ferguson (1853–1912) added anterior wall repair in 1899[112].

P. Turner in 1912[113] and A. MacLennon in 1914[114] advised simple removal of the sac to the IIR through a very small incision. In 1925, Robert Hamilton Russell (1860–1933) strongly emphasized surgical removal of the sac solely in infants and children[115]. In 1938, Gertrude Marianne Amalia Herzfeld (1890–1981) used a small incision over the external inguinal ring, pulled down and ligated the sac, and closed the external inguinal ring with a single stitch[116]. In 1945, Jerome S. Coles (1911–1996) advised transfixation and ligation of the proximal end as high as possible[117].

Willis J. Potts performed a high ligation in 1945[104,107,118]; and Potts' method thereafter spread as the standard surgical technique worldwide. Charles Everett Koop (1916–2013) immobilized the edge of a high ligation by suturing the dorsal side of the IAOM. Koop's fixation prevented postoperative infertility due to uterine retroflexion and reduced postoperative recurrence[119]. The bilateral RLs are cut during herniorrhaphy for bilateral inguinal hernias; thus, Koop's fixation should be routinely performed for bilateral inguinal hernias in female patients[120].

In the field of pediatric herniorrhaphy, surgeons also recognize the importance of the PPS. Laparoscopy has been used to assist repair in the PPS[121,122]; and Takehara *et al*[123] first described laparoscopic percutaneous extraperitoneal closure in 2006. Laparoscopic percutaneous extraperitoneal closure is a simple technique that includes ligation around the IIR by means of a unique needle. This surgery avoids opening of the IC and involves minimal dissection around the testicular vessels and vas deferens (VD).

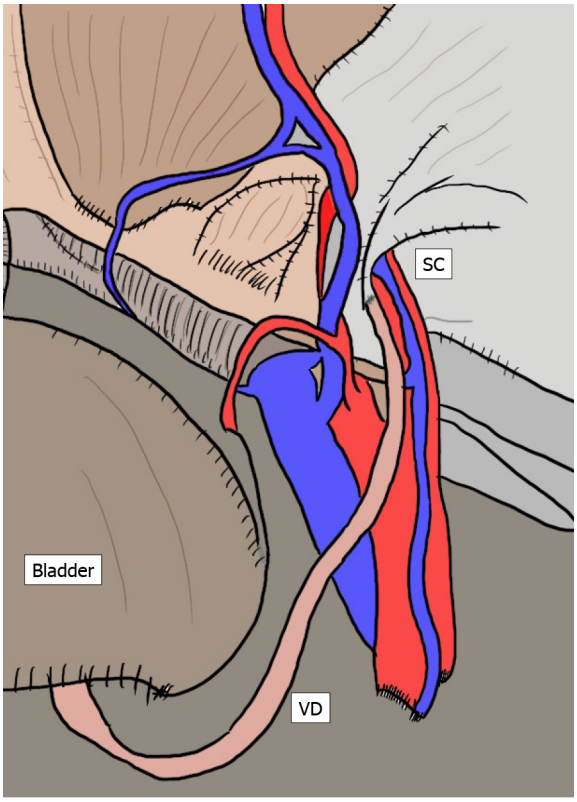

Figure 4 Vas deferens, spermatic cord and bladder. The vas deferens courses as the "preperitoneal loop" in the deeper visceral layer (DVL). The bladder exists in the DVL. DVL: Deeper visceral layer; PPS: Preperitoneal space; SC: Spermatic cord; SPL: Superficial parietal layer; TF: Transversalis fascia; VD: Vas deferens.

HERNIA SURGERY IN THE CURRENT ERA

Beginning in the latter half of the 20th century, prosthetic mesh was routinely used in accordance with the TFR concept; many surgeons recognized the importance of the PPS. Laparoscopy and endoscopy have therapeutic potential for hernia surgeries.

Laparoscopic transabdominal preperitoneal (TAPP) repair is based on the same principle (*i.e.*, therapeutic feasibility of the transperitoneal approach for groin hernia) as the technique published by Lawson Tait (1845–1899) in 1891[124]. P. Fletcher first employed a laparoscope to repair a groin hernia in 1979[64]. Subsequently, Ralph Ger in 1982[125], S. Bogojavalensky in 1989[126], Leonard Schultz in 1990[127], and Maurice E. Arregui in 1992 and 1993[128,129] reported their respective TAPP repair techniques.

Endoscopic totally extraperitoneal repair is based on the preperitoneal anatomy clarified by Henri Fruchaud in 1956[3]. Jean Louis Dulucq first reported mesh implantation into the PPS in 1991[130]; thereafter, George S. Ferzli in 1992[131], Jacques M. Himpens in 1992[132], and John Barry McKernan in 1993[133] performed this procedure. Edward H. Phillips was the first to use the term "totally extraperitoneal" in 1993 [134].

Laparoscopic intraperitoneal on-lay mesh repair does not involve groin dissection. Frederick K. Toy and Roy T. Smoot described this procedure in 1991[135], while Muhammed A. Memon *et al*. employed this procedure beginning in 1991[136].

Surgeons can now choose from among several approaches (*e.g.*, open *vs* laparoscopic/endoscopic and anterior *vs* preperitoneal), planes in which the mesh is placed (*e.g.*, layer in front of the TF *vs* the PPS), fixation devices (*e.g.*, suture, sutureless, tack, or glue), and prostheses (*e.g.*, soft *vs* hard meshes and sheeted *vs* three-dimensional meshes)[137].

TYPES OF MESHES AND TENSION-FREE HERNIOPLASTY

Many meshes are currently available[12]. Hernia repair with mesh is regarded as hernioplasty, while traditional repairs without mesh are regarded as herniorrhaphy.

The TFR concept by Irving L. Lichtenstein was a breakthrough idea[10,11,64], and the use of surgical mesh is superior to other techniques[138]. Mesh is inherently a foreign body;thus, postoperative removal may be required because of complications such as refractory infection[139].

In 1890, Theodor Billroth (1829–1894) used various prosthetic materials for hernia repair[140], although all failed due to infection, rejection, and recurrence[12]. Suitable materials for surgical procedures were seriously needed. The turning point in hernia surgery was the discovery of synthetic polymers (*e.g.*, nylon) by Wallace Hume Carothers (1896–1937) in 1935[11]. Francis Usher (1908–1980) introduced the use of monofilament polypropylene mesh in 1958[85,87]; he later compared nylon, orlon, Darcon, and teflon[141]. Monofilament polypropylene meshes (*e.g.*, Prolene; Ethicon, Inc., Cincinnati, OH USA) are available from many manufacturers in the current era. One of the first synthetic meshes used was Marlex, which comprised crystalline polypropylene and high-density polyethylene. Irving L. Lichtenstein first introduced a mesh plug that consisted of polypropylene mesh in 1986[10,11]. Polypropylene, polyester, and expanded polytetrafluoroethylene were initially used; thereafter, polyglactin 910, cellulose, polyvinylidene fluoride, poliglecaprone 25, omega 3, titanium, and collagen were employed as additional materials.

Current meshes are chemically and physically inert; they are also nontoxic, stable, and nonimmunogenic[12]. However, none are biologically inert, due to the mesh physiology[12]. The introduction of a foreign material into the body triggers a healing response characterized by one of three reactions: destruction, tolerance, or rejection [12]. All meshes have their own characteristics with respect to elasticity (tensile strength), pore size, weight (density), constitution, and material absorption[12].

Double-sided polypropylene mesh is designed as a bilayer polypropylene mesh with a connector between the layers; this mesh is fixated with fewer sutures than a monolayer polypropylene mesh. This system enables coverage of the MO and can repair IIHs, DIHs, and femoral hernias from an anterior approach. The on-lay patch covers the entire floor of the IC, while the under-lay patch is placed into the PPS. Robert Kugel placed a sutureless mesh in the PPS in 1999, and this mesh is known as the "Kugel hernia patch"[142]. This patch was later modified to a so-called "direct Kugel patch" for placement of the mesh by means of a minimal incision in the IL. Double-sided polypropylene mesh (*e.g.*, Prolene Hernia System; Ethicon, Inc.) and the direct Kugel patch have become widespread on a commercial basis. Furthermore, polypropylene mesh itself is currently employed in surgeries for other diseases[143].

Inguinal hernia repair is associated with a low incidence of complications that can be influenced by the type of mesh[144]. In terms of postoperative complication, lightweight and heavyweight meshes showed no differences regarding seromas, infections, erosion, and testicular atrophy[145-147]. Lightweight mesh may contribute to recurrence in patient with inguinal hernias[146], but has advantages in terms of chronic pain and foreign body sensations[145-147]. Moreover, partially absorbable synthetic mesh improves postoperative chronic pain, functional outcomes, and quality of life[148,149].

Surface modification methods and nanofiber-based technology are actively under exploration to retain material strength and biocompatibility[12]. Biological mesh has superior biocompatibility to the above-described meshes and does not trigger an inflammatory response from the body, although higher cost has hampered its widespread acceptance[12]. In patients who experience complications, biological meshes can be placed for temporary or permanent closure of defects after mesh removal due to chronic pain or infection[150,151].

Hence, mesh materials are currently well-developed, but each mesh should be used in the correct manner12 Many types of meshes are available, and surgeons should follow the manufacturers' instructions to avoid malfunctions[12]. Surgeons must also ensure that their knowledge is regularly updated regarding mesh applications[12].

RECURRENT HERNIA

Postoperative recurrence is a critical issue[47,152]. The reasons for inguinal hernia recurrence are most likely multifactorial and include both technical and nontechnical patient-related risk factors[47]. In one study, the overall reoperation rate was reportedly 3.8%[47], while the reoperation rates for IIH and DIH were 2.7% and 5.2%, respectively[47]. Notably, the right side has a higher recurrence rate than the left side [4,47,152].

Iatrogenic recurrence caused by lack of anatomical knowledge and inappropriate techniques should be avoided[47]. All surgeons, including trainees and residents, should ensure professional technique in clinical practice to reduce the risk of recurrence. Nontechnical patient-related factors that influence the risk of recurrence after surgery have not been studied in detail[47]. Female sex, DIH at the time of initial surgery, surgical treatment of a recurrent inguinal hernia, and smoking are considered risk factors for postoperative recurrence[47]. A significant relationship between the type of hernia at the time of initial surgery and reoperation has been identified with respect to hernia recurrence[47].

Surgical repair is generally indicated[58,59], and a laparoscopic approach is strongly recommended for surgical repair of recurrent hernia[59].

OBTURATOR HERNIA

Obturator hernias are internal herniations through the obturator foramen, bordered by the obturator vessels and nerve. This type of hernia was first described by Pierre Roland Arnaud de Ronsil in 1724[153], although a patient had been described by Le Maire in 1718[153]. Notably, the Howship-Romberg sign [named after John Howship (1781–1841) and Moritz Heinrich Romberg (1795–1873)] is associated with obturator hernia[154]; however, this rare hernia generally exhibits nonspecific signs and symptoms[155]. Hence, the usefulness of computed tomography for diagnosis was suggested in 1983[156]. Actual image findings are shown in Figure 5A.

Obturator hernia should be considered a bilateral disease[157]; thus, unilateral repair may be inadequate[158,159]. The normal diameter of the obturator foramen is approximately 1.0 cm[160]. Hence, the bilateral obturator foramina should be routinely checked during surgery[157-159]; bilateral repair is required if even only a subtle dilation of the contralateral obturator foramen is observed during surgery[157-159].

SPECIAL SITUATIONS

Lorenz Heister was the first to successfully repair a strangulated hernia.67 Thereafter, August Gottlieb Richter (1742–1812) produced a two-volume treatise regarding hernias from 1777 to 1779, which included the first description of a strangulated hernia involving only part of the intestine.

Incarcerations of the ovary and appendix are often observed. Although ovarian resection is not required for ovary incarceration, incarceration of the appendix (known as "Amyand's hernia", which is an inguinal hernia that traps the appendix) sometimes requires appendectomy. In 1735, Claudius Amyand (1660–1749) performed the first successful appendectomy[161], which concerned an incarcerated hernia involving a swollen and perforated appendix. The reported incidence of Amyand's hernia among patients with appendicitis is < 0.1%[162], and left-sided Amyand's hernias have rarely been reported[163]. Amyand's hernia leads to further complications (*e.g.*, strangulation and perforation)[162], with a mortality rate of 14% to 30%[162]. Incarceration of the appendix may result in delayed perforation. Appendectomy should be considered[162,164], although it is not always necessary if a normal, uninflamed appendix is observed [162].

Pathophysiological hypotheses for prolapse of the uterus and its appendages have been proposed for both girls and women[165,166]. However, groin hernias involving the uterus and/or its appendages have not been described. The appendix has characteristic features and is completely distinct from the ileum, colon, and rectum[167]. In patients who exhibit a giant hernia involving incarceration of the ileocecal portion of the intestinal tract, only the appendix does not recover from ischemic changes, despite resolution of the strangulation (Figure 6). Thus, incarceration of the appendix has several reasons for a distinctive name (*i.e.*, Amyand's hernia).

In women and girls, the RL is attached to the uterus, near the origin of the fallopian tubes, and the extension of the parietal peritoneum follows the RL as it passes to the IC through the IIR[168]. A hydrocele of the canal of Nuck is a differential diagnosis for groin hernia[51,168,169], although this hydrocele is not conclusively diagnosed until surgery is performed on a suspected inguinal hernia[168]. Ultrasound is a powerful tool for making an accurate diagnosis and determining indications for surgical treatment[169]. Although hydrocele in infancy may resolve without surgical treatment [48], such treatment is generally indicated because of symptoms (*e.g.*, swelling and pain)[52,168,169]. Moreover, hydrocele of the canal of Nuck often accompanies with

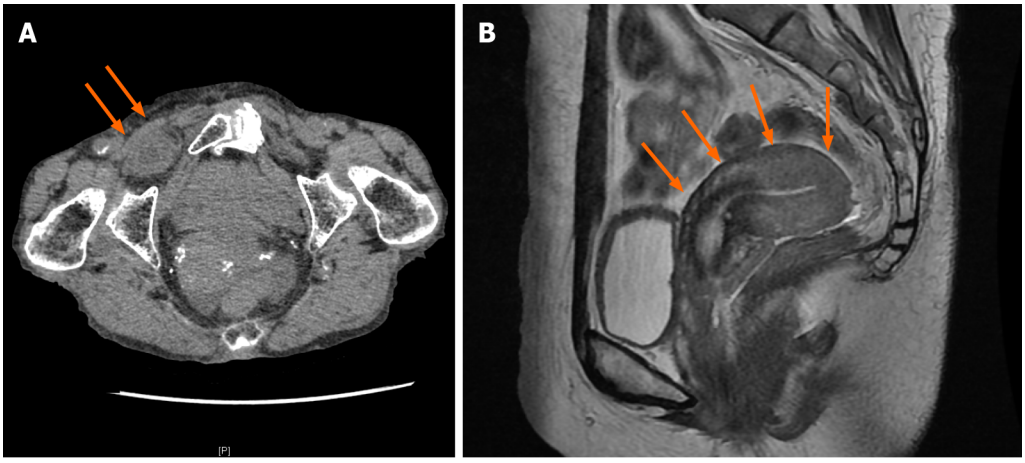

Figure 5 Actual image findings. Actual image findings of obturator hernia in computed tomography (A, orange arrows) and retroflexion of the uterus in magnetic resonance imaging (B, orange arrows) are shown, respectively.

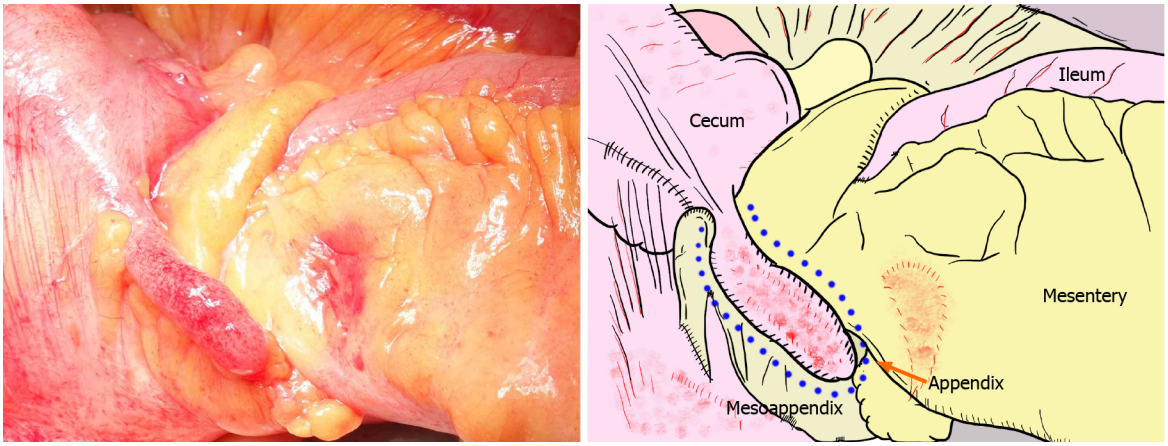

Figure 6 Amyand's hernia. Amyand's hernia is considered as an inguinal hernia that traps the appendix. In patients who exhibit a giant hernia involving incarceration of the ileocecal portion of the intestinal tract, only the appendix does not recover from ischemic changes, despite resolution of the strangulation.

ectopic endometriosis[52,168,169]. Hydrocele with persistent patency of the processus vaginalis requires resection[168,169]; this surgery prevents recurrences[168]. The genital branch of the genitofemoral nerve (Gb-GFN) and RL should be preserved[169]. The IIR is often enlarged by hydrocele; therefore, intentional closure of the IIR by Marcy's repair[70] is generally required[168,169].

POSTOPERATIVE COMPLICATIONS

Recurrence of groin hernias is extremely challenging for general surgeons, and neuropathy may be intractable[161,170,171]. Injury of the SC or VD results in refractory pain with burning[172]. Potential complications include testicular ischemia[173], testicular atrophy[174], bowel obstruction and/or necrosis due to mesh adhesion[175,176], vascular injury[177], visceral injury[176,177], wound infections[173], and hematomas[173]. Rarely, patients with groin hernias have experienced fatal outcomes [178,179].

AGENESIS AND STERILITY

Postoperative agenesis is a critical issue. In female patients, Koop's fixation should be routinely performed for bilateral inguinal hernias to prevent retroflexion of the uterus, which may cause female agenesis[120]. It is suggested to avoid division of the round ligament in open repair[60,61]. In male patients, both the surgical technique and mesh

material can influence the integrity of the SC and testicular function[180-182]. Contact with the mesh material may cause sterility in male patients[183]. Meshes inherently cause varying degrees of postoperative atrophy; therefore, biomechanical stability is extremely important[184]. Soft and hard meshes may result in unidirectional or matrix-like atrophy[12,185,186]. Soft mesh reduces chronic pain, but increases atrophic changes[12]. Biological mesh is predicted to become a powerful tool in the near future, although its cost remains high[12,187,188]. Testicular necrosis induces the formation of autoimmune antibodies to the body's own sperm[173], subsequently causing male sterility[50].

TOPOGRAPHIC NERVES AND NEUROPATHY

Thorough knowledge of peritoneal innervation is important because neurarchy has clinical implications. Some authors have extensively described the anatomy of the nerves located in the groin[22-31] (Figures 7 and 8). The peritoneum has both somatic and autonomic innervations, which are involved in various abdominal pathologies [29]. The parietal peritoneum receives its innervation from the spinal nerves of the 10th thoracic spine through the 1st lumbar spine[29-31]. This innervation is somatic and allows for the sensation of pain and temperature[29-31]. The visceral peritoneum receives autonomic innervation from the vagus nerve and sympathetic innervation that results in difficulty localizing abdominal sensations triggered by organ distension [29-31].

Precise knowledge of the topography of these nerves is essential for performance of high-quality repair with optimal patient outcomes[4,22-31,34]. Six nerves are of particular interest in the field of groin hernia repair[4,22-31,34], and the inguinal neuroanatomy should be thoroughly understood by all surgeons[34] (Figures 7 and 8). These six nerves of interest are the iliohypogastric, ilioinguinal, femoral (including the anterior cutaneous branch), genitofemoral (GFN) (femoral and genital branches), lateral femoral cutaneous (LFCN), and obturator nerves (Figure 8). The reported incidence of postoperative pain and/or discomfort ranges from 14.7% to 17.3%, but the intensity of persistent chronic pain or discomfort after surgery is not sufficiently severe to disturb daily activities in most patients[34,189]. However, a lack of anatomical knowledge and the use of an inadequate surgical technique may result in poor outcomes with refractory neuropathy and intractable chronic pain[4,22-31,34]. Notably, intractable and refractory pain may be an indication for removal of mesh and/or resection of entrapped nerves[190].

The femoral nerve is generally well protected by the psoas tendon. Therefore, injury to this nerve during surgery is extremely rare[34]. Additionally, intraoperative injury to the obturator nerve is only anecdotal because this nerve is well hidden[34].

Although branches of the GFNs course to the lower limbs, more common nerve injuries are observed in GFN branches in the trunk, as well as the LFCN[4,34] (Figure 8). The estimated risks of intraoperative injuries are 58.2% in the LFCN, 31.2% in the femoral branch of the GFN (Fb-GFN), and 4.7% in the Gb-GFN[34]. Although the courses of the obturator and femoral motor nerves are largely predictable and consistent, the courses of the sensory nerves (*i.e.*, GFN and LFCN) demonstrate great variability and are involved in refractory symptoms (*e.g.*, chronic and continuous pain) [34]. Wide variation in the number and course of sensory nerves that traverse the PPS creates considerable potential for overlap with the Gb-GFN, Fb-GFN, and LFCN[4,34]. Notably, the ilioinguinal nerve has a wide area in which injury can occur[4,34]. Respecting these proper dissection planes and ensuring knowledge of relevant neuroanatomy will minimize contact and corresponding risk of injury[4,34].

Injury to the iliohypogastric nerve results in postoperative neuralgia and muscular atrophy[191] (Figure 8). Additionally, injury to the ilioinguinal nerve may cause refractory pain[4,34] (Figure 8).

Nerve preservation during surgery requires a carefully considered approach[4,34,192]. Subtle factors during surgery (*e.g.*, skeletonization, direct detection, countertraction, and mesh contact) may cause postoperative neuropathy and chronic pain[34,190,192,193]. Unnecessary procedures for nerve identification should be avoided if possible; anatomical recognition of the route of each nerve (without direct exposure or complete skeletonization) is generally sufficient during surgery[4,34,192].

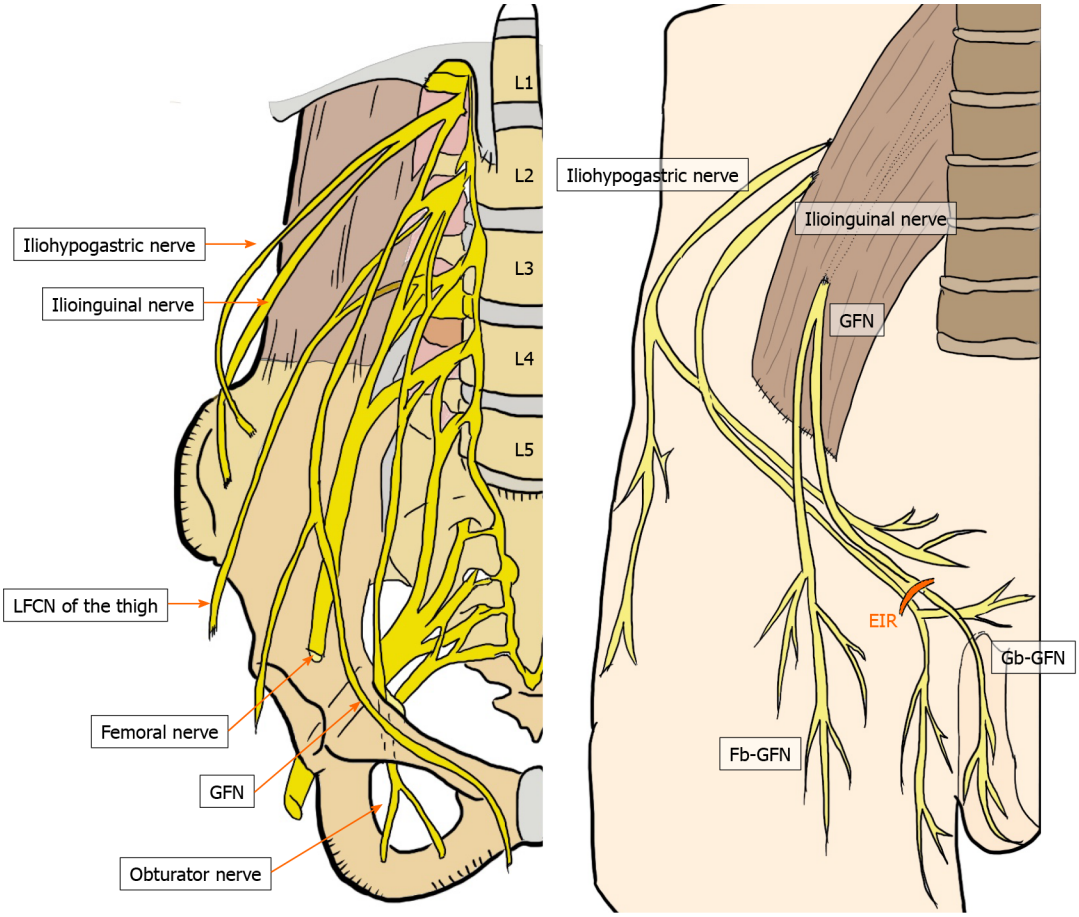

Figure 7 Topographic nerves located in the groin. These six nerves of interest are the iliohypogastric, ilioinguinal, femoral (including the anterior cutaneous branch), genitofemoral (femoral and genital branches), lateral femoral cutaneous of the thigh, and obturator nerves. EIR: External inguinal ring; GFN: Genitofemoral nerve; Fb-GFN: The femoral branch of the GFN; Gb-GFN: The genital branch of the GFN; LFCN: Lateral femoral cutaneous nerve.

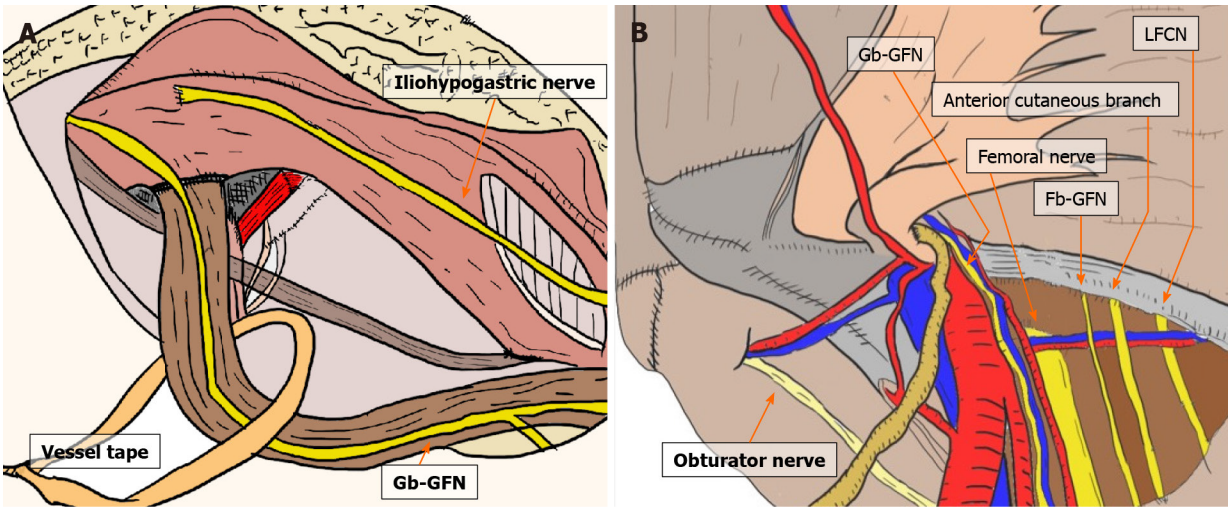

Figure 8 Topographic nerves located in the groin. Respective anterior (A) and laparoscopic (B) views are shown. Fb-GFN: The femoral branch of the genitofemoral nerve; Gb-GFN: The genital branch of the genitofemoral nerve; LFCN: Lateral femoral cutaneous nerve.

PLICAE AND FOSSAE

The initial laparoscopic view of the groin reveals five plicae (perineal folds) that serve as guiding landmarks[4,34]. The median umbilical plica, observed at the midline, contains the obliterated urachus and is less clinically relevant to surgical repair[4,34]. The medial umbilical plica (MUP) is the most prominent landmark present on the

initial view[4,34]. This plica is easily recognized and contains remnant umbilical vessels[4,34]. The MUP should not be routinely cut because the umbilical vessels may still be patent and cause bleeding[4,34]. Although the lateral umbilical plica may be difficult to identify depending on the body habitus and fat distribution, recognition of this plica is important[4,34]. This plica contains the inferior epigastric vessels, which divide the groin into a medial compartment (*i.e.*, space of Retzius) and a lateral compartment (*i.e.*, space of Bogros)[4,34]. External palpation of the surface anatomy allows precise localization of the anterior superior iliac spine and pubic tubercle, thereby delineating the IPT that divides the groin into an upper and a (critical) lower part[4,34]. The space of Bogros extends laterally from the space of Retzius toward the anterior superior iliac spine[4]. These spaces must be developed to allow adequate room for hernia repair and mesh placement[4].

These plicae create three flat fossae recognizable on each side, corresponding to possible hernia defects[4,34]. The hernia presentation can be more easily evaluated by a laparoscopic view than by endoscopic or anterior view[4,34] (Figure 9). The lateral fossa, located in the triangle between the lateral umbilical plica and IPT, corresponds to the point of the IIR from which an IIH originates[4,34]. The medial fossa is located between the lateral umbilical plica and MUP; this fossa is inferiorly limited by the IPT [4,34]. A DIH is located in this region, passing through Hesselbach's triangle[4,34]. The supravesical fossa is located medial to the MUP and cranial to the IPT, pubic bone, and urinary bladder[4,34]. This weak point may rarely become the origin of a supravesical hernia[4,34]. A femoral hernia develops within the region of the femoral canal (*i.e.*, the triangle below the IPT, medial to the femoral vein, and superior to the pubic bone and Cooper's ligament)[4,34].

The pubic symphysis, a cartilaginous joint between the superior pubic rami, symphysis denotes the midline[4]. Cooper's ligament, a lateral extension of the lacunar ligament, forms the periosteum of the superior pubic rami[4].

SIGNATURE TRIANGLE

Although the IIH enters the IIR lateral to the inferior epigastric vessels, a DIH protrudes through Hesselbach's triangle medial to the inferior epigastric vessels[4]. Important nerves are located on the lateral side of the IIR and travel from the pelvic interior to the thigh, coursing under the IPT[4,34]. In contrast, most important vessels course on the internal side of the IIR[4,34]. The VD travels downward, crossing the iliac vessels medially[4,34]. Hence, the VD comprises the "preperitoneal loop" in the DVL. Thereafter, the VD changes its direction at a 90-degree angle and dives down to the urogenital space to join the prostate gland[4,34].

The basic anatomical principles of the laparoscopic view were first described by Albert T. Spaw and Lynn P. Spaw in 1991, based on human cadaveric dissections[194]. They coined the term the "triangle of doom," which delineates the region between the VD and spermatic vessels. However, the neuroanatomy in the PPS was not considered [194]. Thereafter, James Rosser first described the inguinal neuroanatomy in 1994 and roughly delineated the anatomical course of the inguinal nerves[22]. Arnold S. Seid and Edwin Amos provided a more precise description of the nerves[23]; they postulated that the "triangle of doom" should be extended further laterally to the anterior superior iliac spine. Currently, the "triangle of doom" is regarded as an inverted V-shaped area bound laterally by the gonadal vessels (in both sexes) and medially by the VD (in men and boys) or RL (in women and girls)[4,32-34]. The external iliac artery and vein, deep circumflex iliac vein, Gb-GFN, and femoral nerve are involved in this area[4,32-34] (Figure 10).

In 1993, the most comprehensive analysis of inguinal neuroanatomy was performed by Riccardo Annibali, Thomas Quinn, and Robert J. Fitzgibbons Jr.[24,25] They defined the "triangle of pain" as the area lateral to the testicular vessels and inferior to the IPT [24,25]. Reinhard Bittner used the term "trapezoid of disaster" for this area[34]. The course of the nerves and their variations were recently described in detail[26-28]. The "triangle of pain" involves the Fb-GFN, LFCN, femoral nerve, and anterior cutaneous branch of the femoral nerve[4,32-34]. Notably, subtle injury (or greater) to the nerves located within the "triangle of pain" is a risk factor for intractable pain[4,32-34] (Figure 11).

The iliac vessels are accompanied by fatty tissue and lymph nodes[4,32-34], and over-dissection may lead to bleeding, potential nerve injury, or lymphatic leakage[4, 32-34]. Dissection of this region should be performed with substantial caution regarding identification of the corona mortis, a vascular connection between the epigastric

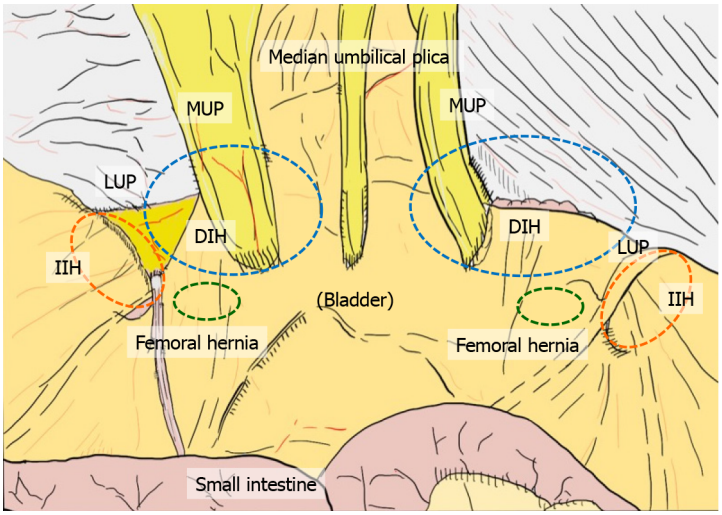

Figure 9 Plicae and fossae. Plicae create three flat fossae recognizable on each side, corresponding to possible hernia defects. Hernia presentation can be more easily evaluated by a laparoscopic view than by an endoscopic or anterior view. DIH: Direct inguinal hernia; IIH: Indirect inguinal hernia; LUP: Lateral umbilical plica; MUP: Medial umbilical plica.

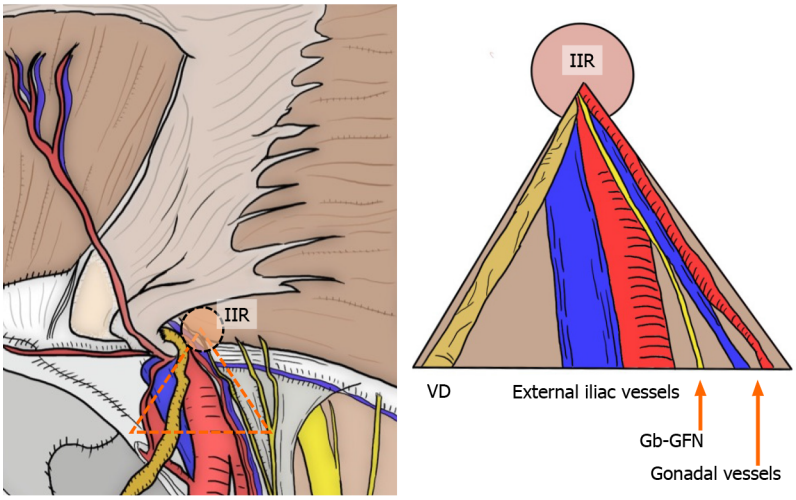

Figure 10 "Triangle of doom". The "triangle of doom" (orange dotted triangle) delineates the region between the VD and spermatic vessels. Currently, the "triangle of doom" is regarded as an inverted V-shaped area bound laterally by the gonadal vessels (in both sexes) and medially by the VD (in men and boys) or RL (in women and girls). Gb-GFN: The genital branch of the genitofemoral nerve; IIR: Internal inguinal ring; RL: Round ligament; VD: Vas deferens.

and obturator vessels[4,32-34]. The corona mortis is classically defined as an arterial anastomosis between the obturator and inferior epigastric arteries by means of the ectopic obturator artery[4,32-34]. The existence of the obturator artery results in annular communication among the inferior epigastric, common iliac, internal iliac, external iliac, and obturator arteries[4,32-34] (Figure 12). The frequency of this variant ranges from 20% to 30%[34]; moreover, several variants of anastomosing vascular branches may exist between the pubic artery/vein and the epigastric and obturator vessels[4,32-34]. Collectively, this variable deep venous circle is regarded as the "circulation of Bendavid." It is composed of the suprapubic, retropubic, deep inferior epigastric, and rectusial veins.4 These small vascular tributaries may form a network investing the pubic bone, Cooper's ligament, and the direct and femoral spaces[4,32-34]. These vessels and the underlying pubic bone are covered by a very thin membrane (*i.e.*, DVL) that should not be disrupted[4,32-34]. Brisk bleeding is difficult to control because of the dual vascular supply from the obturator and iliac vessels[4,32-34].

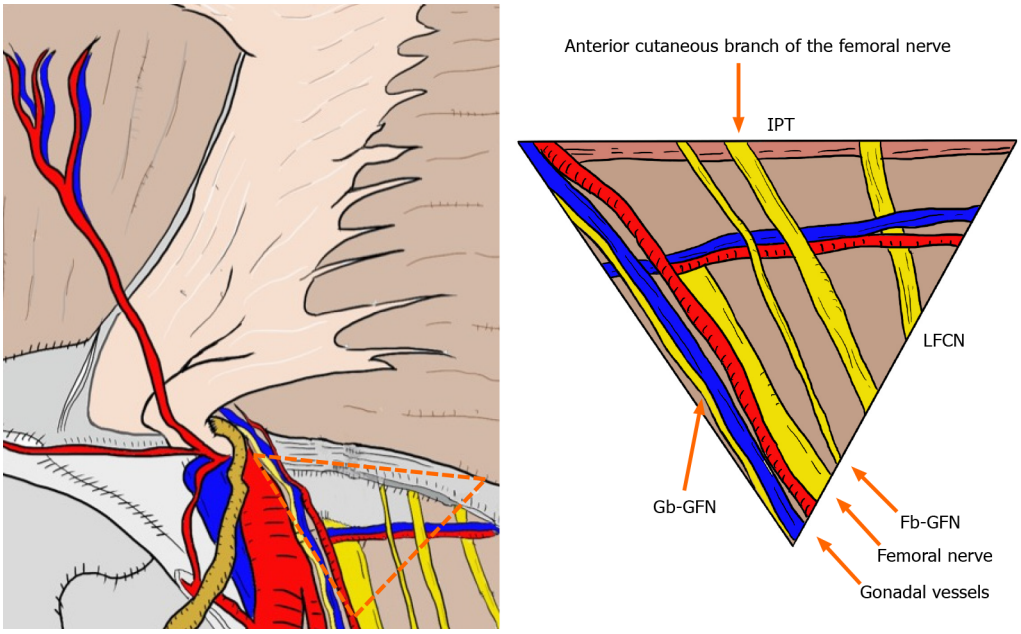

Figure 11 "Triangle of pain". The "triangle of pain" (orange dotted triangle) is defined as the area lateral to the testicular vessels and inferior to the iliopubic tract. The "triangle of pain" involves the femoral branch of the genitofemoral nerve, lateral femoral cutaneous nerve, femoral nerve, and anterior cutaneous branch of the femoral nerve. Subtle injury (or greater) to the nerves located within the "triangle of pain" is a risk factor for intractable pain. Fb-GFN: The femoral branch of the genitofemoral nerve; Gb-GFN: The genital branch of the genitofemoral nerve; IPT: Iliopubic tract; LFCN: Lateral femoral cutaneous nerve.

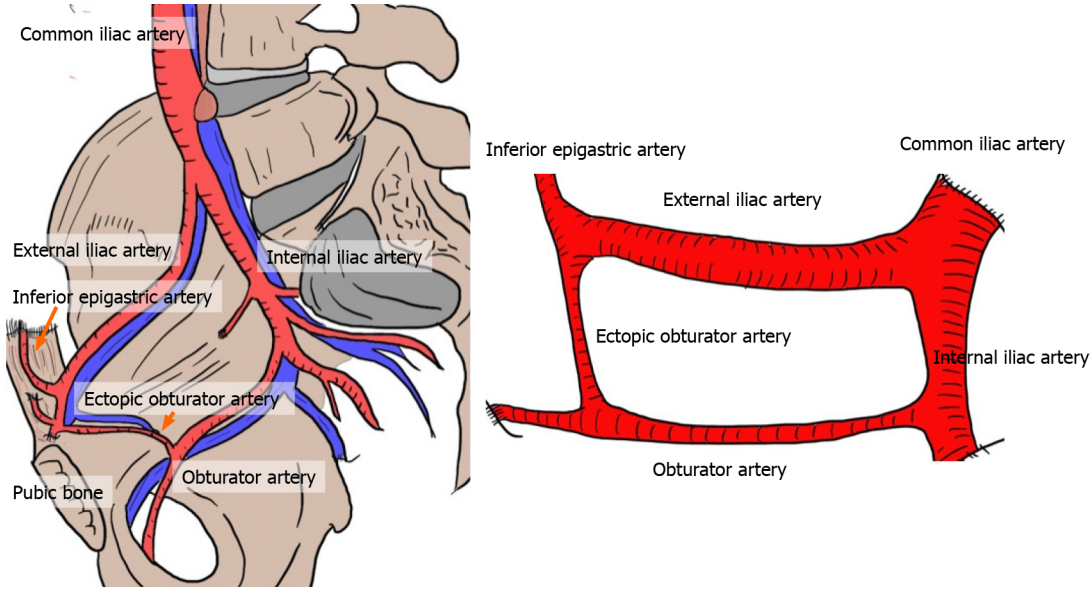

Figure 12 Corona mortis. The corona mortis is classically defined as the arterial anastomosis between the obturator and the inferior epigastric arteries by means of the ectopic obturator artery. The existence of the obturator artery results in annular communication among the inferior epigastric, common iliac, internal iliac, external iliac, and obturator arteries. Brisk bleeding is difficult to control because of the dual vascular supply from the obturator and iliac vessels.

PERSPECTIVE FOR THE FUTURE

Although TAPP and totally extraperitoneal repairs have a higher cost than conventional repair[4,195], the cost-effectiveness of TAPP repair has been documented in other medically advanced countries (*e.g.*, nations in Europe, as well as the United States and Japan)[196,197]. The direct cost and contribution margin are nearly equivalent between robotic and laparoscopic surgery[198], although robotic surgery results in a higher cost for unilateral groin hernia[199].

Robotic surgery is employed in the field of hernia repair[200-202]; the articulate robotic arms are advantageous for approaches without any visual disturbance by the medial umbilical ligament and bowels. Moreover, a singleport robotic surgery system

(da Vinci SP system; Intuitive Surgical, Inc., Sunnyvale, CA, United States) is currently available. General surgeons thus have a very promising frontier in this field.

Although the cost of biological mesh is still high[12,187,188], this mesh may resolve critical problems (*e.g.*, female agenesis, male sterility, neuropathy, and chronic pain) associated with synthetic mesh[203,204]. Currently, surgeons await less expensive, ethically responsible biological mesh to arrive on the world market. The advantages of biological mesh, compared with synthetic mesh, require long-term assessment in a large, multicenter, well-designed, and randomized controlled trial[205,206].

DISCUSSION

Some organizations (*e.g.*, the European Hernia Society and the Society of American Gastrointestinal and Endoscopic Surgeons) currently provide comprehensive inguinal hernia guidelines, and well-known surgeons have discussed herniology in detail[207-214]. However, many physicians may feel that no definitive criteria are available for the selection of surgical procedures. Indeed, the physician's choice, commercial basis, or cost-effective reasoning may be unchallenged in most instances. The surgical procedure should be carefully chosen based on sex (male or female) and age (pediatric, reproductive adult, or older adult). From the perspective of female fertility and male virility, female agenesis and male sterility should be avoided. Mesh material inherently causes varying degrees of postoperative atrophy[12,101,184-186]; moreover, mesh contact is associated with female agenesis, male sterility, neuropathy, and chronic pain[101,139,180-183]. Direct contact with mesh may also cause obstruction of the VD and SC[101]. Retroflexion of the uterus may cause female agenesis (Figure 5B). Only biological mesh can resolve these critical problems[203,204]. Although the TFR concept is important[11,64,90], careless use of synthetic mesh should be avoided in younger patients of reproductive age[180-183]. Potts' repair (accompanied by Koop's fixation in female patients) may be the optimal first choice for younger patients of reproductive age, as well as adolescents and children.

Based on its TFR concept and technical simplicity, so-called "mesh-plug hernioplasty" has spread worldwide[91-93]. However, the mesh should entirely reinforce the MO. Incomplete coverage of the MO results in long-term hernia recurrence after hernioplasty[94]. A DIH or femoral hernia may become a recurrent hernia, especially in female patients, who intrinsically have a wide pelvic space[94]. Female patients should undergo surgical repair at the PPS that fully covers the entire MO, as well as the obturator foramen[94], although "mesh-plug hernioplasty" is effective in men of advanced age[92].

Notably, from the perspective of technical skills, pediatric herniorrhaphy actually reflects the individual ability of each surgeon[8]. Antibiotics are not routinely administered after surgery[215]. Although antibiotics are not indicated for elective surgery using synthetic material[185], antibiotics may be appropriate if a trainee or resident performed surgery with a prolonged operative time and using synthetic material[216]. Administration of antibiotic prophylaxis in open mesh repair in high-risk patients in a low-risk environment is suggested[60,61].

Thorough knowledge of the inguinal anatomy is mandatory for successful herniorrhaphy and hernioplasty, and high-quality repair is required in the treatment of groin hernias. Surgical repair performed solely to prevent prolapse is inadequate and places patients with groin hernia at high risk. Important nerves and vessels should be carefully preserved. Hernia repair without careful consideration (*i.e.*, hernia repair that involves reinforcement of the abdominal wall alone) causes intractable symptoms, such as neuropathy and agenesis. Complacency may lead surgeons to presume that the learning curves for herniorrhaphy and hernioplasty are very short. High-quality repair is of utmost importance for groin hernias. Additionally, a combination of anatomical knowledge and high surgical skill level is crucial and indispensable for successful treatment of groin hernias. The frontier is large for general surgeons including residents.

CONCLUSION

Both anatomical recognition and a skillful technique are essential for successful herniorrhaphy and hernioplasty for groin hernias. In particular, in-depth anatomical knowledge of the nerves and vessels at the PPS and MO is a critical consideration. Surgeons must also avoid female agenesis, male sterility, neuropathy, and chronic

pain. The optimal surgical technique should be carefully chosen based on sex and age (pediatric, reproductive adult, or older adult).

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 July 20; 11(4): 187-198

DOI: 10.5662/wjm.v11.i4.187

ISSN 2222-0682 (online)

MINIREVIEWS

Evidence based review of management of cardiorenal syndrome type 1

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Author contributions: Ong LT designed and performed the literature search, analyzed the data, wrote the paper, and approved the final manuscript.

Conflict-of-interest statement: The author declares no conflict of interests for this article.

Manuscript source: Invited manuscript

Specialty type: Medical laboratory technology

Country/Territory of origin: Malaysia

Peer-review report's scientific

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Abstract

Cardiorenal syndrome (CRS) type 1 is the development of acute kidney injury in patients with acute decompensated heart failure. CRS often results in prolonged hospitalization, a higher rate of rehospitalization, high morbidity, and high mortality. The pathophysiology of CRS is complex and involves hemodynamic changes, neurohormonal activation, hypothalamic-pituitary stress reaction, inflammation, and infection. However, there is limited evidence or guideline in managing CRS type 1, and the established therapeutic strategies mainly target the symptomatic relief of heart failure. This review will discuss the strategies in the management of CRS type 1. Six clinical studies have been included in this review that include different treatment strategies such as nesiritide, dopamine, levosimendan, tolvaptan, dobutamine, and ultrafiltration. Treatment strategies for CRS type 1 are derived based on the current literature. Early recognition and treatment of CRS can improve the outcomes of the patients significantly.

Key Words: Cardiorenal syndrome; Heart failure; Acute kidney injury; Renal insufficiency; Management

Core Tip: Cardiorenal syndrome (CRS) type 1 is defined as the development of acute kidney injury or worsening renal function in patients with acute decompensated heart failure. Impaired renal function in acute decompensated heart failure is often associated with prolonged hospitalization, a higher rate of rehospitalization, high morbidity, and high mortality. The aim of this paper is to discuss the different treatment strategies and provide a guideline for the management of CRS type 1. Early recognition and treatment of CRS can improve the outcomes of the patients significantly.

quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: December 25, 2020
Peer-review started: December 25, 2020
First decision: May 6, 2021
Revised: May 9, 2021
Accepted: May 20, 2021
Article in press: May 20, 2021
Published online: July 20, 2021

P-Reviewer: Greenway SC
S-Editor: Gao CC
L-Editor: Filipodia
P-Editor: Yuan YY

Citation: Ong LT. Evidence based review of management of cardiorenal syndrome type 1. *World J Methodol* 2021; 11(4): 187-198
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/187.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.187

INTRODUCTION

Cardiorenal syndrome (CRS) type 1 is defined as the development of acute kidney injury or worsening renal function in patients with acute decompensated heart failure (HF)[1]. Acute CRS occurs in approximately 25% to 33% of patients admitted with acute decompensated HF[1]. CRS has been associated with adverse outcomes, increased risk of hospitalization, and death[2]. Impaired renal function is a stronger predictor of mortality compared to left ventricular ejection fraction or New York Heart Association class[3]. Besides that, the development of renal dysfunction in HF patients may worsen the preexisting HF[4].

The causes of CRS in hospitalized patients include venous renal congestion due to hemodynamic changes, neurohormonal activation, hypothalamic-pituitary stress reaction, inflammation, and infection[1]. Moreover, administration of intravenous diuretics in patients with acute decompensated failure may lead to worsening renal function[5]. The common underlying risk factors of developing CRS in the setting of acute decompensated HF include severe atherosclerotic disease, hypertension, diabetes mellitus, elderly age, and a history of renal insufficiency or HF[6]. Furthermore, the presence of renal dysfunction is one of the major risk factors that contribute to refractory congestive HF[7]. There is a lack of high-quality evidence or guidelines on the management of CRS, and the management of CRS remains empirical and deduced from the treatment of HF, acute kidney injury, or chronic kidney diseases (CKDs)[8,9]. Therefore, the aim of this paper is to review randomized controlled trials and observational studies to describe the clinical efficacy of different therapeutic options in managing patients with CRS type 1.

METHODLOGY

A systematic search was conducted using the two major electronic medical literature databases, PubMed and ScienceDirect. Search terms included the following keywords and word combinations: “cardiorenal syndrome type 1”, “heart failure”, “kidney injury”, and “renal failure”. Relevant articles published in English from 2005 to 2010 were identified. Additional articles of interest were retrieved from the reference list of selected papers. Review articles and case reports were excluded from this review. PRISMA guidelines were used as a basis for reporting the results of this systematic review.

The inclusion criteria for this review were randomized control trials and observational studies that investigated the efficacy of different therapeutic options for CRS and reported at least one biochemical datum. The exclusion criteria include studies on biochemical markers of CRS, prognosis studies, and prevalence studies. Review articles and case reports were also excluded. The outcomes used in this study were changes in renal function tests such as creatinine levels, glomerular filtration rate (GFR), blood urea nitrogen, cystatin C, urine output, and weight. The flow diagram of the study selection process is shown in Figure 1.

CHARACTERISTICS OF INCLUDED STUDIES

The main characteristics of the studies included in this review are shown in Table 1.

The study by Owan *et al*[10] enrolled 35 patients to standard therapy arm and 37 patients to standard therapy plus nesiritide arm. All the patients received standard therapy for HF as determined by the attending cardiologist and standardized diuretic therapy based on renal function. The patient in the nesiritide was administered intravenous nesiritide of a bolus of 0.2 mcg/kg followed by 0.01 mcg/kg per min[10].

The study by Bart *et al*[11] enrolled 94 patients in each pharmacologic therapy and ultrafiltration arm. Loop diuretics were discontinued in the ultrafiltration arm, and intravenous diuretics were used in pharmacologic therapy. The median duration of

Table 1 Characteristics and main findings of included studies

Ref.	Population	Sample size	Intervention	Duration of follow-up	Main findings
Owan *et al* [10], 2008	ADHF with renal dysfunction	72	Standard therapy *vs* standard therapy plus nesiritide (bolus of 0.2 mcg/kg followed by 0.01 mcg/kg per min)	72 h	Nesiritide produced greater reduction in blood pressure and preserved renal function
Bart *et al*[11], 2012	ADHF with worsened renal function	188	Ultrafiltration therapy *vs* stepped pharmacologic therapy (intravenous diuretics)	96 h	Stepped pharmacologic-therapy with intravenous diuretics was superior to ultrafiltration
Fedele *et al* [12], 2014	ADHF and renal impairment	21	Levosimendan (loading dose 6 μg/kg + 0.1 μg/kg per min) for 24 h *vs* placebo	72 h	Levosimendan improves the laboratory markers of renal function and renal hemodynamic parameters
Chen *et al* [13], 2013	AHF and renal dysfunction	360	Low dose dopamine (2 μg/kg per min for 72 h) *vs* low dose nesiritide (0.005 μg/kg per min for 72 h) *vs* placebo	72 h	Neither low dose dopamine nor low dose nesiritide improved renal function when added to diuretic therapy
Inomata *et al* [14], 2017	HF with diuretic resistance and renal impairment	81	Additive tolvaptan (≤ 15 mg/d) *vs* increased furosemide (≤ 40 mg/d)	7 d	Additive tolvaptan increased urine volume compared with patients receiving an increased dose of furosemide
Lannemyr *et al*[15], 2018	Chronic HF and impaired renal function	32	Levosimendan (loading dose 12 μg/kg + 0.1 μg/kg per min) *vs* dobutamine (7.5 μg/kg per min) for 75 min	60 mo and 75 mo after treatment	Levosimendan is the preferred inotropic agent compared to dobutamine

ADHF: Acute decompensated heart failure; HF: Heart failure.

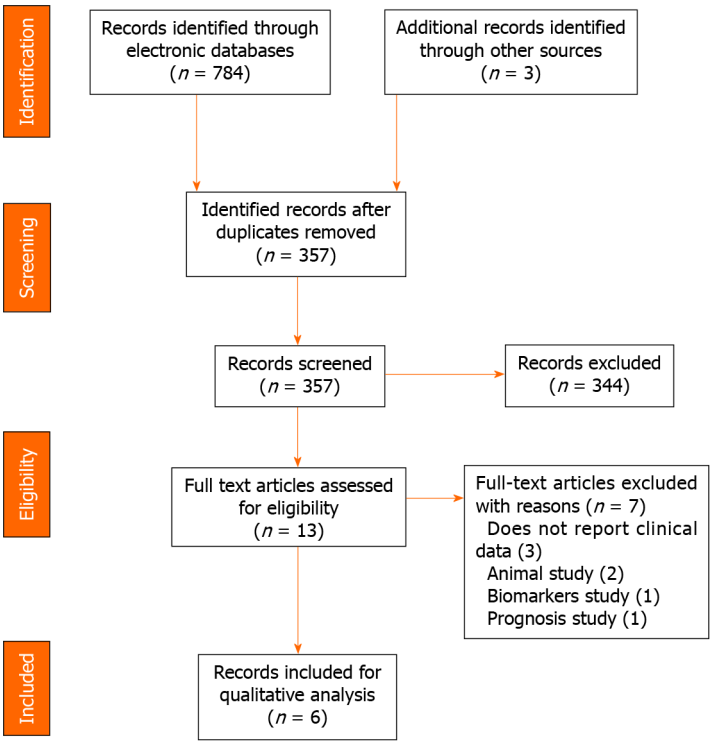

Figure 1 Flow diagram of the study selection process.

the pharmacologic therapy was 92 h (interquartile range, 56 to 138), while the median duration of ultrafiltration was 40 h (interquartile range, 28 to 67)[11].

The study by Fedele *et al*[12] enrolled 14 patients in levosimendan arm and 7 patients in the placebo arm. The patients in the levosimendan arm received 10 min intravenous loading dose of levosimendan (6 μg/kg) followed by an infusion (0.1 μg/kg per min) for 24 h. All the patients were on other drugs, which included angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), aldosterone blocking agents (spironolactone), and beta-blockers (bisoprolol or carvedilol). Placebo group patients also received loop diuretics in addition[12].

The study by Chen *et al*[13] randomized 360 patients to nesiritide strategy and dopamine strategy in a 1:1 allocation ratio. In nesiritide strategy, 119 patients were allocated to low dose nesiritide, while 58 patients were allocated to placebo. In dopamine strategy, 122 patients were allocated to low dose dopamine while 61 patients were allocated to placebo. Patients in the nesiritide strategy were administered with either 0.005 μg/kg per min for 72 h infused or placebo. Patients in the dopamine strategy were administered 2 μg/kg per min for 72 h infused or placebo[13].

The study by Inomata *et al*[14] enrolled 40 patients in additive tolvaptan arms and 41 patients in the increased furosemide arm. The mean dose of furosemide received by the patients before the study was 51 ± 25 mg/d, and the patients were also treated with pharmacotherapy such as ACE inhibitors, angiotensin II receptor blockers, β-blockers, and mineral corticoid receptor blockers. The patients were either administered additive tolvaptan arms of ≤ 15 mg/d or increased furosemide dose of ≤ 40 mg/d. The treating physician determined the dose of added tolvaptan or increased furosemide[14].

The study by Lannemyr *et al*[15] enrolled 16 patients in each levosimendan and dobutamine arm. The patients in the levosimendan arm were given a loading dose of 12 μg/kg given over 10 min, followed by a continuous infusion of 0.1 μg/kg per min for 65 min. The patients in the dobutamine arm were given as a continuous infusion started at 5.0 μg/kg per min for 10 min and thereafter increased to 7.5 μg/kg per min for 65 min[15].

CHANGES IN CLINICAL PARAMETER

The changes in the clinical parameters of the studies included in this review are shown in Tables 2-7.

The study by Owan *et al*[10] showed that nesiritide patients had less increase in creatinine and blood urea nitrogen compared to patients on standard therapy. The cumulative weight loss was greater in patients on standard therapy than in nesiritide patients, however, the difference was not significant[10].

In the study by Bart *et al*[11], the mean change in the serum creatinine level from the baseline was a decrease of 0.04 ± 0.53 mg/dL and an increase of 0.23 ± 0.70 mg/dL in the pharmacologic-therapy group and ultrafiltration, respectively. There was no significant difference in weight loss in both intervention groups[11].

The study by Fedele *et al*[12] showed that levosimendan was beneficial, which was confirmed by the decrease in blood urea nitrogen, serum creatinine, and cystatin C. Besides that, levosimendan increased the GFR and urine output significantly compared to placebo[12].

The study by Chen *et al*[13] showed that low dose dopamine had no significant effect on cumulative urine volume in 72-h, change in creatinine, and change in cystatin-C compared to placebo. Similarly, low dose nesiritide also had no significant effect on cumulative urine volume in 72-h, change in creatinine, and change in cystatin-C compared to placebo[13].

In the study by Inomata *et al*[14], the changes in urine volume between baseline in the tolvaptan group were significantly higher compared to the furosemide group. Besides that, the tolvaptan group had a smaller increase in serum creatinine on day 7 from baseline compared to the furosemide group. However, there were no significant changes in body weight in both groups[14].

The study by Lannemyr *et al*[15] showed that levosimendan and dobutamine had similar increases in renal blood flow. However, the levosimendan group showed an increase in GFR by 22% but remained the same in the dobutamine group. Filtration fraction remained unchanged in levosimendan group but decreased by 17% in the dobutamine group[15].

DISCUSSION

The treatment strategy for CRS type 1 is shown in Figure 2.

Diuretics and diuretic resistance

Loop diuretics are the primary class of diuretics in the management of acute HF with or without CRS[16]. Loop diuretics lead to natriuresis and volume loss in HF due to the inhibition of $Na^+K^+2Cl^-$ cotransporter in the thick ascending limb of the loop of

Table 2 Changes in clinical parameters of the included studies

Intervention	Ref.	Creatinine (mg/dL)		Change in BUN (mg/dL)		Cystatin C (mg/L)		Weight loss (kg)	Cumulative urine volume (mL)
		Baseline	Mean changes	Baseline	Mean changes	Baseline	Mean changes	Mean changes	
Nesiritide	Owan *et al*[10], 2008	1.85 ± 0.71	0.04 ± 0.44	44.8 ± 23.3	-1.3 ± 12.8	NA	NA	-2.75 ± 3.27	NA
	Owan *et al*[10], 2008 (placebo)	1.65 ± 0.42	0.09 ± 0.25	38.3 ± 16.6	2.4 ± 6.8	NA	NA	-4.25 ± 3.42	NA
	Chen *et al*[13], 2013	1.65	0.02	NA	NA	1.66	0.07	NA	8574
	Chen *et al*[13], 2013 (placebo)	1.70	0.02	NA	NA	1.86	0.11	NA	8296

BUN: Blood urea nitrogen; NA: Not available.

Table 3 Changes in clinical parameters of the included studies

Intervention	Ref.	Creatinine (mg/dL)		Change in BUN (mg/dL)		Cystatin C (mg/L)		Weight loss (kg)		Urine output (mL/d)	
		Baseline	Mean changes	Baseline	Mean changes	Baseline	Mean changes	Baseline	Mean changes	Baseline	Mean changes
Furosemide	Inomata *et al*[14], 2017	1.6	0.20 ± 0.27	NA	NA	NA	NA	61	-2.1 ± 2.6	1251 ± 540	79 ± 341

BUN: Blood urea nitrogen; NA: Not available.

Table 4 Changes in clinical parameters of the included studies

Intervention	Ref.	Creatinine (mg/dL)		Change in BUN (mg/dL)		Cystatin C (mg/L)		GFR (mL/min)		Urine output (mL/d)	
		Baseline	72 h	Baseline	72 h	Baseline	72 h	Baseline	72 h	Baseline	72 h
Levosimendan	Fedele *et al*[12], 2014	1.76 ± 0.37	1.51 ± 0.5	45.08 ± 22.19	33.14 ± 16.63	2577.5 ± 700.6	2083 ± 731.4	38.71 ± 7.94	53.34 ± 14.93	1766.4 ± 514.2	2663.5 ± 721.2
	Fedele *et al*[12], 2014 (placebo)	1.6 ± 0.2	1.7 ± 0.2	44.4 ± 13.1	47 ± 12.8	2498.5 ± 262	2470 ± 409.9	43.33 ± 7.99	40.24 ± 6.58	1571.4 ± 125.3	1778.51 ± 798.1
	Ref.	**Creatinine (mg/dL)**		**Change in BUN (mg/dL)**		**RBF (mL/min)**		**GFR (mL/min)**		**FF**	
		Baseline	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**
	Inomata *et al*[14], 2017	NA	NA	NA	NA	426 ± 197	518 ± 276	36.5 ± 18.3	44.5 ± 19.0	0.146 ± 0.080	0.143 ± 0.069

BUN: Blood urea nitrogen; FF: filtration fraction; GFR: glomerular filtration rate; NA: Not available; RBF: renal blood flow.

Henle[16]. Studies suggested that torsemide is a more effective decongestive therapy compared with furosemide in patients with HF because torsemide has more predictable oral bioavailability and a longer half-life[16].

The study by Felker and Mentz[5] suggested that there were no significant differences in observed symptoms or change in renal function in acute HF patients when

Table 5 Changes in clinical parameters of the included studies

Intervention	Clinical parameters evaluated									
	Ref.	Creatinine (mg/dL)		Change in BUN (mg/dL)		Cystatin C (mg/L)		Weight loss (kg)		Cumulative urine volume (mL)
		Baseline	Mean changes	Baseline	Mean changes	Baseline	Mean changes	Baseline	Mean changes	
Dopamine/dobutamine	Chen *et al* [13], 2013 (dopamine)	1.59	0.00	NA	NA	1.71	0.12	NA	NA	8524
	Chen *et al* [13], 2013 (placebo)	1.63	0.02	NA	NA	1.66	0.11	NA	NA	8296
	Ref.	**Creatinine (mg/dL)**		**Change in BUN (mg/dL)**		**RBF (mL/min)**		**GFR (mL/min)**		**FF**
		Baseline	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**
	Lannemyr *et al*[15], 2018 (dobutamine)	NA	NA	NA	NA	397 ± 121	499 ± 154	47.1 ± 14.5	47.3 ± 16.9	0.193 ± 0.070	0.161 ± 0.075

BUN: Blood urea nitrogen; FF: filtration fraction; GFR: glomerular filtration rate; NA: Not available; RBF: renal blood flow.

Table 6 Changes in clinical parameters of the included studies

Intervention	Clinical parameters evaluated									
	Study	Creatinine (mg/dL)		Change in BUN (mg/dL)		Cystatin C (mg/L)		Weight loss (kg)		Urine output (mL/d)	
		Baseline	Mean changes	Baseline	Mean changes	Baseline	Mean changes	Baseline	Mean changes	Baseline	Mean changes
Tolvaptan	Inomata *et al*[14], 2017	1.5	0.06 ± 0.32	NA	NA	NA	NA	62	-2.1 ± 1.8	1306 ± 494	459 ± 514

BUN: Blood urea nitrogen; NA: Not available.

Table 7 Changes in clinical parameters of the included studies

Intervention	Clinical parameters evaluated								
	Study	Creatinine (mg/dL)		Change in BUN (mg/dL)		GFR (mL/min per 1.73 m^2)	Weight loss (lb)		Urine output (mL/d)	
		Baseline	Mean changes	Baseline	Mean changes	Mean changes	Baseline	Mean changes	Baseline	Mean changes
Ultrafiltration	Bart *et al*[11], 2012	2.09	-0.04 ± 0.53	50.5	5.68 ± 18.29	1.67 ± 10.94	234	12.1 ± 11.3	NA	NA
	Bart *et al*[11], 2012 (pharmaco-logic therapy)	1.90	+0.23 ± 0.70	48.7	12.54 ± 24.81	0.93 ± 14.60	207	12.6 ± 8.5	NA	NA

BUN: Blood urea nitrogen; GFR: Glomerular filtration rate; NA: Not available.

the furosemide therapy was administered as a bolus compared with continuous infusions or at a low-dose compared to high-dose regimen. Nevertheless, the study by Palazzuoli *et al*[17] showed that continuous infusion of loop diuretics was associated with worsened renal filtration function even though the treatment resulted in greater reductions in brain natriuretic peptide from admission to discharge.

Diuretics use can increase systemic vascular resistance, plasma renin, aldosterone activity, norepinephrine, and arginine vasopressin and indirectly lead to deterioration

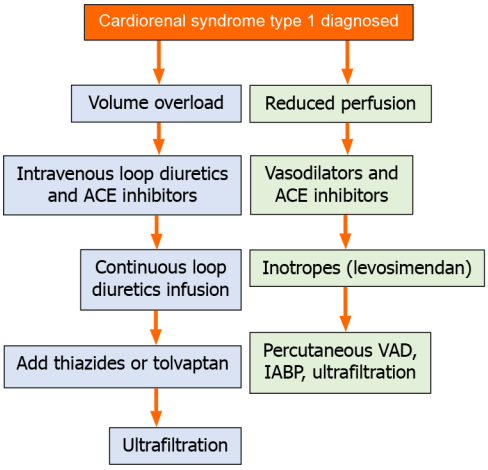

Figure 2 Management strategy for cardiorenal syndrome type 1. The management strategy in left arm is for patients presented with volume overload (decompensated heart failure, venous congestion, venous hypertension, edema, ascites, weight gain). The management strategy in right arm is for patients presented with reduced perfusion (decreased cardiac output, effective circulating volume, renal blood flow and renal plasma flow, arterial hypotension). ACE: Angiotensin-converting enzyme; IABP: Intra-aortic balloon pump; VAD: Ventricular assist device.

of left ventricular function[18]. Diuretics use can result in renal dysfunction through the above mechanisms[18]. However, the study by Ahmad *et al*[18] showed that kidney tubular injury detected by biomarkers in aggressive diuresis of patients with acute HF was not associated with worsening renal function. Furthermore, the study by Mentz *et al*[19] showed that high-dose loop diuretic therapy did not result in renin-angiotensin-aldosterone system (RAAS) activation greater than that with low-dose diuretic therapy. Ultrafiltration resulted in a greater increase in plasma renin activity compared with stepwise pharmacological care[19].

Loop diuretic resistance in HF can occur due to a decrease in renal perfusion, likely from low cardiac output[20]. Besides that, CKD reduces the excretion of diuretic into the tubular lumen thereby reducing and diminishing the filtered load of sodium[16]. HF can also increase proximal reabsorption of sodium through RAAS activation and increased expression of $Na^+K^+Cl^-$, which then limits the peak effect of drug delivered to the lumen[21]. However, increased furosemide dose in loop diuretic resistance can cause aggressive fluid removal, which leads to depletion of intravascular volume without refilling from the extravascular space[22]. Moreover, hyperdiuresis can lead to prerenal renal dysfunction due to the potential risk of hypotension[23].

Diuretic resistance can be managed by continuous infusion of furosemide starting at 5 mg/dL to 10 mg/dL followed by an intravenous thiazide diuretic[6]. This combination therapy can result in a sequential nephron blockade of sodium reabsorption, but it may cause excessive sodium and potassium loss[6]. The systematic review by Salvador *et al*[24] showed that continuous infusion of loop diuretic showed greater urine output, shorter duration of hospitalization, and better safety profile compared with bolus injections in patients with congestive HF. The study by Bart *et al*[11] also showed that stepwise pharmacological care including thiazide diuretics, inotropes, and vasodilator therapy was more effective compared to ultrafiltration for preserving renal function and relieving congestion. In addition, the study by Inomata *et al*[14] suggested that additive tolvaptan increased urine volume and prevented renal dysfunction in HF patients with diuretic resistance and renal impairment.

ACE inhibitors and ARBs

Clinical data have shown that RAAS inhibitors can slow CKD progression and are one of the components in managing patients with left ventricular systolic dysfunction in HF[6,16]. However, the use of RAAS inhibitors in acute CRS with underlying renal disease may lead to an increase in serum creatinine levels[16]. The Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS) enrolled patients with severe HF with renal dysfunction with serum creatinine concentrations less than 3.4 mg/dL [25]. The study showed that patients who were in the enalapril arm had a reduction in symptom burden and HF-associated mortality compared to placebo but were associated with doubling the serum creatinine by 11%[25]. Besides that, the subgroup of HF patients with creatinine levels higher than 2 mg/dL showed improvement in symptoms and outcomes when treated with an ACE inhibitor[25].

A *post hoc* analysis of Study of Left Ventricular Dysfunction (SOLVD) study showed that patients with HF and CKD in the enalapril group had higher mortality benefits even with more advanced stages of CKD[26]. A study by Ahmed *et al*[27] followed 1165 patients age ≥ 65 years, with systolic HF (ejection fraction < 45%) and CKD (eGFR < 60 mL/min per 1.73 m^2) where 1046 received ACE-inhibitors or ARBs for 8 years. The results showed that patients receiving ACE-inhibitors or ARBs had a significant reduction in all-cause mortality and HF hospitalization[27]. Therefore, patients with CRS should be started on the lowest dose of an ACE inhibitor, and the dosage titrated up carefully. Concomitant use of NSAIDs should be avoided to prevent further deterioration of kidney function[6]. ACE-inhibitors or ARB therapy should be continued in patients with CRS unless there is the development of severe renal dysfunction and hyperkalemia[6].

Vasodilators and inotropes

Nesiritide can reduce afterload and increase cardiac output *via* the coronary, arterial, and venous vasodilatory properties without inotropic effects[16]. The study by O'Connor *et al*[28] randomized 7141 patients with acute HF to receive either intravenous nesiritide or placebo for 1 d to 7 d in addition to standard care. The result showed that nesiritide had a small and non-significant effect on dyspnea improvement [28]. However, nesiritide therapy was associated with increased rates of hypotension and no differences in renal function, rate of death, and hospitalization compared to the placebo group[28]. The study by Owan *et al*[10] showed that the recommended dose of nesiritide can lower blood pressure more compared to the standard therapy but had no adverse effect on changes in creatinine or cystatin C levels. Furthermore, the study by Wang *et al*[29] also suggested that nesiritide did not improve renal function in patients who had decompensated HF with renal insufficiency. Therefore, nesiritide does not improve clinical outcomes, decongestion, or renal function in recommended dose[29].

The study by Chen *et al*[13] showed that patients treated low dose nesiritide did not enhance decongestion or improve renal function. Besides that, the study also showed that low dose dopamine neither improved decongestion nor preserved renal function [13]. However, the findings of the study are contrary to the guidelines for the management of acute HF that suggest the use of low dose dopamine can be considered to improve diuresis and preserve renal function[30]. Observational studies also indicate that the use of nesiritide and dopamine in acute HF is associated with longer length-of-stay, higher mortality, and higher costs[31]. Therefore, use of low dose dopamine or low dose nesiritide as renal adjuvant therapies is not recommended in patients with CRS as it does not provide benefits on renal function, decongestion, and clinical outcome[13].

Levosimendan is a positive inotrope with Ca^{2+} sensitization that improves the calcium sensitivity of cardiac muscle cells and therefore provides hemodynamic and symptomatic improvement[32]. Besides that, levosimendan has a vasodilatory effect on vasculature *via* activation of ATP-sensitive K^+, voltage-dependent K^+, and Ca^{2+}-activated K^+ channels[33]. Levosimendan can achieve maximal improvement in hemodynamic parameters at 1 to 3 d after starting the infusion and the effects can be sustained for at least a week[34,35]. Levosimendan can also improve renal function through the increased cardiac output[12]. Moreover, levosimendan can reduce the right-sided pressures, pulmonary artery wedge pressure, and central venous pressure, thereby improving the function of the right ventricles[36,37].

The study by Bragadottir *et al*[38] showed that levosimendan also increases both renal blood flow and GFR by inducing pre-glomerular vasodilation. The study by Lannemyr *et al*[15] showed that the renal filtration fraction remained unchanged in levosimendan group but decreased in dobutamine. This is due to levosimendan preferentially vasodilating the afferent arterioles, while dobutamine has balanced vasodilation of both afferent and efferent arterioles[15]. Furthermore, levosimendan increases glomerular capillary surface area by inhibiting angiotensin II-mediated mesangial cell contraction[39].

The study by Yilmaz *et al*[40] also suggested that levosimendan offered more beneficial effects in terms of ejection fraction, systolic pulmonary artery pressure, 24-h urine output, and creatinine compared to dobutamine in patients with biventricular HF. The study by Packer *et al*[41] showed that levosimendan provided rapid and durable symptomatic relief in the first 5 d but was associated with an increased risk of adverse cardiovascular events such as hypotension, cardiac arrhythmias, and a numerically higher risk of death at 90 d[41]. However, inotropic therapy should be reserved for patients with severe low cardiac output where vasodilatory agents cannot be used to avoid a further decrease in systemic pressure or systemic vascular resis-

tance[42,43].

Ultrafiltration

Ultrafiltration can be an effective decongestion strategy because of the ability to remove the isotonic plasma and therefore more sodium for the same amount of water [44]. The study by Costanzo *et al*[45] showed that weight loss and net fluid loss were greater in the ultrafiltration group compared to intravenous diuretics. Moreover, the rate of re-hospitalized for HF at 90 d was significantly lower in the ultrafiltration group[45]. However, there were no differences in episodes of hypotension within the first 48 h and serum creatinine at 90 d between the two groups[45]. On the contrary, the study by Bart *et al*[11] showed that there was a significant increase in serum creatinine level 96 h after enrollment in the ultrafiltration group compared with the pharmacologic therapy group, but there were no significant differences in weight loss. Patients who had ultrafiltration experienced an early rise in the creatinine level due to a transient decrease in intravascular volume[11]. GFR in patients with pharmacological therapy improved significantly after 60 d[11]. Besides that, a higher percentage of patients in the ultrafiltration group experienced a serious adverse event compared to the pharmacologic-therapy group over the 60-d period of follow-up[11]. The most common adverse events associated with ultrafiltration treatment included kidney failure, complications, and catheter-related complications[11]. Therefore, ultrafiltration treatment is not justified for patients with CRS due to the complexity and high cost of treatment[11]. Pharmacological therapy is recommended as the first-line therapy, and ultrafiltration should only be reserved in cases of refractory congestion[8].

CONCLUSION

CRS in patients with decompensated HF is associated with several cardiovascular and renal adverse events such as myocardial infarction, stroke, need for hemodialysis, high rates of hospitalization, and mortality[16,46,47]. However, management of CRS type 1 is often challenging due to the various underlying mechanisms of renal impairment and the lack of novel therapeutic options targeting renal impairment in HF patients[6, 8]. Therefore, early recognition of the condition by using different novel biomarkers and imaging techniques is important to initiate optimal treatment and care of the patients[16]. Moreover, patients with underlying HF should be educated to manage their condition well to prevent decompensation. A multidisciplinary team approach with cooperation between internists, cardiologists, and nephrologists is important to establish an effective treatment plan for patients with CRS to improve their quality of life[6]. Further research on drugs targeting the pathophysiological mechanism CRS, which includes both cardiac and renal dysfunction, can be conducted to improve the survival of the patients.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 July 20; 11(4): 199-207

DOI: 10.5662/wjm.v11.i4.199

ISSN 2222-0682 (online)

MINIREVIEWS

Isolation of lymphocytes from the human gastric mucosa

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Author contributions: Iwamuro M organized the report and drafted the article; Takahashi T and Watanabe N critically revised the article for important intellectual content; Okada H approved the final article.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest associated with this article.

Manuscript source: Invited manuscript

Specialty type: Medical laboratory

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Abstract

Flow cytometry is widely used for lymphocyte immunophenotyping in clinical settings. However, few studies have applied it for analyzing lymphocytes of the gastric mucosa. This review offers an overview of methodologies for isolating lymphocytes from the human stomach. Previously reported articles were reviewed, focusing on procedures for isolating human gastric mucosal lymphocytes. *Helicobacter pylori*-associated peptic diseases and gastric cancer are two major subjects of research in this field. Enzymatic dissociation, mechanical dissociation, or a combination of the two have been used to isolate lymphocytes from the stomach. Intra-epithelial and lamina propria lymphocytes were separately isolated in several studies. We also summarize the history and present trends in analyzing lymphocytes in patients with gastric disease.

Key Words: Gastrointestinal biopsies; Gastrointestinal endoscopy; Gastric cancers; *Helicobacter pylori*; Gastric ulcer; Flow cytometry

Core Tip: This review provides an overview of methodologies used to analyze lymphocytes in the stomach. *Helicobacter pylori*-associated peptic diseases and gastric cancer are two major subjects of research in this field. Previously reported articles were reviewed, focusing on procedures for isolating human gastric mucosal lymphocytes. The history and present trends in analyzing lymphocytes in patients with gastric disease are also summarized.

technology

Country/Territory of origin: Japan

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: December 31, 2020
Peer-review started: December 31, 2020
First decision: April 6, 2021
Revised: April 9, 2021
Accepted: July 9, 2021
Article in press: July 9, 2021
Published online: July 20, 2021

P-Reviewer: Di Mario F
S-Editor: Gao CC
L-Editor: A
P-Editor: Li X

Citation: Iwamuro M, Takahashi T, Watanabe N, Okada H. Isolation of lymphocytes from the human gastric mucosa. *World J Methodol* 2021; 11(4): 199-207
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/199.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.199

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is responsible for most peptic ulcers and chronic inflammation in the stomach. Such prolonged inflammation causes mucosal damage and regeneration, in turn leading to carcinogenesis of the gastric epithelium and lymphomagenesis. Additionally, recent advances in cancer immunology and immunology-based anticancer therapies highlight the contribution of tumor-infiltrating lymphocytes in gastric cancer treatment. Thus, chronic inflammation with lymphocyte infiltration in the stomach may be involved in various gastric diseases[1,2].

Flow cytometry is widely used for lymphocyte immunophenotyping. An advantage of flow cytometry over immunohistochemistry is its multicolor analysis, providing an accurate characterization of the surface antigen profile of specific cells. Despite its superiority, few studies have assessed gastric mucosal lymphocytes by flow cytometry. We believe that its widespread use will promote a better understanding of surface marker expression and function of lymphocytes in the stomach. Here, we review relevant basic studies and summarize the methods of isolating lymphocytes from the human stomach for flow cytometry. Additionally, the history and current trends in analyzing lymphocytes in patients with gastric disease are summarized.

LITERATURE SEARCH

We performed a literature search on October 13, 2020, in the PubMed database. The search terms used were "lymphocyte isolation", "stomach", and "flow cytometry". We retrieved 66 titles[1-66], and no previous systematic review of lymphocyte isolation from the gastric mucosa was identified (Figure 1). Articles not written in English (n = 4) were excluded from this review. A further 20 articles were excluded as the research subjects were cell lines (n = 4) or non-human mammals, including mice (n = 14), pigs (n = 1), or alpacas (n = 1). Among the remaining 42 articles, lymphocyte isolation from the stomach was not performed in 16 articles. Finally, we reviewed 26 articles and summarized the methodologies for lymphocyte isolation from the human gastric mucosa.

Lymphocyte isolation was performed using endoscopic biopsy specimens (n = 15), surgically resected specimens (n = 10), or both (n = 1). The number of endoscopically biopsied specimens was 1 (n = 2), 1–2 (n = 1), 2 (n = 3), 4 (n = 5), 5 (n = 1), or 6 (n = 1). The number used for lymphocyte isolation was not specified in the remaining three articles. Gastric mucosal lymphocyte analysis was performed in association with *H. pylori*-related peptic diseases (n = 12), gastric cancer (n = 9), or other diseases (n = 5).

During lymphocyte isolation, enzymatic degradation and/or mechanical shredding and grinding are performed to remove the epithelial cells and connective tissue of the stomach. Collagenase is commonly used for enzymatic processing, while mesh strainers or glass slides are used for mechanical processing. Hereafter, we review representative methods for lymphocyte isolation from the stomach.

Combination of enzymatic and mechanical dissociation

In previously reported studies, sequential processing with enzymatic and mechanical dissociation is most frequently used for lymphocyte isolation from the gastric mucosa. In one protocol[2,6,16], fresh tissues were obtained from the surgically resected stomach and washed three times with Hank's solution containing 1% fetal calf serum. The specimen was cut into small pieces and collected in RPMI 1640 medium containing collagenase IV (1 mg/mL) and deoxyribonuclease I (10 mg/mL). Subsequently, mechanical separation was performed using gentleMACS Dissociator and the dissociated cell suspensions were incubated for 1 h under continuous rotation at 37 °C. After passing the cell suspension through a 70 μm cell strainer, cells were deemed ready for flow cytometry.

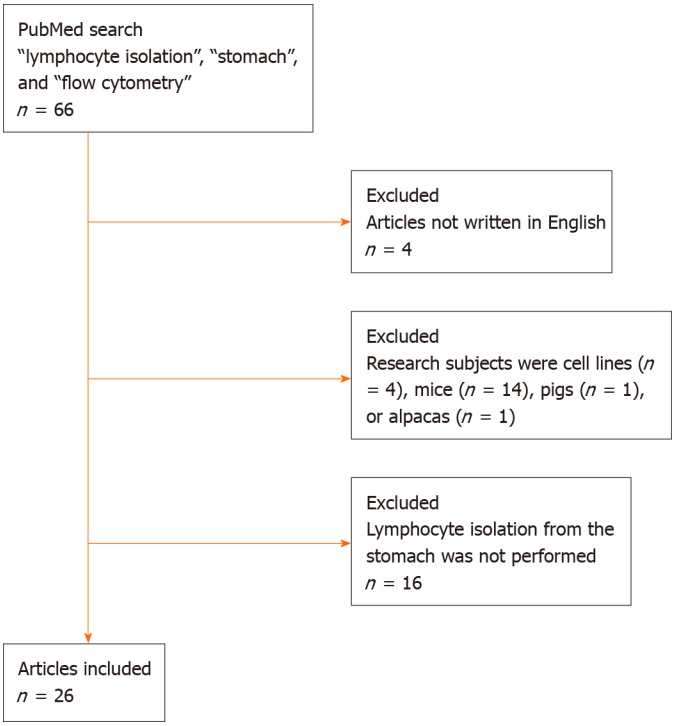

Figure 1 Selection of articles for the review.

In another protocol[10], two specimens were obtained from the gastric mucosa by endoscopic biopsy. The specimens were placed on ice in RPMI 1640 medium containing 10% fetal calf serum, glutamine, and penicillin/streptomycin. The biopsy specimens were transferred to a dithiothreitol/EDTA solution and incubated for 15 min at 37 °C. The samples were finely sliced and digested in a collagenase A solution for 1 h at 37 °C. The digested cell suspensions were filtered through a 70 μm cell filter and collected in RPMI 1640 medium. After centrifugation, the pellet was resuspended in phosphate-buffered saline.

For enzymatic dissociation, other reagent combinations are also used, such as deoxyribonuclease 1 (0.02 mg/mL), collagenase (0.25 mg/mL), and hyaluronidase (0.1 mg/mL)[28]; collagenase (300 μg/mL) and Dispase II (500 U/mL)[33]; or collagenase (5 mg/mL), DNase (0.1 mg/mL), and protease (2 U/mL)[43,49,58,62].

Enzymatic dissociation

Isolation of lymphocytes can be performed by enzymatic dissociation alone. For instance[60], fresh gastric tissue can be digested in a rotating chamber (60 rpm, 37 °C, 12 h) with NaCl (8 mg/mL), KCl (0.4 mg/mL), $CaCl_2$ (0.56 mg/mL), $NaHPO_4$ (60 μg/mL), Na_2HPO_4-$12H_2O$ (151 μg/mL), HEPES (2.3 mg/mL), *Clostridium hystolyticum* collagenase (0.5 mg/mL), and soybean trypsin inhibitor (5 μg/mL). The lymphocytes are then dispersed and enriched on Ficoll-Paque density gradients.

Mechanical dissociation

Without enzymes, lymphocytes can be obtained by mechanical mincing of surgically resected or endoscopically biopsied specimens, followed by passing through a 40-100 μm filter to exclude tissue fragments[23,24,52] or by pressing and grinding in a coarse glass grinder[48].

Isolation of intra-epithelial lymphocytes and lamina propria lymphocytes

The predominance, heterogeneity, and distribution of lymphocytes are diverse at different locations within the gastric mucosa[22]. Therefore, intra-epithelial lymphocytes and lamina propria lymphocytes have been investigated separately in several studies[22,47,54,55]. Briefly, biopsy samples were rotated at 37 °C for 1 h in calcium- and magnesium-free Hanks' balanced salt solution supplemented with 5% fetal calf serum, dithiothreitol (1 mmol/L), and EDTA (1 mmol/L). At this step, the epithelial layer is removed, leaving the lamina propria intact and attached to the basement membrane. Subsequently, the resulting single cell suspension was washed in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin/streptomycin.

The remaining tissue was placed in 5 mL of RPMI 1640 medium containing collagenase Type 1A (130 U/mL) and rotated at 37 °C for 3 h to obtain lamina propria cells.

Single-step lymphocyte isolation from an endoscopic biopsy specimen

We recently introduced a simplified, one-step procedure for lymphocyte isolation from an endoscopic biopsy sample[67]. To isolate lymphocytes, we used a porcelain bowl with a spout and a wire mesh tea strainer. First, the porcelain bowl and wire mesh strainer were sterilized by autoclaving. During esophagogastroduodenoscopy, enteroscopy, or colonoscopy, a single tissue sample was collected from the gastrointestinal tract with a standard biopsy forceps. The collected gastrointestinal sample was put into a plastic centrifuge tube containing 5 mL of isotonic saline solution. Next, a wire mesh strainer was placed in a porcelain bowl and the specimen-containing saline solution was poured through the mesh. The solid specimens were crushed using the rubber portion of a 10 mL injection syringe plunger. The wire mesh tea strainer was then removed and thrown away. Subsequently, the saline solution containing lymphocytes was decanted into a plastic centrifuge tube and the cells were deemed ready for flow cytometry.

As far as we know, this is the easiest procedure reported to date; lymphocyte isolation by this method is achieved within 2 min. Besides, laboratory wares and apparatus are not necessary for this method. Therefore, our approach for lymphocyte separation can be completed in an endoscopy unit right after taking a biopsy sample. This method would allow widespread evaluation of lymphocytes in the field of gastroenterology.

HISTORY AND PRESENT TRENDS OF LYMPHOCYTE ANALYSIS IN THE STOMACH

During the early development stages of flow cytometry for gastric disorders (1995-1999), the association between peptic ulcers and inflammation induced by mucosal lymphocytes was investigated in patients with *H. pylori* infection or duodenal ulcers [52,54,55,58,61,62]. Activation markers such as CD25 [interleukin (IL)-2 receptor alpha chain], CD69 (activation inducer molecule), CD71 (transferrin receptor protein 1), HLA-DR, and adhesion and emigration-related molecules, such as CD11a/CD18 (lymphocyte function-associated antigen 1: LFA-1), CD11b (integrin alpha-M), CD54 (intercellular adhesion molecule-1: ICAM-1), CD106 (vascular cell adhesion molecule-1: VCAM-1), and CD49d (very late antigen-4: VLA-4), were analyzed by flow cytometry in these patients. Also analyzed were the pan T cell markers CD2 and CD3, T-helper cell marker CD4, and T-cytotoxic cell marker CD8. Cytokines, such as interferon-gamma, tumor necrosis factor beta (TNF-β), IL-2, IL-4, and IL-5, were also examined by flow cytometry[52,55].

In the year 2000, research was focused on CD95, also known as Fas or TNF receptor superfamily member 6[47,48]. CD95 is a death receptor localized on the surface of cells that triggers a signal transduction pathway upon binding its ligand, leading to programmed cell death (apoptosis). In *H. pylori*-associated gastritis, epithelial cell damage is mediated through Fas/Fas ligand interactions[47]. Simultaneously, apoptotic depletion of invading mucosal lymphocytes occurs with Fas ligand expression, providing a mechanism of immune privilege evading severe destruction of gastric epithelium in the *H. pylori*-infected stomach[48]. Meanwhile, flow cytometry was also performed for gastric mucosal lymphocytes in patients with duodenal ulcers[33,43] and in children[38].

To our knowledge, lymphocyte infiltration in gastric cancer was first assessed in 1996 in patients with lymphocyte-rich, Epstein-Barr virus-associated gastric carcinoma [60]. In 2006, tumor-infiltrating lymphocytes were investigated in two major types of gastric adenocarcinoma (Lauren classification): intestinal type or diffuse type[28]. The number of B cells was significantly higher, while the number of T cells was significantly lower in intestinal type compared with diffuse type tumors. Furthermore, cloning and characterization of tumor-infiltrating T cells isolated from gastric cancers revealed a specific type-1 T cell response to gastric cancer antigens[21]. Analysis of $CD8^+$ cells that produce IL-17 (Tc17 cells) showed that the percentage of Tc17 cells increased with tumor progression and was associated with overall survival time[15]. Tc17 cells induce CXCR4-dependent chemotaxis of myeloid-derived suppressor cells and impair cytotoxic functions of anti-tumor $CD8^+$ cells, promoting tumor progression.

Since 2008, regulatory T (Treg) cells have gained attention in association with gastric disorders owing to their involvement in immune regulation[9,23,24]. In these studies, Tregs were defined as $CD4^+CD25^{high}$[24], $CD4^+CD25^+CD127^{low/-}$[23], or $CD4^+FOXP3^+$ cells[9]. Studies show that local Treg cells in gastric cancer express a suppressive cytokine profile characterized by high IL-10, low transforming growth factor-beta (TGF-β), and interferon-gamma production[9]. Thus, it is believed that Tregs suppress effector T cell proliferation and contribute to gastric cancer progression[23]. Furthermore, increase of IL-10 secretion by Tregs was confirmed in *H. pylori*-infected gastric mucosa[24]. IL-10 inhibits IL-8 expression, activates nuclear factor kappa B in the gastric epithelium, and enhances *H. pylori* growth *in vitro*, suggesting the participation of Tregs in gastric ulcer formation and persistent *H. pylori* infection.

In one study, T cells expressing natural killer cell receptors, defined as $CD3^+CD56^+$, $CD3^+CD161^+$, or $CD3^+CD94^+$ cells, were quantified in *H. pylori*-positive and -negative patients[22]. $CD3^+CD161^+$ cells were higher in the epithelium of *H. pylori*-infected gastric mucosa, whereas $CD3^+CD56^+$ cells were lower in the lamina propria, indicating a site-specific distribution of T cells bearing natural killer receptors. In another study, mast cells, defined as $CD45^+CD117^+Fc\varepsilon RI^+$ cells, were investigated in patients with gastric cancer[2]. A significantly higher number of mast cells exist in gastric cancer tissues, and the mast cell levels increase with tumor progression and independently predict a reduced survival. Besides lymphocytes, tumor-infiltrating neutrophils were examined in gastric cancers by cell sorting against CD66b, which allows for the enrichment of mature neutrophils[6].

Using flow cytometry, specific lymphocyte subsets are defined based on their lineage-, developmental stage-, and function-specific cell surface markers. In addition, fluorescence-activated cell sorting technology enables diversion of individual cells from the fluid stream and collection into viable, homogeneous fractions. Efficient isolation of a lymphocyte population enables characterization of the specific fractions in *in vitro* and animal studies[6,9,15]. More recently, mass cytometry and single-cell RNA sequencing are available. These cutting-edge technologies may reveal distinct immune cell signatures of gastric disorders, such as gastric cancers and *H. pylori*-related peptic diseases.

DIAGNOSIS OF GASTRIC LYMPHOMA

In addition to the aforementioned technologies of evaluating gastric diseases, flow cytometry has been used for the diagnosis of lymphoma of the stomach. Flow cytometry is a rapid and practical diagnostic tool for B-cell lymphoma. Analysis of the distribution of surface immunoglobulin light chain kappa and lambda using flow cytometry offers evidence for the monoclonality of B-cell neoplasms because these lymphomas that typically arise from an expansion of a B-cell clone expressing only one class of immunoglobulin light chain, either a kappa or lambda chain. Thus, isolation of lymphocytes from the gastric mucosa and detection of monoclonality using flow cytometry lead to the prompt diagnosis of lymphoma of the stomach. This approach may be particularly useful for the detection and therapeutic monitoring of extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma), which is the most common non-Hodgkin lymphoma subtype arising in the stomach [68-70].

We have reported the utility of a single-step lymphocyte isolation procedure from an endoscopic biopsy specimen, which is described above, for the diagnosis of gastrointestinal lymphoma[67,71]. Our previous study included patients with gastric extranodal marginal zone MALT lymphoma (n = 8), duodenal follicular lymphoma (grade 1; n = 5), and benign lymphoid hyperplasia (ileum, n = 1, and rectum, n = 1). Lymphocytes were successfully isolated from 14 (93.3%) patients. The sensitivity and specificity of flow cytometric analysis of immunoglobulin light chain expression for the diagnosis of B-cell lymphoma were 83.3% and 100%, respectively. These results suggest that a single endoscopic biopsy specimen contains enough lymphocytes for flow cytometric analysis and can be used for the diagnosis of gastrointestinal lymphoma.

CONCLUSION

In this review, we provided methodologies for lymphocyte isolation from the gastric mucosa that are reported in the literature. We also described the history and current

trends of lymphocyte analysis in the stomach. Owing to the multicolor analysis that accurately defines the surface antigen profile of specific lymphocyte populations, flow cytometry will continue to be a powerful tool for revealing the pathogenesis of gastric disorders. We believe that the methodologies described herein will provide a better understanding of the application of flow cytometry.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 July 20; 11(4): 208-221

DOI: 10.5662/wjm.v11.i4.208

ISSN 2222-0682 (online)

MINIREVIEWS

One-day seminar for residents for implementing abdominal pocket-sized ultrasound

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Author contributions: Naganuma H and Ishida H collected the data and wrote the paper.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Manuscript source: Invited manuscript

Specialty type: Medical laboratory technology

Country/Territory of origin: Japan

Peer-review report's scientific quality classification

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Abstract

Despite its proven high utility, integration of pocked-sized portable ultrasound (US) into internal medicine residency training remains inconsistent. For 10 years, we have held a 1-d seminar biannually, consisting of lecture (half-day) and hands-on training (half-day) on pocket-sized US of the abdomen and lungs. The lecture consists of training on US physics and clinical applications of pocket-sized US, followed by a lecture covering the basic anatomy of the abdomen and lungs and introducing the systemic scanning method. Given the simple structure of pocket-sized US devices, understanding the basic physics is sufficient yet necessary to operate the pocket-sized US device. It is important to understand the selection of probes, adjustment of B mode gain, adjustment of color gain, and acoustic impedance. Basic comprehension may have a significant positive impact on the overall utilization of pocket-sized US devices. The easiest and most reliable way to observe the whole abdomen and lungs is a combination of transverse, sagittal, and oblique scanning, pursuing the main vascular system from the center to the periphery of the organ in the abdomen and systemic scanning of the pleura. There is usually a marked change in knowledge and attitudes among the program participants, although skill gaps remain among them. We discuss the limitations and problems to this education system as well.

Key Words: Pocket-sized ultrasound; Abdomen; Lung; Medical education; Resident; Ultrasound physics

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 27, 2021
Peer-review started: February 27, 2021
First decision: March 31, 2021
Revised: April 10, 2021
Accepted: June 1, 2021
Article in press: June 1, 2021
Published online: July 20, 2021

P-Reviewer: Deshwal H
S-Editor: Fan JR
L-Editor: A
P-Editor: Wang LYT

Core Tip: Despite its high utility, there is no standardized method to integrate pocked-sized ultrasound (US) into daily clinical settings. We present here our 1-d seminar for residents that consists of lecture and hands-on training on pocket-sized US. The lecture consists of training on US physics and clinical applications of pocket-sized US, covering basic anatomy, and introducing the systemic scanning method. Understanding of some basic physics is necessary to operate the pocket-sized US device. Although the residents' skill gaps remain, the seminar yields a marked change in knowledge and attitude towards pocket-sized US.

Citation: Naganuma H, Ishida H. One-day seminar for residents for implementing abdominal pocket-sized ultrasound. *World J Methodol* 2021; 11(4): 208-221
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/208.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.208

INTRODUCTION

As the worldwide population rapidly ages, a concurrent rapid increase in the global financial healthcare burden has been observed[1]. The resources available for daily clinical practice are generally limited. Thus, simple, effective, and realistic systemic methods in patient care and medical education are urgently required. Actual clinical scenarios are varied, complex, and require a prompt response. Thus, the traditional medical approaches (inspection, palpation, auscultation, or other components of the physical examination and laboratory data analysis) cannot immediately answer the questions raised during clinical practice[2,3]. Addition of a "scientific eye" in the hand is expected to resolve such problems to a certain degree.

Recent advances in ultrasound (US) technology have made US instruments continuously smaller[4]. Among all of the medical imaging tools, the US device is the only instrument to be miniaturized. In the past, US examination was performed by radiologists using a bulky, expensive, and specialized machine with sophisticated functions in a specialized room[5]. US is currently utilized as an important adjunct to physical examination in a wide range of clinical situations due to the development of small-sized devices[6]. Portable US devices were first used in cardiology[7,8] then in emergent medicine[9,10]. A comparative study between fifth-year medical students using US and cardiologists not using US showed that the former achieved a correct diagnosis 75% of the time, while the latter achieved a correct diagnosis 49% of the time, indicating the importance of visual information in the diagnosis[8]. It also suggests that practitioners unexperienced with US can obtain adequate proficiency with minimal training.

The benefits of portable US are that it is widely available, and carries no risk of radiation exposure. However, an important disadvantage to using this tool is that its diagnostic accuracy is highly operator-dependent, and errors occur during image acquisition and image interpretation (like in traditional US examination)[4]. Furthermore, there is no standardized technique among examiners for observing the abdomen by portable US[11]. However, the benefits of portable US must be emphasized against such inconveniences, and US education is important to extend the benefit of portable US devices. Recent studies have reported favorable results of US education for medical students[12-14]. Though in our opinion, US education for new residents are more meaningful than for medical students. At the residency level, there is a deeper understanding of balancing portable US results and patient characteristics to establish clinical decision making. This background motivated us to begin a portable US education seminar intended for new internal medicine residents.

In this review, we present our US education 1-d seminar for new (post graduate year-1 or -2) internal medicine residents that focuses on the use of pocket-sized US on the abdomen and lungs. The goal of the seminar is for residents to have the confidence to integrate pocket-sized US results into their clinical decision making.

THE POCKET-SIZED US EDUCATION SEMINAR

The use of portable US devices has recently been adopted more frequently in clinical settings, and the market is flourishing[15]. Portable US devices have passed through three generations: Laptop-associated devices (personal computer-sized machine coupled with multiple applications), hand-carried devices (book-sized machine carried by hand), and hand-held devices (portable and able to fit in the pocket of a clinician's white coat, thus referred to as pocket-sized US). Each US company has different built-in applications, such as Doppler display, puncture guidance, and distance or volume measurement[16].

Pocket-sized US devices are usually used by unexperienced non-radiologist general clinicians under the name of "US stethoscope"[4] to complement clinical examination and provide immediate visual correlates of clinical findings. The concept of a US stethoscope is expanding worldwide, but like high-end US machines, pocket-sized US devices are effective in the hands of experienced examiners. Furthermore, comparative studies between pocket-sized US and high-end US have stressed some important points: the image quality of pocket-sized US is slightly inferior to high-end US but still satisfactory for clinical use[17,18]. US measurement by pocket-sized US devices is feasible, and acoustic power of the US machine strongly affects the penetration and resolution of the US image [mechanical index: 0.9-1.0 (pocket-sized US) *vs* 1.8 (high-end US)]. Pocket-sized US devices will not replace a high-end US machine and the need for detailed US examination by experienced radiologists will always be present, but there is value in the pocket-sized US examination.

Since 2010, we have biannually hosted a 1-d seminar consisting of lecture (half-day) and hands-on training (half-day) on portable US devices to increase the number of portable US operators and to develop the examination skills among new residents. For this hands-on training, we use pocket-sized US devices because of their portability and affordability (the most important characteristic of portable US devices when thinking of expected future applications). The aim of this seminar is to familiarize residents with pocket-sized US devices but not to immediately increase their US capability.

In the lecture, we begin with a simple lecture about fundamental US physics to optimize pocket-sized US application and to minimize errors in device manipulation and US image interpretation. The next lecture covers the basic anatomy of the abdomen and lungs as well as introducing systemic scanning procedures (Figure 1). In the lecture, we present both high-end US images and pocked-sized US images simultaneously in order to compare these images. This comparison is necessary to become familiar with the slightly inferior image quality of pocket-sized US (Figure 2). In the remaining time, we present a few model cases where pocked-sized US information helped create a patient care strategy followed briefly by a lecture on built-in applications including color Doppler US.

HALF-DAY LECTURE

Mini lecture on simple US physics and instrumentation

Compared with sophisticated and complex high-end applications, such as contrast-enhanced US and shear wave elastography, the pocket-sized US device has a simple structure. Understanding basic physics is sufficient but necessary to begin to operate the pocket-sized US device. During this lecture, we stress four basic points of US physics needed to prevent device manipulation errors[19]: Selection of probes (high-frequency linear probe for superficial areas, such as the gastrointestinal tract and pleura or conventional sector (convex) probe for deep areas, such as liver and abdominal vessels), adjustment of B mode gain, adjustment of color gain, and problems related with acoustic impedance/the reason why US detection of stones (biliary, renal, and others) and fluid collection (ascites, pleural effusion, and others) is highly sensitive. This basic comprehension has a significant positive impact on the utilization of pocket-sized US devices.

Probe selection: With the transducer, the US beams are steered at varying angles from one side to the other to produce a sector format. This format permits a large, deep field of view, but its near field focus is reduced. Therefore, this format is unsuitable for observing superficial areas. The linear probe activates a group of elements to generate perpendicular US beams, which provide a high resolution in the near field[20]. As a result, probe selection is dependent on the type of organ to be observed (Figure 3).

Figure 1 Lecture by a clinician who specializes in ultrasound. The lecture covers basic ultrasound physics and simple ultrasound anatomy of the abdomen.

Adjustment of B mode gain: The echo signal amplitudes are usually compressed in order to be accommodated by the display. This accommodation serves to visualize a wide range of echo signals on the display but reduces the real differences in the echo signal amplitude. As a result, when B mode gain is too low or too high, we cannot recognize small differences in echogenicity (echo signal amplitude). Thus, B mode gain should be adjusted in order to not overlook the abnormality (Figure 4).

Adjustment of color gain: In color Doppler US, flow velocity in each point is indicated by color brightness: the higher the velocity, the brighter the color. Color Doppler gain is the receiver end amplification of the Doppler signal. The Doppler gain is usually increased to the maximum limit just before the background noise is seen. If color Doppler gain is too high, then the field of view is filled with noise. If it is too low, then useful Doppler data is not seen on the display. Thus, color Doppler gain must be properly adjusted to gain useful hemodynamic information (Figure 5).

US physics related to stones and fluid: Acoustic impedance is an important property of tissues. It is defined as the tissue density multiplied by its propagation velocity of sound[21]. Acoustic impedance changes according to the tissue (*e.g.*, water: 1.48×10^6 $kg/m^2/s$, liver: 1.65×10^6 $kg/m^2/s$, bone: 7.80×10^6 $kg/m^2/s$)[21]. When passing through two tissues of different acoustic impedance, some portion of the US is reflected. Its reflection degree depends on the difference in acoustic impedance between two tissues. The reflection of the US is larger between water (fluid collection) and soft tissue than between two soft tissues, leading to the fact that fluid collection is clearly margined and easily detected[22]. The reflection of the US is larger between soft tissue and stone. Almost all the US is reflected at the stone surface. Therefore, stones are easily and correctly detected by US[23] (Figure 6).

Mini lecture on fundamental scanning methods

For detailed US diagnosis, the examiner is expected to make a differential diagnosis of a wide spectrum of diseases. Meticulous scanning techniques are required for this purpose. However, because most participants do not have sufficient prior knowledge of US examination, our initial effort is focused on understanding the global normal anatomy. The combination of transverse, sagittal, and oblique (intercostal) scanning (Figures 7 and 8) permits the efficient observation of the abdominal organs. The most reliable way to observe each organ (liver, pancreas, spleen, and kidneys) is to pursue the main vascular system (landmark) from the center to the periphery of the organ. After the training in each organ, the parenchymal echostructures around the vessel are

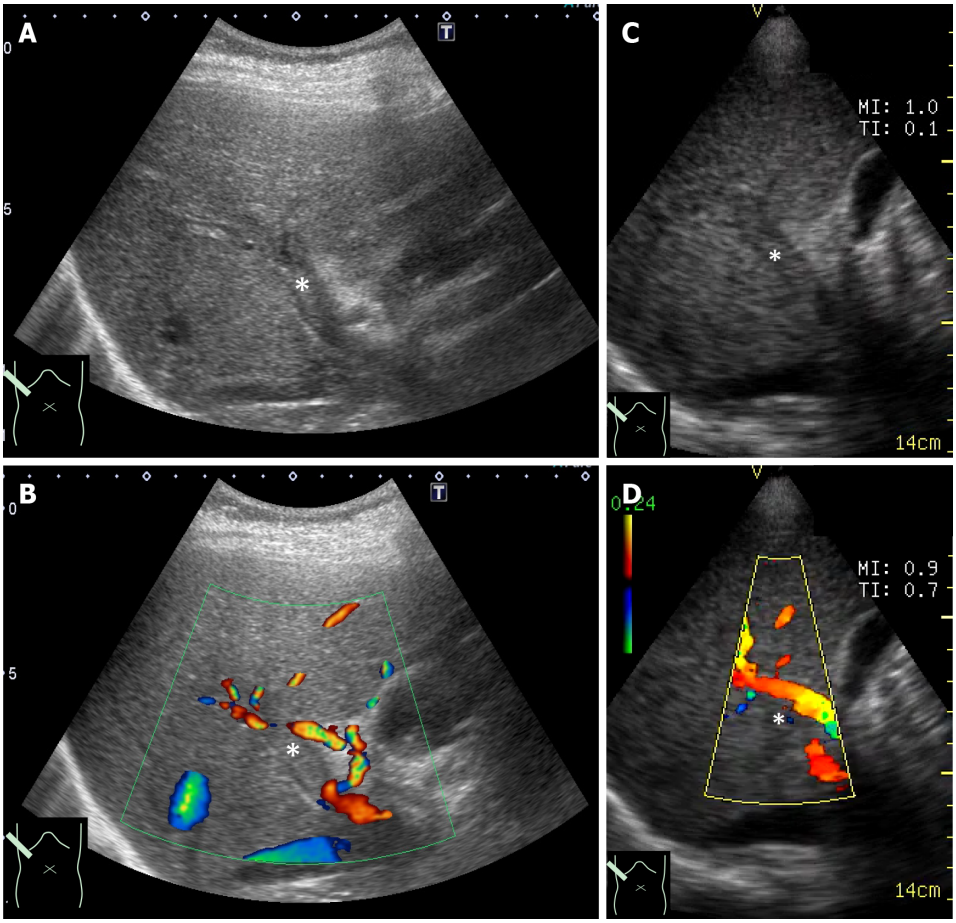

Figure 2 Simultaneous presentation of ultrasound images of a portal thrombus. A: High-end ultrasound B mode; B: High-end ultrasound color Doppler; C: Pocket-sized ultrasound B mode; D: Pocket-sized ultrasound color Doppler. These comparisons are used to understand the difference in image quality between the two machines. It also confirms that pocket-sized ultrasound is sufficient for diagnoses. *: Thrombus in the portal vein.

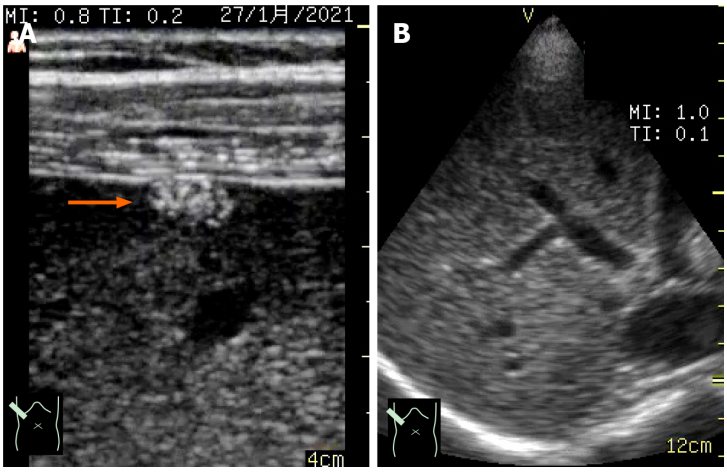

Figure 3 Ultrasound image by different probes on a pocket-sized ultrasound device. A: High-frequency linear probe; B: 1.7-3.8 MHz sector probe. The linear probe is used to visualize superficial areas, and the sector probe is used for observing deep areas. In this case of a small liver tumor situated at the hepatic surface, the lesion was detected by the linear probe (→) but not the sector probe.

presented. Recognition of these landscapes serves to detect abnormal lesions. Use of the high-frequency linear probe permits the observation of the pleura (Figure 9). The hands-on training following this short lecture reinforces the anatomical knowledge of the abdomen/Lungs and the technical skills (transducer handling, portable US device manipulation, and confidence in abdominal observation by pocket-sized US).

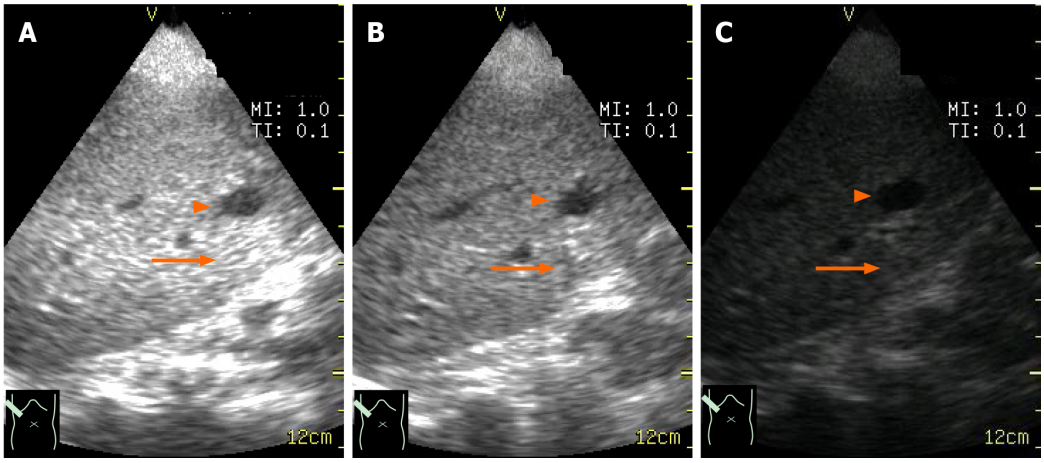

Figure 4 B mode gain setting (hepatic cyst). A: B mode is too high; B: B mode is well-adjusted; C: B mode is too low. The lesion anechoic mass (arrowhead) with acoustic enhancement (arrow) is clearly seen only when the B mode gain is well-adjusted.

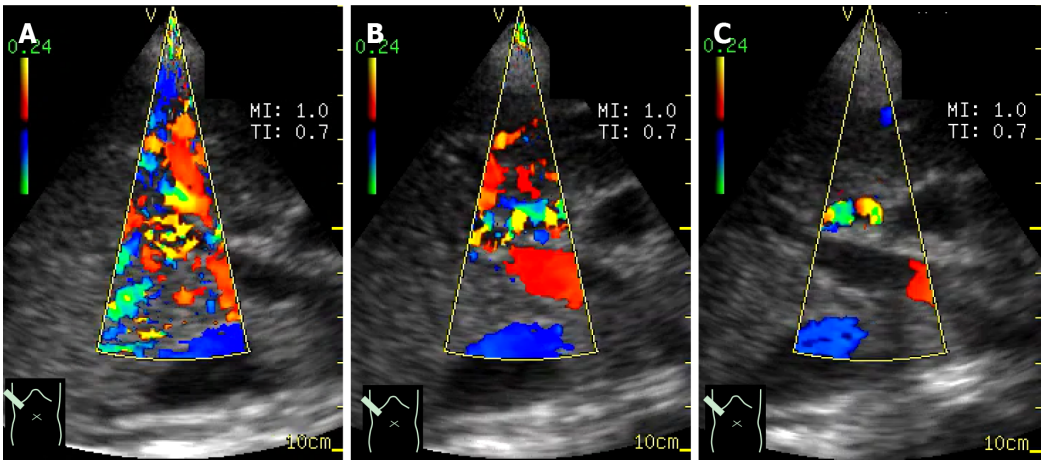

Figure 5 Color Doppler gain setting (normal portal vein). A: Gain setting is too high; B: Gain setting is well-adjusted; C: Gain setting is too low. Portal venous flow is not detected when color gain setting is too low (C) and is covered by color noise when it is too high (A).

Presentation of model cases

Then, we present several clinical cases where portable US was useful for confirming a wide spectrum of clinical applications.

Case 1: A 35-year-old female with known repetitive gastric ulcers presented with abdominal pain and nausea visited our outpatient clinic (Gastroenterology). Initially, the clinician may suspect a recurrence of the gastric ulcers. Though the patient denied the possibility of pregnancy, the use of a pocket-sized US device confirmed the pregnancy. The proper use of the pocket-sized US device led to reduced ionizing radiation exposure and allowed to the transfer of the patient to the gynecology section (Figure 10).

Case 2: A 72-year-old female with severe abdominal pain visited our emergency department. The pain was so severe that she could not move from the ambulance. A pocket-sized US device revealed an impacted gallbladder stone. The pain worsened upon probe compression of the gallbladder, which led to the diagnosis of acute stone-impact-induced cholecystitis. The patient was immediately treated. The pocket-sized US device significantly reduced the time to diagnosis (Figure 11).

Case 3: A 41-year-old female was admitted to our hospital with a chief complaint of hepatic dysfunction. She was almost asymptomatic. A pocket-sized US device was used as part of the physical examination during rounds. The US revealed a small amount of ascites around the liver, and her gallbladder collapsed. Biochemical examination was immediately ordered and showed markedly elevated transaminases and coagulopathy. She was diagnosed with severe acute hepatitis, and energic

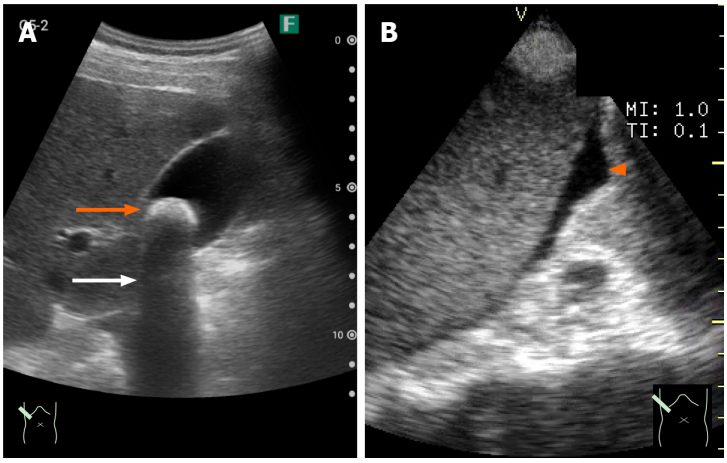

Figure 6 Pocket-sized ultrasound images of gallbladder stones and ascites. A: A 1-cm stone; B: A small amount of ascites. A 1-cm stone (A) and a small amount of ascites (B) is clearly visualized as a strong echo (orange arrow) with acoustic shadowing (white arrow) in the former and an echo-free space in the latter (arrowhead).

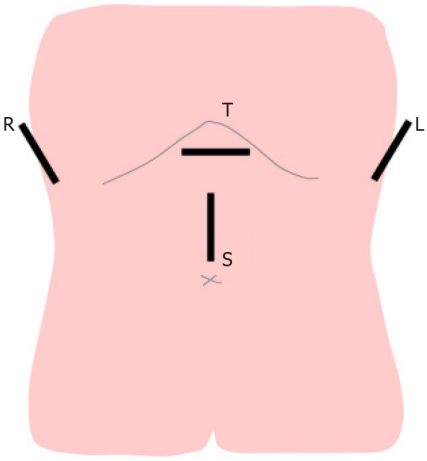

Figure 7 Schematic drawing of basic scanning planes of the abdomen. The combination of these indicated planes permits quick observation of the whole abdomen. The transverse plane of the upper abdomen is for observation of the pancreas. The right upper abdominal plane is for observation of the liver and the gallbladder. The left upper abdominal plane is for observation of the spleen and left kidney. The sagittal plane is for observation of the abdominal aorta and its branches. L: Left upper plane; R: Right upper plane; S: Sagittal plane; T: Transverse plane.

treatment began immediately (Figure 12).

Although these US examinations were successfully performed by skillful pocket-sized US practitioners, the program participants can image how to utilize a pocket-sized US device in their own clinical setting[22-24].

Built-in applications in pocket-sized US devices

Each US company has different built-in applications, such as a Doppler display (Figures 2 and 5), embedded in almost all devices. Puncture guidance is embedded in about half of all devices[25], and distance or volume measurement function is embedded in a small number of devices (Figure 13)[16,26]. Biplane imaging is embedded in a limited number of devices[27], and wireless function is embedded in a small number of devices (Figure 14).

HALF-DAY HANDS-ON TRAINING

Following the half-day lecture, the participants are divided into five or six groups (4-5 participants/group). Each participant receives hands-on training for 30 min under the supervision of experienced US physicians (5 min each for liver, biliary system, pancreas, vascular system, digestive tract, and lungs) with both a high-end machine

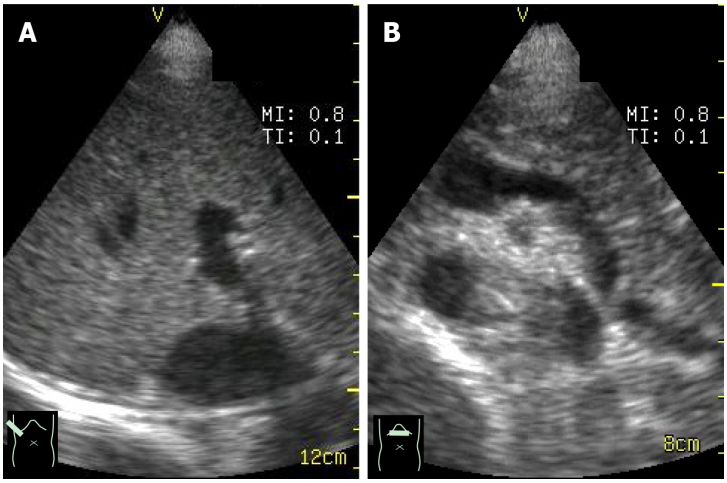

Figure 8 Representative ultrasound images of the abdominal scanning procedure. A: The right upper abdominal scanning plane permits the observation of the liver (right lobe); B: Through the transverse scanning plane, the pancreas and the neighboring vessels are clearly demonstrated.

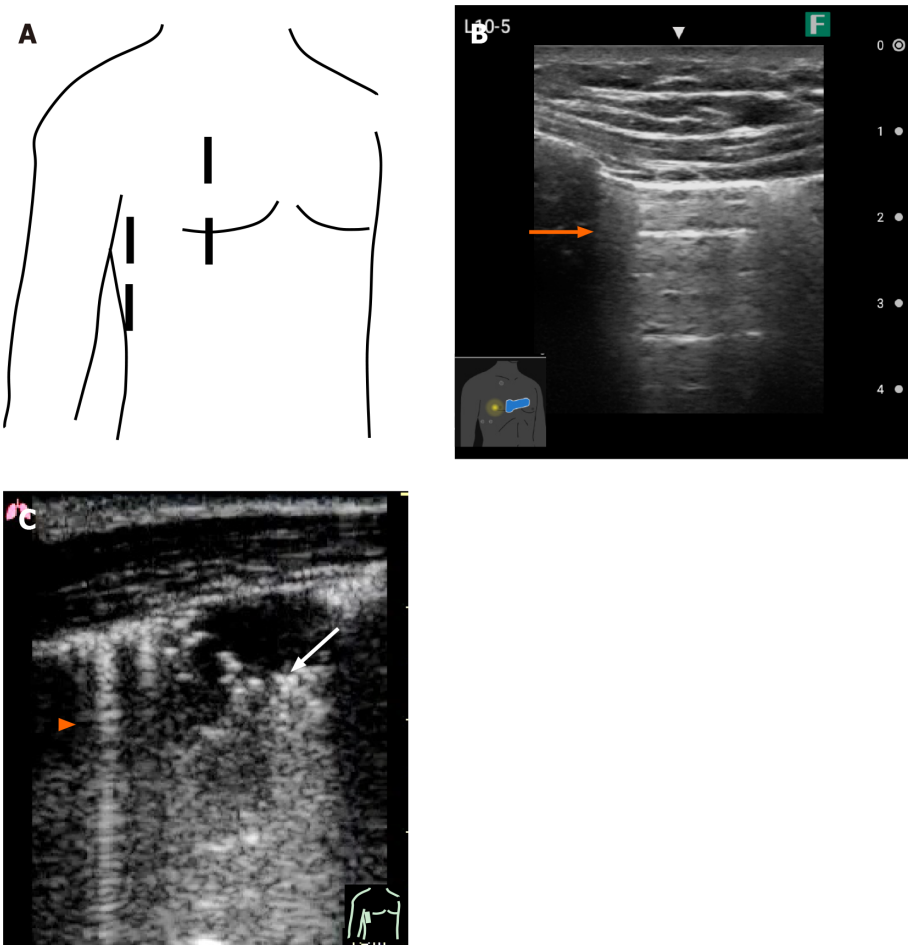

Figure 9 Pocket-sized ultrasound image of the pleura. A: Basic scanning planes of the chest (right) by ultrasound; B: Ultrasound image of normal pleura showing a typical A line (orange arrow); C: Ultrasound image of B line (arrowhead) and consolidation of the lung (white arrow).

and a pocked-sized US device for comparison. The abdomen is generally observed by a medium frequency transducer (Figure 8), and the pleura is observed by a high-frequency linear probe with a focus on pleura (Figure 9)[28-32]. All instructors are certified as registered Senior Medical Sonographers and fellowship-obtained highly diagnostic doctors of the Japan Society of Ultrasonics in Medicine. The participants are required to register in advance because the numbers of instructors and US machines are limited.

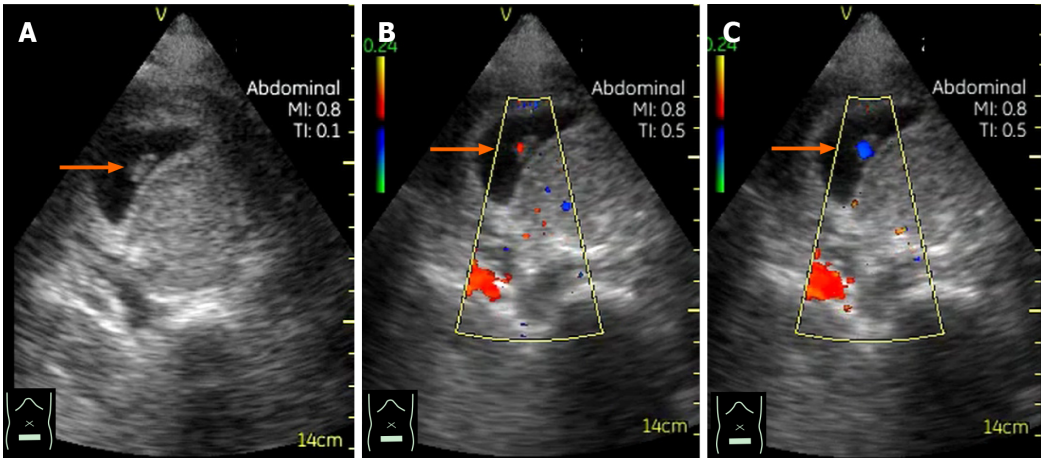

Figure 10 Case presentation 1 of undesirable pregnancy causing abdominal symptoms. A: At presentation, the patient denied the possibility of pregnancy. However, pocket-sized B mode performed as part of a general physical examination revealed an embryo (arrow) in her lower abdomen. B and C: Color Doppler was particularly useful for confirming the baby's cardiac movement. Daily use of pocket-sized ultrasound can lead to a reduction in ionizing radiation and time to correct diagnosis.

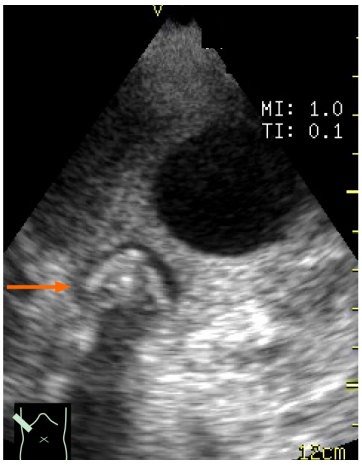

Figure 11 Case presentation 2 of abdominal pain so severe that the patient could not move. The patient's abdominal pain was so severe that she could not move from the ambulance. Pocket-sized ultrasound performed in the ambulance revealed a gallbladder stone impact (arrow), leading to the diagnosis of acute stone-impact-induced cholecystitis.

After the seminar, participants answer a questionnaire to determine whether: (1) The 1-d seminar was useful; (2) The lecture on basic US physics helped in understanding pocket-sized US application; (3) Their general attitude toward the clinical utility of US and pocket-sized US improved; (4) Presentation of clinical cases was meaningful; and (5) They feel that their skill increased after the hand-on training. Thus far, the responses have been unanimously the same: stressing the efficacy of the seminar, especially the lecture on US physics and case presentation, but that their skill gap remains. We have performed one year later a post-seminar questionnaire to the participants, to determine whether (1) Their general attitude towards the clinical utility of pocket-sized US has changed; (2) They perform pocket-sized US in daily practice; and (3) If not, what is the most important barrier. The responses have unanimously stressed that their understanding of the utility of pocket-sized US continue after the seminar. However, no participants use pocket-sized US device in daily practice despite their desire to perform it. The most important barrier is the cost of device (about 8000-10000 US dollars in Japan).

USE OF POCKET-SIZED US DEVICES IN CLINCAL SETTINGS

There are a wide range of possible applications for pocket-sized US devices in offering advanced diagnostic capability to benefit patient care in the clinical setting. They

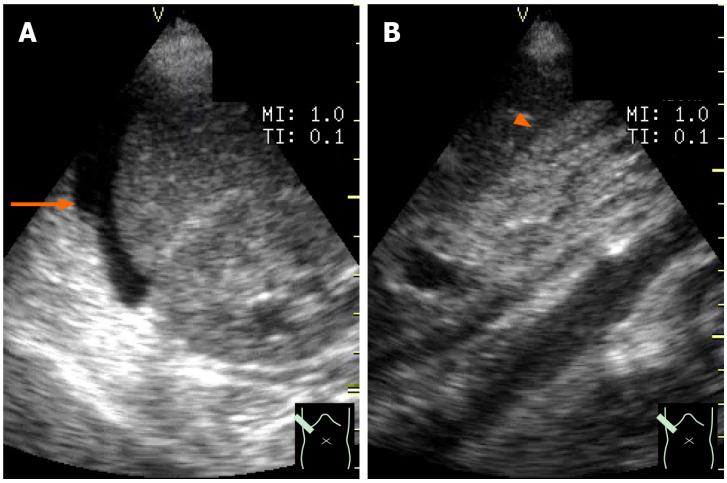

Figure 12 Case presentation 3 of asymptomatic patient with acute hepatitis. The patient was asymptomatic upon admission. A: Pocket-sized ultrasound performed as part of a physical examination during medical rounds revealed a small amount of ascites (arrow) around the liver; B: The gallbladder was collapsed (arrowhead). She was diagnosed with severe acute hepatitis, and an energic treatment began immediately.

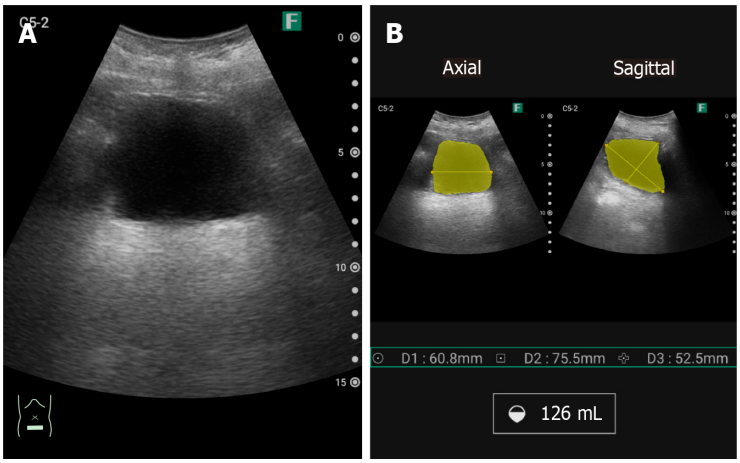

Figure 13 Built-in volume measurement application in pocket-sized ultrasound device. This function is useful for evaluating urine volume in elderly patients. A: Scanning with pocket-sized ultrasound device; B: Urine volume display.

include the following situations.

Emergency

Due to recent advances in US technology and informatics, wireless probes are now a reality (Figure 14). Remote telesonography has the potential to improve the quality of patient care. Pocket-sized US devices are particularly important in situations where the time of examination is urgent (emergency room, intensive care) or the location favors the use of pocket-sized devices (remote locations)[33,34]. More advanced technologies will guarantee rapid transfer of US information to hospitals to provide the best medical care. Becoming wireless is a meaningful function[35]. Furthermore, remote telesonography will improve the quality of US applications in underserved communities[36]. Lesser trained examiners will be able to obtain and interpret US images that impact patient care immediately[37].

Outpatient clinics

Clinicians at outpatient clinics see a range of abdominal problems. The differential diagnosis includes digestive tract, hepatobiliary and pancreatic diseases, and gynecological and urogenital diseases. Portable US provides clinically significant visual information that is not obtainable by physical examination and helps decrease the diagnosis time[38,39].

Figure 14 Built-in wireless function application in pocket-sized ultrasound device. This function is particularly important when sending ultrasound information from distant locations (ambulance, *etc.*) or infection zones.

Inpatient care

The biggest advantage of pocket-sized US devices is the time savings (booting time, transfer, bedside positioning). The most useful function of portable US is the global estimation of fluid volume (ascites, pleural effusion, pericardial effusion) under drug therapy[4]. Pocket-sized US may be repeated due to clinical need but is typically performed for monitoring physiologic or pathologic changes of admitted patients[40]. In addition, US-guided procedures provide safety to a wide variety of punctures (ascites, bile duct, abscess) (Figure 13).

Decontamination assessment must never be compromised. Some recent studies have shown that pocket-sized US performed efficiently as a tool for screening a variety of diseases[16,20,29,30,34,41]. The pocket-sized US devices were also used successfully by other healthcare providers, such as nurses and physical therapists. Additionally, pocket-sized US may be feasible for guiding aspiration needles for the drainage of abscesses, ascites aspiration, *etc.*

Family medicine

The compact size of the pocket-sized US device makes it possible to carry in a doctor bag when visiting patients[4,42,43]. The clinicians feel confident using the pocket-sized US when caring for patients in limited medical conditions[44]. Physicians proficient in its use can quickly answer specific questions at the bedside[45].

CONCLUSION

In this review, we have presented our 1-d method for implementation of pocket-sized US into the clinical setting. The biannual nature of the seminar is insufficient for completely integrating the pocket-sized US device into frequent clinical use. However, this method contributes to new residents gaining confidence in using the pocket-sized US despite the skill gap remaining. However, there are many limitations. There are a small number of US experts who can correctly and efficiently teach US physics and abdominal anatomy to new doctors and supervise their hands-on training. The "teach-the-teacher" system is important for training new US practitioners[46,47]. Costs related to US equipment may present additional (and the most important) obstacles to develop and continue pocket-sized US training programs. We recommend that all the US leaders and experts find the means to integrate pocket- sized US into clinical setting through the training of new clinicians.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 July 20; 11(4): 222-227

DOI: 10.5662/wjm.v11.i4.222

ISSN 2222-0682 (online)

MINIREVIEWS

Wound irrigation for preventing surgical site infections

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Author contributions: Marios Papadakis was involved in the study conceptualization, literature research, project administration, and writing of the original draft.

Conflict-of-interest statement: Author has nothing to disclose.

Manuscript source: Invited manuscript

Specialty type: Surgery

Country/Territory of origin: Germany

Peer-review report's scientific quality classification

Grade A (Excellent): 0

Grade B (Very good): B

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Abstract

Wound irrigation (*i.e.* washing out a wound before wound closure) aims to reduce the microbial burden by removing tissue debris, metabolic waste, and tissue exudate from the surgical field before site closure. Although it is a popular procedure in every day surgical practice, the lack of procedure standardization, leads to studies with high heterogeneity and often controversial results. Thus, there are studies that advocate its use, while others discourage its implementation in clinical practice to reduce the risk of surgical site infection. The present article reviews the current literature on wound irrigation for preventing surgical site infections. Several irrigants are presented. Chlorexidine is generally considered to be less effective than povidone-iodine, while antibiotics are not that common nowadays, as they require prolonged exposure with the target to act. Hydrogen peroxide has several potential complications, which eliminate its use. Any differences in the incidence of surgical site infections between different irrigants, especially between antibacterial and non-bacterial ones, should be viewed sceptically. More randomized controlled studies are needed to provide better quality of evidence regarding the irrigants' effectiveness and safety.

Key Words: Wound irrigation; Surgical site infections; Antiseptics; Antibiotics; Patient Safety

Core Tip: Chlorexidine is generally considered to be less effective than povidone-iodine, while antibiotics are not that common nowadays, as they require prolonged exposure with the target to act. Hydrogen peroxide has several potential complications, which eliminate its use. Any differences in the incidence of surgical site infections between different irrigants, especially between antibacterial and non-bacterial ones, should be viewed sceptically.

Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 21, 2021
Peer-review started: February 21, 2021
First decision: May 6, 2021
Revised: May 6, 2021
Accepted: June 25, 2021
Article in press: June 25, 2021
Published online: July 20, 2021

P-Reviewer: Serban D
S-Editor: Liu M
L-Editor: A
P-Editor: Zhang YL

Citation: Papadakis M. Wound irrigation for preventing surgical site infections. *World J Methodol* 2021; 11(4): 222-227
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/222.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.222

INTRODUCTION

Surgical site infections (SSI) arise from contamination of the surgical site in the period of time between incision and closure[1]. According to the Centers for Disease Control (CDC), SSI are defined as infections occurring at the site of surgery within 30 d after surgery, or, within 1 year if an implant is placed and the infection appears to be related to surgery[2]. CDC classifies SSI as incisional SSI (superficial or deep) and organ/space SSI. Superficial incisional SSI typically involve only the skin and subcutaneous tissue, while deep incisional SSI involve deep soft tissues, such as fascial and muscle layers. The term "organ/space" refers to any part of the anatomy (*e.g.*, organs or spaces), other than the incision, opened or manipulated during the operative procedure. Infections here are called organ/space SSI[2]. When organ/space SSI drain through the incision, they do not require reoperation and are classified as deep incisional SSI.

The CDC definitions have been criticized because they always end with the disclaimer that an infection exists if the surgeon or the attending physician declares that an infection exists. Similarly, as Fry states[3], "an infection may not exist if the surgeon says that an infection does not exist". Given the pressure for early discharge, it is likely that a substantial number of patients leave the hospital without having their SSI reported.

Wound irrigation (*i.e.* washing out a wound before wound closure) aims to reduce the microbial burden by removing tissue debris, metabolic waste, and tissue exudate from the surgical field before site closure[4]. Interestingly, although it has been shown to be beneficial in selected surgical disciplines, not only it is not a universally established standard-of-care preventive measure but there are guidelines that do not recommend its use to reduce the risk of SSI[5]. The present article the current literature on wound irrigation for preventing SSI.

IRRIGANTS

Irrigants are classified to normal saline, antiseptic agents and antibiotic agents. According to a Cochrane metaanalysis, there is only low-quality evidence available and, therefore, any differences in the incidence of SSI between different irrigants, especially between antibacterial and non-bacterial ones, should be viewed sceptically [6].

Normal saline

Warmed physiologic saline is universally considered the irrigation fluid of choice. It is widely available and safe for all surgical site surfaces, including the peritoneal and pleural cavities (serosal mesothelium)[4]. However, recent metaanalyses could not identify an advantage of irrigation with normal saline over no irrigation in patients undergoing abdominal surgery[7,8].

Pressurized (< 15 psi) pulse irrigation of subcutaneous tissue with saline may reduce bacterial counts by removing the desiccated tissue. It is considered a cost-effective infection prevention strategy, when applied in major laparotomy wounds in prolonged operative procedures (> 2 h) as it reduces SSI[9].

Antiseptics

Chlorhexidine: The most common antiseptic agent used is chlorhexidine gluconate (CHG), as it covers a broad spectrum of pathogens, including gram-negative, gram-positive and non-spore forming bacteria[10]. CHG disrupts the bacterial cell membrane within 30 s and in concentrations of 0.05%, it kills biofilm-based S. epidermidis in less than a minute.

However, there are limited data regarding its effectiveness when used for intraoperative irrigation. In vitro, CHG is found to be less effective than povidone-iodine (see below)[4,11].

Goztok *et al*[10] compared 0.05% CHG to saline in patients undergoing temporary loop ileostomy closure. This was a retrospective study of a prospectively collected database. Irrigation of the surgical site with CHG was associated with significantly lower rates of incisional SSI (32% *vs* 5%), incision dehiscence (32% *vs* 5%) and seroma formation (14% *vs* 2%). The authors also observed an earlier site healing in the CHG group (10 d *vs* 7 d)[10]. In pilonidal disease, wound irrigation with CHG is associated with lower SSI rates but it does not prevent seroma formation or incision dehiscence [12].

In plastic surgery, CHG is considered unable to penetrate the biofilm forming on the breast implants' surface. An in-vitro model assessing SSI prophylaxis after breast implant surgery showed that a 0.05% CHG solution can achieve sterility after 15 min exposure, while its effectiveness against Pseudomonas was absent in 40% of the cases [13]. Some authors advocate the usage of hypochlorous solution as an irrigant, as hypochlorous acid has a wide spectrum of antibacterial efficacy against Gramnegative, Gram-positive bacteria and fungi. This solution has the advantage of bypassing the need for neutrophils to be present in the wound implant interface[14].

Moreover, CHG is superior to saline in resolving MRSA biofilm-mediated polypropylene mesh infections[4].

Iodophors: Iodine has traditionally been used for prevention and treatment of wound infection, as it is effective against a broad spectrum of microorganisms, including gram-negative and gram-positive bacteria, spores, mycobacteria, fungi, viruses *etc.* However, it is toxic to vital tissues. For this reason it is almost always combined with povidone.

Most povidone-iodine (PVP-I) solutions contain 10% iodine, although iodine has been shown to preserve its full effect even in solutions with 1:100 dilution of the full-strength (10%) solution[11]. PVP-I is effective against highly resistant gram-positive microorganisms as it not only destroys the cell wall but also inhibits the release of endotoxins, exotoxins and tissue-destroying enzymes[11]. Some authors let the solution soak in the wound for 3 min before been suctioned[15].

PVP-I can kill biofilm-forming strain of Staphylococcus but this requires concentrations as high as 10% for 1 min or 3.5% for 10 min. However, the 10% solution is recommended only for external use and 10-min irrigations can not easily be implemented in clinical practice, as irrigations usually last 1-2 min[4].

Irrigation with diluted PVP-I is very popular among surgeons. It is used from more than 50% of visceral surgeons and one third of orthopaedic surgeons[16]. It is more effective in preventing SSI in abdominal surgery compared to no irrigation, but less effective compared to antibiotics[7]. A randomised controlled trial in women undergoing ceasarean section showed that povidone-iodine irrigation prior to skin closure does not prevent SSI[17]. Surprisingly, elective cesarean section was associated with higher infection rates compared to emergency cesarean section during labour[17].

Hydrogen peroxide: Hydrogen peroxide (H_2O_2) is a highly reactive oxidizing agent, effective against a broad range of microorganisms. H_2O_2 forms oxygen species that react with macromolecules such as membrane lipids and destroy bacteria. Its disadvantages include its rapid decomposition upon contact with organic material and its low effectiveness against catalase-producing bacteria (11-Ulivieri 2011). It is commercially available at concentrations of 3% and 30%, which can be diluted with saline solution to any desired concentration[18]. The 3% solution is found to be cytotoxic but it does not seem to affect wound reepithelialization[19]. Apart from cytotoxicity, another potential serious complication of H_2O_2 is air embolism, especially in closed cavities, as large volumes are pressurized into small vascular channels. For this reason, wound irrigation with H2O2 should be followed by copious irrigation with normal saline or other liquid and accompanied by placement of a surgical drain [19]. Its potential complications are the main reason why it is in most cases used in combinations with other antiseptics (see below), making it difficult to estimate its effect alone. However, there is limited high-level evidence supporting its use as a wound irritant, as most available studies are small-scale case series.

Soap: Soap has also been studied as a wound irritant. It mainly acts as an emulsifier, dispersing one liquid into another one. It has several advantages, i.e. it is widely available, cheap, less toxic and not prone to antibioc resistance. However, according to a randomized controlled study, normal saline has proven superior to soap in terms of reoperation rates, when used in th initial management of patients with open fractures [20].

Combinations: Several antiseptic combinations have been shown to have synergistic

effect. The combination of CHG with H_2O_2 is synergistic against species of Streptococcus and Staphylococcus. The combination of PVP–I with H_2O_2 is reported to reduce the rate of post-operative infection in spine surgery from 1.5% to zero[11]. In single stage exchange arthroplasty for hip and knee periprosthetic joint infections, a combination of 1% PVP-I and a 50:50 dilution of 3% H_2O_2 can prevent from recurrences of infection[19]. The effect can be explained by the fact that the combination is bacteriocidal, while both substances are bacteriostatic when used separately[19].

Antibiotic agents

Antibiotic agents are still widely used in irrigation fluids in almost all surgical disciplines, with rates as high as 22% in plastic surgery and 50% in general surgery. Main reason for this wide use is the failure to appreciate the mechanistic nature of how antibiotic agents work. Antimicrobial activity requires sufficient contact time for the antibiotic agent to bind to its target site. A second requirement is a persistent drug concentration above the MIC90, *i.e.* the concentration of the antibiotic agent that is required to kill 90% of the microbial population. These requirements are not met during antibiotic irrigation, as the irrigating fluid is rapidly removed[4]. In fact, antibiotic activity should be present in the tissue at the time of contamination of the surgical incision for infection to be prevented[3].

On the contrary, the use of antibiotics for wound irrigation may cause harm. Except for the risk of potential development of antimicrobial resistance, it may induce severe anaphylaxis, whereas some antibiotics, *i.e.*, neomycin and vancomycin, have been associated with tissue irritation or systemic toxicity when added in the irrigation fluid. Bacitracin for injection, an agent that is mostly used off-label for wound irrigation, has found to have severe side effects, i.e. nephrotoxicity and anaphylaxis, that outweigh its potential benefits. The FDA requested its voluntary withdrawal from the market on January 31, 2020[1].

In open appendectomy, layer-by-layer wound irrigation is shown to decrease the rates of incisional SSI compared to the no-irrigation group. However, adding gentamicin to saline solution did not further decrease SSI rates[21]. A recent meta-analysis showed no benefit of irrigation with antibiotic agents in reducing incisional SSI and discourages its use[22]. A network metaanalysis found that antibiotic and antiseptic irrigation had the lowest odds of SSI. Aminoglycosides had the lowest OR of SSI compared to non-antibacterial irrigation, followed by penicillin. However, there was high heterogeneity and irrigation of antibiotic agents was more likely to enroll patients undergoing operations with higher levels of contamination[23].

Although antimicrobial wound irrigation is reported to be superior to placebo for surgical prophylaxis in some studies, no study supports its superiority over parenteral administration of antimicrobials[1].

The combination of intrawound vancomycin powder and betadine irrigation was found to reduce SSI rates after posterior spinal fusion in patients with idiopathic scoliosis[24]. In another study of spine surgery patients, the same combination was found to reduce the proportion of gram positive cultures from 53% to 80% and MRSA infections from 7% to 30%. Multibacterial infections also decreased from 27% to 37% and were found to consist of just 7 different organisms, compared to 15 organisms without intervention. Based on these findings, the authors recommend adding one more prophylactic agent targeted for further reduction of the proliferation of gram positive bacteria. However, the addition of antibiotic agent that reduce gram negative bacteria is also important, as such organisms are found in SSI[25].

On the contrary, intraoperative irrigation with ceftriaxone did not reduce SSI in clean neurosurgical procedures when prophylactic intravenous antibiotics are administered (Okunlola 2020). Rifampicin has also been tested as a washing and irrigation solution in spinal instrumentation. However, both were found to be ineffective in preventing or reducing spinal implant infections[26].

In plastic surgery, combined antibiotic solutions are proven effective to *in vitro* eliminate MRSA and MSSA after breast implant reconstruction. Interestingly, adding of vancomycin did not increase in their effectiveness. However, all combinations required prolonged irrigation time to achieve sterility of the experimental surgical site [13].

CONCLUSION

Although wound irrigation is a popular procedure in every day surgical practice, the lack of procedure standardization, leads to high heterogeneity that downgrades the

level of evidence of the available studie. The existing studies have often controversial conclusions. Any differences in the incidence of SSI between different irrigants, especially between antibacterial and non-bacterial ones, should be viewed sceptically. Chlorexidine is generally considered to be less effective than povidone-iodine, while antibiotics are not that common nowadays, as they require prolonged exposure with the target to act. Hydrogen peroxide has several potential complications, which eliminate its use. More randomized controlled studies are needed to provide better quality of evidence regarding the irrigants' effectiveness and safety.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 July 20; 11(4): 228-230

DOI: 10.5662/wjm.v11.i4.228

ISSN 2222-0682 (online)

LETTER TO THE EDITOR

Simplified figure to present direct and indirect comparisons: Revisiting the graph 10 years later

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Author contributions: Fadda V and Messori A were the main contributors of this paper; The other authors were involved in checking the manuscript, identifying inconsistencies, and generating the figure.

Conflict-of-interest statement: The authors declare no conflicts of interests.

Manuscript source: Unsolicited manuscript

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Abstract

A "simplified" figure was proposed in 2011 to summarize the results of controlled trials that evaluate different treatments aimed at the same disease condition. The original criteria for classifying individual binary comparisons included superiority, inferiority and no significance difference; hence, they did not differentiate between no proof of difference *vs* proof of no difference. We updated the criteria employed in the original "simplified" figure in order to include this differentiation. A revised version of the simplified figure is proposed and described herein. An example of application is also presented. The example is focused on first-line treatments for paroxysmal atrial fibrillation. Three treatments (medical therapy, cryoballoon ablation, radiofrequency ablation) are compared with one another through direct and indirect comparisons.

Key Words: Randomised controlled trials; Outcome research; Meta-analysis; Direct comparisons; Indirect comparison; Statistics

Core Tip: A "simplified" figure was proposed in 2011 to summarize the results of controlled trials that evaluate different treatments aimed at the same disease condition. This graphical tool presents the network geometry along with the results of the analysis. The original criteria for classifying individual binary comparisons (direct or indirect comparisons) did not differentiate between no proof of difference *vs* proof of no difference. We have therefore updated the criteria employed in the original "simplified" figure to include this differentiation.

Citation: Fadda V, Bartoli L, Ferracane E, Trippoli S, Messori A. Simplified figure to present direct and indirect comparisons: Revisiting the graph 10 years later. *World J Methodol* 2021;

Specialty type: Health care sciences and services

Country/Territory of origin: Italy

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 19, 2021
Peer-review started: March 19, 2021
First decision: May 6, 2021
Revised: May 9, 2021
Accepted: May 27, 2021
Article in press: May 27, 2021
Published online: July 20, 2021

P-Reviewer: Tsikopoulos K
S-Editor: Ma YJ
L-Editor: Filipodia
P-Editor: Wang LYT

11(4): 228-230
URL: https://www.wjgnet.com/2222-0682/full/v11/i4/228.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i4.228

TO THE EDITOR

In 2011, Fadda and coworkers published in the BMJ the proposal of a simplified graph that, in the context of a network meta-analysis, presents the results of direct and indirect comparisons[1]. In 2019, another graph with very similar characteristics was proposed by De Vecchis *et al*[2]. Both of these graphs adopt the symbol "+" for superiority, "-" for inferiority, and "=" for the remaining cases.

Differentiating between no proof of difference (with $P > 0.05$) and proof of no difference (with $P > 0.05$ and $P_{equivalence} < 0.05$) is increasingly recognised to be important [3]; the same applies to differentiation between no proof of difference and proof of non-inferiority (with $P > 0.05$ and $P_{non\text{-}inferiority} < 0.05$, respectively). Since the two graphs of Fadda *et al*[1] and De Vecchis *et al*[2] do not include this differentiation, we propose to limit the symbol "=" to cases of equivalence and to adopt the symbol "NI" for non-inferiority or "ND" for the remaining cases. The suffix "t" remains useful because it identifies cases where the binary comparison shows a trend in favour of a treatment though in the absence of a statistically significant difference.

An example of the revisited graph is presented in Figure 1 that compares three first line treatments in paroxysmal atrial fibrillation[4-8].

In the field of network meta-analysis, the issue of graphical communication is complex, and the debate is still ongoing[9-15]. While the objective of describing the network geometry is quite straightforward[9,10], communication becomes more complex when it comes to presenting the results of the analysis[11-15]. The graphical proposal described herein is aimed at presenting the network geometry along with the results of the analysis. In our view, despite some unavoidable aspects of complexity, this tool deserves to be used particularly when the number of comparators is small.

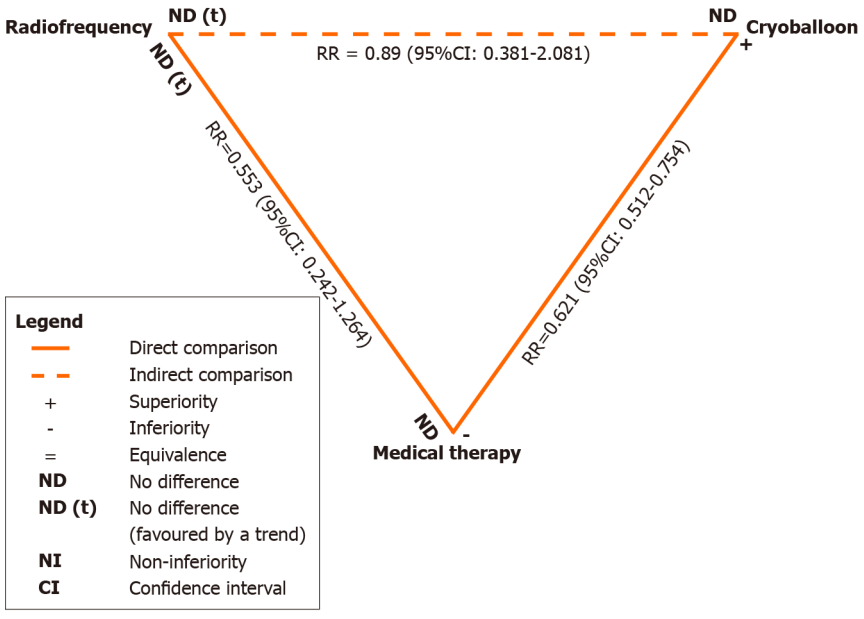

Figure 1 Direct and indirect comparisons across three first-line treatments for patients with paroxysmal atrial fibrillation. The comparisons of radiofrequency *vs* medical therapy and cryoballoon *vs* medical therapy are based on three[4-6] and two trials[7,8], respectively.

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Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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Help Desk: https://www.f6publishing.com/helpdesk
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ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2021 September 20; 11(5): 231-277

Published by Baishideng Publishing Group Inc

World Journal of Methodology

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AIMS AND SCOPE

The primary aim of *World Journal of Methodology* (*WJM*, *World J Methodol*) is to provide scholars and readers from various fields of methodology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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INDEXING/ABSTRACTING

The *WJM* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Gerhard Litscher

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2222-0682/editorialboard.htm

PUBLICATION DATE
September 20, 2021

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World J Methodol 2021 September 20; 11(5): 231-242

DOI: 10.5662/wjm.v11.i5.231

ISSN 2222-0682 (online)

FRONTIER

Genomics in medicine: A new era in medicine

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Author contributions: Pattan V, Kashyap R and Surani S were involved with idea origination, writing and review of manuscript; Bansal V, Candula N and Koritala T were involved in writing and review of literature.

Conflict-of-interest statement: None of the authors have any conflict of interest.

Manuscript source: Invited manuscript

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Abstract

The sequencing of complete human genome revolutionized the genomic medicine. However, the complex interplay of gene-environment-lifestyle and influence of non-coding genomic regions on human health remain largely unexplored. Genomic medicine has great potential for diagnoses or disease prediction, disease prevention and, targeted treatment. However, many of the promising tools of genomic medicine are still in their infancy and their application may be limited because of the limited knowledge we have that precludes its use in many clinical settings. In this review article, we have reviewed the evolution of genomic methodologies/tools, their limitations, and scope, for current and future clinical application.

Key Words: Genomic medicine; Medical genetics; Gene sequencing; DNA sequencing; RNA sequencing; Clustered regularly interspaced short palindromic repeat; Gene based therapy; Genomic tools; Genome editing

Core Tip: The field of Genomics is the future of medicine, as evidenced by the

Specialty type: Medical laboratory technology

Country/Territory of origin: United States

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 12, 2021
Peer-review started: January 12, 2021
First decision: June 17, 2021
Revised: June 18, 2021
Accepted: July 19, 2021
Article in press: July 19, 2021
Published online: September 20, 2021

P-Reviewer: Taheri S
S-Editor: Gao CC
L-Editor: A
P-Editor: Guo X

unprecedented research and clinical application which pushed the time boundaries for the coronavirus disease 2019 mRNA vaccines. However the path to unleashing the potential from genomic tools is far from perfect. A thorough research with international collaboration and cooperation is a necessity and the need of the hour.

Citation: Pattan V, Kashyap R, Bansal V, Candula N, Koritala T, Surani S. Genomics in medicine: A new era in medicine. *World J Methodol* 2021; 11(5): 231-242
URL: https://www.wjgnet.com/2222-0682/full/v11/i5/231.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i5.231

INTRODUCTION

Understanding the human genome has come a long way since the initial discovery of DNA structure by Watson and Crick in 1953[1]. The genome study and reference used to be a very specialized area, but lately with the advent of the messenger based RNA vaccine have brought the concept of genetics even to the lay public. In the 1970s, the ability to manipulate DNA with recombinant DNA technology increased the horizon. Our understanding of medical genetics began with inheritance patterns of single-gene diseases. The database of Mendelian Inheritance in Man (MIM) was initiated in the early 1960s by McKusick[2]. As of January 5, 2021, 4368 genes were mapped to phenotype-causing mutations[3]. However, only a small portion of diseases have a monogenic cause. The majority of the common diseases are polygenic, and elucidation of their mechanism has remained elusive.

The human genome project, which was completed in 2003, revolutionized the understanding of the human genome and served as a turning point to fast forward the genomic methodologies. However, the clinical application of findings from these genomic studies is still in its infancy. This is largely because we still have not understood or made complete sense of the available information. That is, the sequence data have been difficult to correlate to functional outcomes, making it difficult to understand the genetic basis of diseases and the complex gene-lifestyle-environment influences or their interaction. Moreover, most of the initial focus of the research had been on coding regions of DNA which comprises approximately 2% of the DNA and the knowledge about specific implications of non-coding DNA regions (98% of DNA) are largely unknown[4,5].

Remarkably, the human genome and the closest related species chimpanzees differ in single nucleotide alterations by a mere 1.23% and in deletions, insertions, and copy number variations by 3%[6]. In humans, the genomes of any two individuals are about 99.9% identical. However, a mere 0.1% variation allows for changes in a massive number of nucleotides because the human genome has approximately 30 billion base pairs (3.3×10^9)[7].

In this review, we will discuss the evolution in genomic methodology, limitations, and their scope for current and future clinical application.

GENOMIC TOOLS AND THEIR EVOLUTION

DNA sequencing

After the initial DNA sequencing method by Maxam and Gilbert[8] in 1977, the chain-termination DNA sequencing method developed by Sanger *et al*[9] in 1977 was used for the next few decades. It relied on the template DNA strand and had limited capacity for sequencing gene panels. Subsequently, with commercial production of high throughput technologies or next-generation sequencing (NGS) revolutionized the DNA sequencing by 2007[10]. Also called as massively parallel sequencing, NGS does parallel sequencing of millions of small DNA fragments. Each DNA fragment is fixed at a unique location on the solid support. While the sample of the patient's DNA which serves as a template in NGS is amplified and fragmented, the third-generation sequencing uses single DNA molecules rather than the amplified DNA as a template thus eliminating errors from DNA amplification processes. The NGS can be used for whole-genome sequencing, exome sequencing, or targeted gene panels comprising

Table 1 Characteristics of commonly used genomic tools

Tools for genomics	Principle of use	Pros and application	Limitation
Genome-wide association studies (GWAS)	Gene mapping study using DNA microarray to identify the association between SNP and specific risk alleles that are more prevalent in cases than in controls, *via* linkage disequilibrium	Has potential for population-based application. Example — The Severe COVID-19 GWAS Group[34] studied patients with respiratory failure from severe COVID-19 and narrowed down the genetic susceptibility locus to a gene cluster on chromosome locus 3p21.31. They also verified the potential involvement of the ABO blood group system	Does not establish causality but only an association with SNP; Missing heritability- cannot explain variance in complex traits or genes with a small effect size; Does not account for epigenetic changes and epistasis (gene-gene interaction); GWAS data catalog mostly from individuals of European descent which may limit application in minority population [35]
Expression quantitative trait loci (eQTL) analysis	Links SNPs to changes in gene expression by measuring the expression of many genes simultaneously in microarrays. Helps to narrow down to SNPs more likely to impact the disease condition	Provides better insight into specific causal mechanisms[36]; Liver eQTL — useful in pharmacogenomic studies by analyzing Epistatic eQTL Interactions [37]	Limited tissue interrogation will give misleading biological interpretations about the gene mediating the regulatory effect to increase disease risk[38]
Deep sequencing or Next-generation sequencing	**Exome sequencing:** 85% of known disease-causing mutations in Mendelian disorders are found in exons. Exome sequencing is a useful tool to find the causal genes for Mendelian disorders	Reduced cost and limited data to interpret; Linkage study design is unsuitable for extremely rare and sporadic Mendelian disorders for which exome sequencing would be more practical[39]	**Exome sequencing**: It can miss pathogenic variants in a non-coding region. Repetitive regions (*e.g.*, pseudogenes) can confound results in whole-exome sequencing[41]; Potentiate technical biases regarding exon capture limiting its use in detecting copy-number variants as well as in genomic regions where capture is less efficient[42]
	Whole-genome sequencing: Can sequence every nucleotide base in the human genome (approximately 3.3×10^9 base pairs)	**Whole-genome sequencing**: Avoids inherent biases of exome capture	**Whole-genome sequencing**: Too much data but little clinical knowledge available to interpret; Higher cost compared to clinical utility
	Targeted gene panel: Provides information on prespecified disease-associated genes	**Examples:** Rapid whole-genome sequencing to investigate extensively drug-resistant (XDR) tuberculosis[40]	
RNA-seq	Uses NGS to analyze RNA expression patterns or transcriptome profiling by reverse transcription of RNA sample to complementary DNAs (cDNA) and PCR amplification	**Can be used:** to analyze RNA expression profile at single cell level or quantify gene expression[43]; to obtain data on novel transcripts and is not limited by availability of reference genome data[44]; to identify alternatively spliced genes; to detect allele-specific gene expression[44]	cDNA synthesis and PCR amplification steps can introduce bias and errors[44]
Epigenomics	Epigenomics involves methods used to identify DNA methylation and histone modifications. Sodium bisulfite can identify unmethylated cytosines due to its ability to convert unmethylated cytosines to uracil. However the methylated cytosine is resistant to this conversion. Methylation-dependent restriction enzymes are used for DNA methylation analysis[45]. Chromatin immunoprecipitation (ChIP) is used for the investigation of histone modifications	ChIP allows precise mapping of the DNA-protein interaction in living cells. Cross-linked protein-DNA complex can be treated with exonucleases to remove cross-linked DNA sequences that are not avidly bound to protein of interest. This is called ChIP-Exo. This allows mapping of *in vivo* protein occupancy at single nucleotide-level resolution[47]	Needs design of antibodies specific to DNA-bound protein of interest which could be modified histone or transcription factors
	Immunoprecipitation techniques: ChIP on Chip; ChIP-Seq. Chromatin is isolated from the sample and the DNA involved in DNA protein cross-linked complex is isolated using antibodies specific to the DNA-bound protein. The isolated DNA is amplified using PCR and analyzed using gel electrophoresis imaging, microarray hybridization (ChIP-chip), or direct sequencing with NGS (ChIP-Seq)[46]		
Transcriptomics	**Northern blot:** RNA molecules separated by gel electrophoresis by size and subsequently hybridized with labeled complementary ssDNA and detected using chemic luminescence or autoradiography	Northern blot can both quantify the amount of RNA and also determine the size of mRNA transcript. Can detect transcript variant of genes[49]	Northern blot-need radioactive probes and has lower sensitivity
	Ribonuclease (RNase) protection assay: Differs from northern blot by use of antisense RNA probes	**RNase protection assay:** It can simultaneously detect and quantify	**RNase protection assay:** Does not provide information on transcript

called riboprobes	multiple mRNA targets in a single RNA sample .It has high sensitivity	size[52]
Real-time RT-PCR: cDNA are synthesized by reverse transcription from the sample RNA identified. The resulting cDNA is amplified by using fluorescently labeled oligonucleotide primers. Fluorescence intensity is monitored and correlated with several PCR cycles	**Real-time RT-PCR:** Allows quantitative genotyping, detection of SNPs and allelic variants or genetic variations even when mutation is found in very small fraction of cells in the sample. Has become clinical standard for diagnoses in Infectious diseases and it's role is evolving rapidly in cancer diagnostics [50]	**Real-time RT-PCR:** The process is complex and any errors in choice of reagents, primers or probes will affect accuracy. There could be risk for errors during data analysis and reporting. The process is expensive [53]
In situ hybridization: Tissue specimen is fixed to preserve morphology and then treated with proteases. A labeled probe is hybridized to the sample and detected using chemiluminescence or autoradiography[48]	**In situ hybridization:** Very useful in diagnostic application when there is limited tissue sample (in embryos and biopsy specimen). Several specific hybridizations can be done on the same sample. Tissue samples can be freeze for future use[48]	**In situ hybridization:** Low diagnostic yield when the sample has low DNA and RNA copies[48]
Spotted DNA arrays: Measures relative expression levels between 2 samples. cDNA probes amplified by PCR are spotted on a glass slide and then mRNAs are isolated from the samples. The mRNA from each sample is labeled with different fluorescent dyes. The samples are mixed, co-hybridized with cDNA probes on glass slides to measure relative gene expression	**Spotted DNA arrays:** The major application of DNA array is measurement of gene expression levels [51]	**Spotted DNA arrays:** DNA array can only detect known sequences, that were used to construct the array. It only gives relative estimate of gene expression and not reliable for absolute quantification. When the genome has multiple related sequences then design of array that distinguishes these sequences is challenging. Difficult to reproduce the array[51]

SNP: Single nucleotide polymorphism; NGS: Next-generation sequencing; PCR: Polymerase chain reaction; RT-PCR: Real-time reverse transcription polymerase chain reaction; ssDNA: Single stranded DNA.

tens to hundreds of genes.

Single nucleotide polymorphism

Single nucleotide polymorphism (SNP) is the variation in genetic sequence by a single nucleotide. It is the most common type of genetic variation in man[11]. It was detected in the 1980s using restriction enzymes[12]. With application of the microarray technology to SNPs, the scope of SNP in clinical practice has widened, especially in oncology. The first SNP array analysis was done in 1998 and the first application of SNP array analysis in cancer was done in 2000[13]. SNP array analysis is used to determine loss of heterozygosity, allelic imbalance, genomic copy number changes, frequency of homozygous chromosome regions, uniparental disomy, DNA methylation alterations and linkage analysis of DNA polymorphisms in cancer cells [13,14].

DNA amplification

Kary Banks Mullis successfully demonstrated polymerase chain reaction (PCR) in 1983 [15]. PCR is a cost-effective method that can amplify a single DNA exponentially[16]. It is a rapid, highly specific, and extremely sensitive method. PCR is being used in SNP genotyping, detection of rare sequences, insertion-deletion variants, and structural variants like copy-number variants.

Linkage and association analysis

Linkage studies have been used for mapping of genes for heritable traits to their chromosomal locations. 1st genetic linkage map was done in 1911 by Sturtevant A[17]. Parametric linkage analysis is used to map the disease-causing gene for monogenic diseases. Here, the logarithm of the odds (LOD) scores and recombination fractions are used to map the gene location. Model-free linkage analysis or non-parametric linkage analysis is used for complex or polygenic diseases, or when the model of inheritance is not known[18]. Linkage analysis of the whole genome can identify large regions of the chromosome with evidence of disease containing the gene[19,20], but this large span of chromosomes can have hundreds of candidate genes.

Linkage studies have been used for mapping Mendelian traits with high penetrance in families and relatives[20]. They are especially useful to identify rare alleles that are present in a small number of families[21], for disease genes with weak effects and polygenic diseases, linkage disequilibrium association mapping has proved to be more

useful. In genome-wide association studies (GWAS), genotyping of hundreds or thousands of SNPs is done in cases and control populations and their association with heritability is analyzed. A combination of linkage and association methodologies helps to identify and characterize the wider range of disease-susceptibility variants[22].

Fluorescence in Situ Hybridization (FISH) was developed in 1987. It is a cytogenetic technique which uses fluorescent DNA probes which are designed to label precise chromosomal locations. The advantage of FISH over conventional cytogenetic metaphase karyotype analysis is lack of cell culture requirement. It can rapidly evaluate interphase nuclei in the fresh or paraffin-embedded sample[23]. However, the resolution of this technique is only as good as that of karyotype bands. Cloned DNA FISH probes of about 100 kb, called bacterial artificial chromosomes, are now available. FISH is being utilized more in making clinical diagnosis among Oncology due to its simplicity and reliability to evaluate the key biomarkers in various malignancies.

Comparative genomic hybridization

Comparative genomic hybridization (CGH) was developed in 1992. CGH can detect DNA copy number changes across the entire genome of a patient sample in a single experiment. It compares the hybridization signal intensity of a test sample (for example tumor sample) against a reference sample along the chromosomes[13].

HAPMAP AND 1000 GENOME PROJECTS HAVE CREATED A CATALOG OF SNPS

The HapMap project was started in 2002 to develop a haplotype map of the human genome. It can also describe the common patterns of human genetic variation[24]. The 1000 Genomes Project comprised a total of 26 diverse population set in which whole-genome sequencing was performed. It also used deep exome sequencing and dense microarray genotyping to give a comprehensive description of common human genetic variation[25].

TARGETED GENOME EDITING OR GENOME ENGINEERING

It involves modification of the genome at a precise, prespecified locus using programmable nucleases. Examples of some of the programmable nucleases include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas (CRISPR-associated) system. These programmable nucleases are designed to impart site-specific double-strand breaks (dsBs) in chromosomal DNA. The cell is therefore forced to use one of the endogenous DNA repair mechanisms — homologous recombination or homology-directed repair (HDR) and nonhomologous end-joining (NHEJ). This enables targeted genetic modifications during the repair process in the living cells (*in vivo*) (Table 1)[26]. ZFNs and TALENS recognize the target sequence through protein-DNA interaction. CRISPR-Cas nucleases recognize target sequences through RNA and DNA base pairing[26].

In the year 2013, Cong *et al*[27] and Mali *et al*[28] showed successful genome editing in mammalian cells using the CRISPR system. In the last 5 years, we have seen a leap in the research interest (both animal and human) in CRISPR genomic editing.

While genome editing holds promise to correct the defective genome in vivo, therapies can also be designed to alter the gene expression without altering the genomic code. For example, anti-sense oligonucleotide can be used to alter the splice points of pre-mRNA to correct for a defective gene or suppress its expression. Examples of drugs which use splice modulation and approved by Food and Drug Administration (FDA) are Eteplirsen (exon skipping, approved for Duchenne muscular dystrophy) and nusinersen (exon inclusion, approved for spinal muscular atrophy)[29].

Table 1 summarizes the commonly used genomic tools, their working principle, advantages/applications and limitations (see Table 1). Table 2 summarizes the major genome/gene editing tools their working principle, advantages/applications and limitations. Table 3 summarizes gene-based therapies that are either FDA approved therapies or investigational therapies showing promise.

Table 2 Characteristics of genome-editing technologies using programmable nucleases

Gene editing	Principle of use	Advantages or application	Limitation
CRISPR-Cas9 guided gene editing: (1)NHEJ; and (2)HDR	Cas9 enzyme (an endonuclease) cleaves ds- DNA at a specific site as determined by the specific sequence of the guide RNA. Genome editing is done when the cell tries to repair the dsB (either *via* NHEJ or HDR)	Has the potential to edit genes in almost any cell type *in vivo*; Has potential in every field, notably infections[54], genetic disease [55], cancer[56] *etc.*; CRISPR-Cas9 can also be used for large scale loss-of-function gene screen: Catalytically inactive Cas9 (dCas9) can be directed by guide RNA, bind to specific genes to reversibly suppress or activate gene transcription (by fusion of transcription activators or suppressors with dCas9)[57]; Epigenetic modulators (*e.g.*, DNA methylase) can also be fused with dCas9 to achieve controlled epigenetic modulations. Cas-9 NHEJ is simpler and efficient; Cas-9 HDR is more precise but lower efficiency than NHEJ. The mutant version of the Cas9 called Cas9 nickase can be used to minimize the risk of off-targets	The off-target activity of RNA-guided endonuclease-induced mutations[58]. Off-target mutations with a frequency below 0.5% cannot be detected by current off-target detection techniques[59]
Augmented CRISPR-Cas12a system	Cas12a cuts target ds- DNA. However, unlike Cas9, Cas12a subsequently becomes activated and causes indiscriminate cleavage of ssDNA causing collateral damage. SARS-CoV-2 RNA DETECTR Assay: samples from upper airway swabs are processed using simultaneous reverse transcription and isothermal amplification with loop-mediated amplification (RT-LAMP). Subsequently the Cas12 enzyme is added	CRISPR-Cas12a system can be used to create new drug or cell delivery systems and bio-sensing (*e.g.*, to detect methicillin-resistant Staphylococcus aureus, Ebola virus[60]. Emergency Use Authorization (EUA) Only for qualitative detection of nucleic acid from the SARS-CoV-2 in upper respiratory specimens[61,62]	Limited research data and application. The technology is still in its infancy
CRISPR-Cas 13	CRISPR-Cas 13 system can be used *via* SHERLOCK technique for ultra-sensitive detection of RNA or DNA from the clinical samples	SherlockTM CRISPR SARS-CoV-2 kit: Emergency Use Authorization (EUA) qualitative for detection of nucleic acid fromSARS-CoV-2 in upper respiratory specimens[63,64]	
Prime editors	It uses a catalytically impaired Cas9 which is fused to an engineered reverse transcriptase and prime editing guide RNA. The guide RNA specifies the target site and encodes the desired sequence	Prime editing is associated with fewer off-target edits when compared with conventional CRISPR-Cas system[65]. Anzalone *et al*[66] applied prime editing in human cells to correct the primary genetic causes of sickle cell disease and Tay-Sachs disease. It does not require double-strand breaks or donor DNA templates	Research literature on application of prime editing is limited. Unlike conventional CRISPR-Cas system prime editing may not be able to provide large DNA insertions or deletions[65]
Zinc finger nucleases	Zinc finger nuclease (dimer of zinc finger hybrid bound to restriction endonuclease) is a programmable nuclease that cleaves specific sites in DNA. They recognize the target sequence through protein-DNA interaction	Potential for plant genome editing for crop improvement[67]	Necessity to engineer novel proteins for each target site: Expensive; Difficult to reproduce
TALENS	TAL proteins have TAL effector DNA-binding domain fused to a DNA cleavage domain. TALENs create dsBs that require repair by NHEJ or HDR	The DNA-binding specificity of TALEs is easier to engineer than zinc-fingerProteins[68]	Necessity to engineer novel proteins for each target site. TALENs are large and pose packaging challenge in viral delivery systems[69]

HDR: Homology-directed repair; NHEJ: Nonhomologous end-joining; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TALENs: Transcription activator-like effector nucleases; dsBs: Double stranded breaks; ssDNA: Single stranded DNA; TAL: Transcription activator-like; SHERLOCK: Specific High Sensitivity Enzymatic Reporter UnLOCKing.

DISCUSSION

The newer genomic technology and tools have broadened the scope and pushed the time limits for development of new diagnostic kits, preventive strategies like vaccines, therapeutic strategies like gene modulation and gene therapy. A lot is yet to be studied in terms of the complex interaction of gene-environment-lifestyle-disease. Knowing the impact of genomics on disease pathophysiology and response to medications[30]. expands the scope of research and clinical application. While genome editing holds promise to correct the defective genome in vivo, therapies can also be designed to alter the gene expression without altering the genomic code (example exon skipping, or inclusion discussed above).

The newer genomic editing tools have showed great potential and promise but they need to be studied extensively before clinical application. Also, uniform international ethical guidelines and guiding principles need to be established so that these genomic technologies are not misused.

Table 3 Gene based therapies: List of Food and Drug Administration approved therapies and investigational therapies showing promise

Therapy or drug	Indication	Mechanism of action	Approval status
Janssen COVID-19 vaccine	Prevention of 2019 coronavirus disease (COVID-19) for individuals 18 yr of age and older	Recombinant, humanadenovirus type 26 vector which expresses the SARS-CoV-2 "S" antigen after entering human cells thus eliciting immune response against COVID-19	Emergency use authorization (EUA) on February 27, 2021[70]. Pause placed on vaccine use on April 13, 2021[71]. FDA lifted vaccination pause on April 23, 2021[72]
Pfizer-BioNTech COVID-19 Vaccine [73-75]	Prevention of COVID-19 for individuals 16 yr of age and older	modRNA forumated in lipid particles when delivered to host cells express SARS-CoV-2 "S" antigen, thus eliciting immune response against COVID-19	EUA on December 11, 2020
Moderna COVID-19 vaccine[76-78]	Prevention of COVID-19 for individuals 18 yr of age and older	modRNA forumated in lipid particles when delivered to host cells express SARS-CoV-2 "S" antigen, thus eliciting immune response against COVID-19	EUA on December 18, 2020
Lumasiran[79]	Primary hyperoxaluria type 1	HAO1-directed small interfering ribonucleic acid	Approved in Nov 2020
Viltolarsen[80]	Duchenne muscular dystrophy	Antisense oligonucleotide directed to exon 53 skipping	Approved in August 2020
Brexucabtagene autoleucel[81]	Relapsed/refractory mantle cell lymphoma	Genetically modified autologous CD19 T cells directed against CD19 expressing cancer cells	Approved in July 2020
Golodirsen[82]	Duchenne muscular dystrophy	Antisense oligonucleotide directed	Approved in December 2019
Givosiran[83]	Acute hepatic porphyria	Double-stranded small interfering RNA that degrades the ALAS1 mRNA in hepatocytes *via* RNA interference	Approved in November 2019
Onasemnogene abeparvovec-xioi[84]	Spinal muscular atrophy (SMA)	AAV9-based gene therapy which encodes the human SMN protein	Approved in May 2019
Inotersen[85]	Polyneuropathy of hereditary transthyretin-mediated amyloidosis	Transthyretin-directed antisense oligonucleotide	Approved in October 2018
Axicabtagene ciloleucel[86]	Relapsed or refractory large B-cell lymphoma after two or more lines of systemic therapy	Genetically modified autologous CD19 T cells directed against CD19 expressing cancer cells	Approved in October 2017
Tisagenlecleucel[87]	Refractory or relapsed B-cell precursor acute lymphoblastic leukemia (ALL)	Genetically modified autologous CD19 T cells directed against CD19 expressing cancer cells	Approved in August 2017
Nusinersen[88]	SMA	Survival motor neuron-2 (SMN2)-directed antisense oligonucleotide	Approved in December 2016
Eteplirsen[89]	Duchenne muscular dystrophy	Antisense oligonucleotid that binds to exon 51 of dystrophin pre-mRNA	Approved in September 2016
Talimogene laherparepvec[90]	Genetically modified herpes simplex virus, type 1 used as oncolytic viral therapy	They utilized the local treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with melanoma who had the recurrence after the initial surgery	Approved in October 2015
Giroctocogene fitelparvovec[91]	Moderately severe to severe hemophilia A	Factor VIII gene delivery using recombinant adeno-associated viruses as vectors	Investigational in phase 3 trial
Inclisiran[92]	Heterozygous and possibly homozygous familial hypercholesterolemia	Small-interfering ribonucleic acid which decreases hepatic production of PCSK9	Investigational phase 3 trial
Volanesorsen[93]	Familial chylomicronemia syndrome	Antisense oligonucleotide that targets the messenger RNA for apo-CIII	Conditional approval by European Medicines Agency's (EMA) but not by FDA
CRISPR-Cas9 gene editing[94]	Sickle cell disease and β-thalassemia	CRISPR-Cas9based allele editing of the BCL11A erythroid-specific enhancer in autologous CD34+ cells	Investigational- FDA Fast Track Designation for CTX001 in sickle cell disease

AAV: Adeno-associated virus; ALAS1: Aminolevulinate synthase 1; BCL11A: B cell lymphoma/leukemia 11A; HAO1: Hydroxyacid oxidase (glycolate oxidase) 1; modRNA: Nucleoside-modified messenger RNA; SMN: Survival motor neuron 1; FDA: Food and Drug Administration.

It is very important to include diverse populations and to represent minority population in the genomic studies, so that results could be generalized and more accurate diagnostic, predictive and therapeutic tools can be developed.

Genomics in medicine is indeed a new era in medicine. Even the control of coronavirus disease 2019 pandemic[31] has just begun at the time of writing of this article with gene based therapies eliciting immune response against severe acute respiratory syndrome coronavirus 2 spike proteins. A unified international collaboration[32,33] is needed to continue expanding gene therapy use in opening new frontiers for fight against novel infections and disease.

CONCLUSION

Genomic medicine holds great promise for providing insight into disease pathophysiology, provide better diagnostic or disease predictive tools, preventive therapies and finally for targeted treatment of diseases. Although some of the newer tools (like CRISPR system) have great potential, more research is needed before these tools can be unleashed to clinical use. Hence there is great need for studies to unravel the mystery of complex interaction of both coding and noncoding genomic regions with environment and lifestyle influences on disease occurrence and management.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 September 20; 11(5): 243-262

DOI: 10.5662/wjm.v11.i5.243

ISSN 2222-0682 (online)

REVIEW

IgY technology: Methods for developing and evaluating avian immunoglobulins for the *in vitro* detection of biomolecules

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Author contributions: Karachaliou CE collected the literature articles and drafted the manuscript; Vassilakopoulou V contributed to the literature search and draft writing; Livaniou E designed the draft and edited the final paper; All authors have read and approved the final manuscript.

Conflict-of-interest statement: Authors declare no conflict of interest for this article.

Manuscript source: Invited manuscript

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Abstract

The term "IgY technology" was introduced in the literature in the mid 1990s to describe a procedure involving immunization of avian species, mainly laying hens and consequent isolation of the polyclonal IgYs from the "immune" egg yolk (thus avoiding bleeding and animal stress). IgYs have been applied to various fields of medicine and biotechnology. The present article will deal with specific aspects of IgY technology, focusing on the currently reported methods for developing, isolating, evaluating and storing polyclonal IgYs. Other topics such as current information on isolation protocols or evaluation of IgYs from different avian species are also discussed. Specific advantages of IgY technology (*e.g.*, novel antibody specificities that may emerge *via* the avian immune system) will also be discussed. Recent *in vitro* applications of polyclonal egg yolk-derived IgYs to the field of disease diagnosis in human and veterinary medicine through *in vitro* immunodetection of target biomolecules will be presented. Moreover, ethical aspects associated with animal well-being as well as new promising approaches that are relevant to the original IgY technology (*e.g.*, development of monoclonal IgYs and IgY-like antibodies through the phage display technique or in transgenic chickens) and future prospects in the area will also be mentioned.

Key Words: Animal welfare; Polyclonal IgYs; Egg yolk; IgY technology; Relevant-to-IgY-technology approaches; *In vitro* immunodetection techniques

Core Tip: IgY technology has been widely used during the last decades, especially as a means for the efficient *in vitro* immunodetection of biomolecules in various fields of research and disease diagnosis. Despite the very promising relevant new approaches,

Specialty type: Immunology

Country/Territory of origin: Greece

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 4, 2021
Peer-review started: February 4, 2021
First decision: June 7, 2021
Revised: June 10, 2021
Accepted: July 13, 2021
Article in press: July 13, 2021
Published online: September 20, 2021

P-Reviewer: Neagu M
S-Editor: Zhang H
L-Editor: Filipodia
P-Editor: Guo X

there is still space to further exploit the original IgY technology due to functional, practical, and ethical reasons/advantages associated with the unique features of IgYs, the highly efficient isolation of large amounts of IgYs from the immune egg yolk, and the avoidance of animal bleeding, respectively.

Citation: Karachaliou CE, Vassilakopoulou V, Livaniou E. IgY technology: Methods for developing and evaluating avian immunoglobulins for the *in vitro* detection of biomolecules. *World J Methodol* 2021; 11(5): 243-262
URL: https://www.wjgnet.com/2222-0682/full/v11/i5/243.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i5.243

INTRODUCTION

The term "IgY technology" was introduced in the 1990s to describe a procedure consisting of immunization of birds, especially laying hens, in order to produce polyclonal antibodies of the Y class (IgYs). IgYs can be isolated in large quantities from "immune" egg yolk (thus avoiding the animal bleeding procedure, which is stressful for an animal) and has been applied to various fields of biotechnology and biomedicine[1-3]. To date, IgYs developed in poultry and isolated from the egg yolk as aforementioned have been and are still being used as specific laboratory tools, especially for detecting biomolecules in biological specimens through various *in vitro* techniques (and also as *in vivo* immunotherapeutic agents).

The origins of the IgY technology can be traced back many years, *i.e.* at the end of the 19th century, when Klemperer observed that immunized hens (*Gallus domesticus*) generated antibodies that were present in the egg yolk[2-4]. Subsequently, a new type of immunoglobulin was found in the blood and egg yolk of birds (also in lungfish, amphibians and reptiles), which was called IgY[3,5]. Actually, birds, which do not produce colostrum like mammalian organisms do, use the yolk of their eggs as a very effective source of antibodies through which they can transfer humoral immunity to their offspring, until the latter develops fully mature immune system[6]. Transfer/accumulation of IgY from blood to/in the egg yolk, which is realized by a selective transport mechanism in avian mature oocytes and mediated by specific receptor(s)[7-9], enables the non-invasive isolation of antibodies and eliminates the need to bleed the animal. Isolation and subsequent application of egg yolk–derived antibodies minimize animal suffering and this meets at least one of the three main requirements for animal welfare, *i.e.* "Reduction," "Replacement," "Refinement," as they have been summarized in the "3Rs principle"[10]. As a consequence, in 1996 the European Centre for the Validation of Alternative Methods to animal testing (ECVAM) strongly recommended avian antibodies as alternative to mammalian ones[1]. In parallel, in the mid 1990s the term "IgY technology" was introduced in the literature, as already mentioned; in 1999, the IgY technology was approved as an alternative method for supporting animal welfare by the Veterinary Office of the Swiss Government[3].

Egg yolk is composed mainly of water, which accounts for approximately 50% of its weight, and contains many important nutrients and preservatives, since it serves the role of a protective chamber for the hen embryo. The dry weight of egg yolk is composed mostly by lipids (67%) and also proteins (33%). Egg yolk proteins are distributed between granules and plasma, in which granules are suspended. Granule proteins are divided into α- and β-lipovitellins (70%), phosvitin (16%), and low-density lipoproteins (12%), whereas plasma proteins include α-, β- and γ-livetins and low-density proteins[11]. A precursor of the major egg yolk proteins is vitellogenin, consisting of vitellogenin I (molecular weight [MW]: 260 kDa), vitellogenin II or major vitellogenin (MW: 246 kDa), and vitellogenin III (MW: 210 kDa)[12-14]. IgYs, which are the main constituent of γ-livetin, are among the most important and most abundant egg yolk proteins[11].

IgY is considered to be the functional equivalent and evolutionary precursor of mammalian IgG and probably of mammalian IgE[15]. Due to this functional and evolutionary relationship, some researchers use the term (avian) IgG instead of IgY; however, the first articles in the field have put emphasis on the distinct differences between IgG and IgY and strongly suggested use of the term IgY[5]. In addition to IgYs, there are two more avian immunoglobulin classes, avian IgM and IgA, which are

similar to mammalian IgM and IgA. Mammalian equivalents of IgE and IgD have not been found in hens[16].

Like mammalian IgG, IgY is composed of two heavy (H) and two light (L) polypeptide chains, which are organized in the Y-shaped characteristic "unit," and contains two identical binding sites for the antigen. However, the structure of IgY is actually different than that of IgG and this results in distinct properties, as well. The nucleotide sequence corresponding to the hen upsilon ("υ") heavy chain has revealed that the molecule contains four constant and one variable Ig heavy chain domains; the additional domain (Cυ2) has been conserved in mammalian IgE, but "transformed" into the flexible hinge region in mammalian IgG. As a consequence, the IgY molecule has higher molecular mass (approximately 180 kDa), than mammalian IgG (approximately 160 kDa). Moreover, the Fc part of IgY has a different carbohydrate content compared to the Fc part of IgG. An intact Fc part is necessary for the transfer of IgY from blood serum to egg yolk. In ducks an alternatively spliced form of IgY, the so-called IgY ΔFc, is also present. This variant lacks the Fc region and is mainly found in the blood serum. Hen as well as ostrich and pigeon express only the full-length version of IgY. In some birds, including hen, duck, zebra finch and ostrich, only a single κ light-chain locus has been found. The bursa of fabricius is the site in which immature B-cells are differentiated into mature and competent B-cells, while the spleen is the organ in which plasma cells, *i.e.* the antibody-producing cells, proliferate and memory cells are located. IgY's heavy and light chain loci consist of single functional V, D, and J genes; in addition to the single functional V genes, there are several pseudo-V genes that lack the usual transcription-regulatory and signal-recognition sequences and are not functional. The antibody diversity in avian organisms is mainly achieved by the so-called gene conversion, through which 10 to more than 120 base pairs from not functional pseudo-genes are transferred to the functional V gene[3,16,17].

The distinct structural features of IgY offer several functional advantages to this unique immunoglobulin type, rendering IgY a versatile and invaluable *in vitro* tool in biotechnology research and in disease diagnostics. Moreover, many reports have suggested *in vivo* application of IgYs in various fields of immunotherapy. The advantages of IgYs include: high potential for developing specific IgYs against conserved mammalian proteins due to the evolutionary distance between mammals and birds, avoidance of activating the mammalian (including human) complement system and reaction with mammalian Fc receptors, ability to isolate substantial amounts of IgYs from immune egg yolks, and avoidance of animal bleeding, which fulfills the "refinement" ethical requirement, as already mentioned[3,18,19].

In the last several decades, more complicated technologies associated with the original IgY technology have emerged, such as the development of avian monoclonal antibodies *via* hybridoma and recombinant techniques, mainly through the phage display technique[20]. Although the above antibodies are IgYs (or IgY-like) immunoglobulins and therefore have all (or part of) the consequent advantages, they are isolated from the supernatant of suitable cell cultures and are not egg yolk-derived. Thus, strictly speaking and at least in our opinion, the techniques leading to the development of monoclonal IgYs cannot be classified as a part of the original IgY technology. On the other hand, transgenic chickens[21] have been used for the production of recombinant proteins, including recombinant antibodies (mostly human/humanized ones), which can be isolated mainly from egg white and are recommended especially for *in vivo* therapeutic applications. Though the aforementioned antibodies have not gained wide application yet and their development and evaluation are considered outside the main scope of the present article, they are considered very promising and will be briefly presented.

The present review article will focus on specific aspects of the original IgY technology, such as immunization of laying hens, isolation of the IgYs developed from the immune egg yolk and consequent immunochemical evaluation. Various recent applications of polyclonal IgYs to the *in vitro* immunodetection of various biomolecules will be also presented and discussed.

DEVELOPMENT AND EVALUATION OF EGG YOLK-DERIVED POLYCLONAL IGYS

General aspects

IgY technology has produced a large number of valuable immunochemical tools for biotechnology and medicine since the 1990s. Various parameters that are associated

with and can affect the results of the IgY technology have been reported in the literature such as housing and breeding conditions, line, age, and stage of development of the immunized birds[2,3,18,22]. Laying hens are the avian organisms of choice (*e.g.*, White Leghorn and Rhode Island Red hens) and are used for immunization to produce polyclonal IgYs throughout their egg-laying period. Other types of poultry such as duck, goose, ostrich, and quail have been referred to in the literature, though to a lesser extent[23-26]. Normal hen lines and conventional housing, *e.g.*, in suitable cages[27], are usually adequate to produce IgYs for research purposes; however, when the IgYs are to be applied as human therapeutics, the use of specific pathogen-free hens is considered necessary[1,3]. Administration of specific food supplements during hens' breeding, *e.g.*, carnitine, has been proposed in the literature as a means to improve overall yield of IgY production, but the results are often contradictory[28].

Immunizing protocols

Parameters that may influence the immune response include antigen nature and dose, use of adjuvants, route of administration, and overall immunization schedule[3].

Both, complex antigens, *e.g.*, whole viruses, bacteria and parasites[29-33] and individual biomolecules, *e.g.*, large proteins[34,35], or small peptides conjugated to a suitable carrier protein, such as keyhole limpet hemocyanin (KLH)[36,37], have been used to stimulate development of specific IgYs in hens. Our team tried to develop IgYs against various antigens, including a recombinant protein of high molecular mass, *i.e.* human kallikrein-related peptidase 6[38] as well as peptides of the alpha- and beta-thymosin families isolated from mammalian tissues or synthetically prepared, either conjugated to KLH or not[39-41]. Moreover, we successfully developed IgYs against the olive fruit fly pheromone by using a KLH-conjugate of the synthetic hapten (±)-β-[3-(1,7-dioxaspiro[5.5]undecane)] propionic acid[27].

The antigen dose may be also critical, since too much or too little antigen can lead to an undesirable immune response[2]. Different antigen doses have been reported in the literature. In an early study, a good immune response in hens immunized with bovine serum albumin at doses as low as 0.1-1.0 μg was reported[3]; however, higher doses ranging from 10 to 1000 μg (most often 50-100 μg) have been also used. Information on the doses administered to immunize hens has been presented in a recent review[18].

The outcome of immunization is commonly enhanced by the addition of adjuvants, though successful immunization of hens without any adjuvant has been reported in the literature[3]. Among the adjuvant preparations that have been described till now, Freund's complete adjuvant (FCA) is still considered the gold standard for generating high levels of antibodies in animals, including birds. FCA is a suspension of heat-killed and dried mycobacteria (*Mycobacterium spp.*) in mineral oil, which forms a depot at the injection site and slows down release of the antigen in the host organism, so that long-lasting exposure and a non-specific immune stimulation is achieved. The main problem of FCA is the severe tissue damage it causes at the injection sites, which is usually attributed to the mycobacteria it contains. Although a few studies have reported that hens can better tolerate FCA, in comparison with mammals, other studies have reported contradictory data. For this reason, Freund's incomplete adjuvant, *i.e.* Freund's adjuvant without mycobacteria, is commonly used for booster injections as an alternative to FCA, which is used only in the first immunization[18]. Use of other adjuvants has been also reported in IgY technology, such as the so-called, mineral-oil based Montanide adjuvant, along with oligodeoxynucleotides containing C-phosphate guanosine motifs, which are promising immunoenhancing agents[28]. Research in the area of developing new adjuvants, both highly efficient and animal welfare-friendly, is being continued.

Regarding the route of administration, several approaches have been tested. The most recommended one is the intramuscular injection (i.m.) into the breast tissues[3,29,34,42] in multiple sites; i.m. administration in the thigh muscle has been also used but according to some reports it may cause lameness and has to be avoided[18]. Subcutaneous (s.c.) immunization in the neck has also been used by several research teams including our team[27,38,39]. As reported, i.m. immunization in breast muscle is most suited especially for young hens[18]. The intravenous (i.v.) route has been very rarely used, without adjuvants and at a very slow injection rate. The intraperitoneal (i.p.) route, which Klemperer has followed in his pioneer work, is hardly used these days. Efforts to immunize hens orally have been also reported[3,30,43].

The interval between the first and second (*i.e.* first booster) immunization is considered a critical parameter in hen immunization protocols. Age of hens when first immunized might also be an issue. However, literature information on these specific parameters substantially varies. A general recommendation is to administer a booster immunization when the IgY titer reaches a plateau or begins to decrease[44]. If a

substantial decrease in the antibody titer has been observed, further immunizations can be performed during the entire laying period, which lasts about 72 wk[22], to keep the antibody titer adequately high for as long as possible, in many cases for more than 150 d[18]. As presented in a previous review[3], some immunization protocols have recommended antigen administration at days 0, 14 and 28, or once a week for 7 consecutive weeks, or at day 0, week 10, and week 15. Other protocols propose hen immunization at 10-d intervals, but in most cases, the interval between the first and second immunization is at least 4 wk, while another protocol has reported achievement of a high antibody titer by prolonging the boost interval from 14 to 42 d. Intervals among booster injections also vary, averaging 2 wk[3]. Our team has mainly used 3-mo-old hens for immunization; the first booster was administered 2 wk after first immunization, while several further injections were given, mostly at 4 wk-intervals[27].

In general, eggs are collected weekly, starting 1 wk prior to the first immunization (pre-immune eggs), eggshells are washed or sanitized with 70% ethanol, and stored at 4°C until further processed for IgY isolation. Lyophilization of egg yolk has also been reported, resulting in an easy-to-mix egg yolk powder with an extended shelf-life[45].

Immunization with plasmids: "DNA-designed" IgYs

Apart from the conventional administration of antigen along with adjuvant, the so-called genetic immunization has also been applied to the production of polyclonal IgYs in avian species[46]. In this context, avian organisms have been immunized with plasmid vectors encoding target eukaryotic antigens, *e.g.*, bovine interferon gamma protein[47], prokaryotic antigens, *e.g.*, *Botulinum* toxin A1[48], as well as viral ones, *e.g.*, antigens from Andes virus[23]; in almost all cases, antibodies Y of desired immunochemical characteristics have been developed. A great deal of effort has been put forth to improve DNA-vaccine delivery, and consequently, immunogenicity. The "gene gun" method has garnered much attention, since low doses of DNA applied *via* a gene gun can efficiently induce high antibody titers against the antigen encoded[49]. Although DNA immunization is a promising approach, which prevents costly and tedious preparation of purified antigens or presence of adjuvants in the immunization mixture, it has not yet gained wide application.

Isolation of IgYs from the egg yolk

Hen eggs are an excellent source of high amounts of antibodies[19]. An average hen can lay roughly 325 eggs a year. Given that according to the literature an egg can produce 60-150 mg[50], or 40-80 mg total IgY per egg yolk depending on the hen's age [22], one hen can roughly produce 20-40 g of antibodies a year, with 1%-2% up to 10% of the antibodies being antigen-specific[18,51], which is much higher than that obtained from mammalian sources[11].

Isolation of IgYs from the "immune" egg yolk in pure form is a challenging task. Several protocols have been described, with different characteristics in terms of total yield, purity, duration, convenience, and cost[42]. IgYs account for about 3%-5% of the egg yolk proteins, which are dispersed in a lipid emulsion combined with lipoproteins and glycoproteins. Consequently, in most cases, IgY isolation involves, first, removal of lipids to form a water-soluble fraction ("de-lipidation" step), and then precipitation of the antibodies that are present in the water- soluble fraction with various approaches[3,18].

The most commonly used de-lipidation technique is the "acidified water dilution method"[52], using 6- to 10-fold dilution of egg yolk in water at pH ~5, incubation for several hours at 4°C and then centrifugation, at the end of which the lipid portion is precipitated and the water-soluble portion is collected in the supernatant. Alternatively, lipid removal has been successfully performed by means of organic solvents (chloroform, acetone, isopropanol)[53,54], acids (caprylic acid, trichloroacetic acid)[55] or natural gums (polyanionic polysaccharides, *e.g.*, xanthans)[56]. A de-lipidation solution containing polysaccharides (such as pectin, λ-carrageenan, carboxymethylcellulose, methylcellulose, and dextran sulfate) has been also reported[57].

After de-lipidation, various IgY extraction methods that can be applied either to laboratory- or to large-scale production have appeared in the literature; these methods can be divided into three main groups, *i.e.* precipitation, chromatographic and filtration methods.

Precipitation methods, involving precipitation of IgYs with saturated salt solutions, such as ammonium sulfate, sodium sulfate or sodium chloride[58,59], polyethylene glycol (PEG)[60], caprylic acid[61,62] and carrageenan[63]. PEG precipitation usually involves, first, dilution of egg yolk in phosphate-buffered saline (PBS) containing PEG 6000 at low concentration (3.5%), to facilitate de-lipidation. After centrifugation, the

supernatant is treated with 8.5% and then with 12% PEG 6000 to precipitate IgYs[30]. Among the above methods, ammonium sulfate precipitation is considered one of the best choices for the scale-up purification of IgY[11], with most suitable concentration of ammonium sulfate being 20%[55]. Extracted IgY samples usually undergo a final dialysis step, usually against PBS, to eliminate residual salts from the extraction procedure.

Chromatographic methods include low-pressure chromatography[30], ion exchange chromatography[52,59], highresolution chromatography through multicolumn systems[64] and affinity chromatography[65]. Conventional affinity chromatographic methods using protein A or protein G columns cannot be performed for IgY purification, since IgYs, contrary to IgGs, do not bind to protein A or G[66]. Other types of ligands are therefore required, such as the elastin-like polypeptide-tagged immunoglobulin-binding domain of streptococcal protein G[67]. Still other ligands, such as IgY-binding peptides screened from a random peptide library, have been also proposed as a means of IgY purification[68]. IgY can also be purified with thiophilic adsorption chromatography, usually through commercially available IgY-extraction columns[18,69]. However, chromatographic techniques are generally expensive and impractical for the large-scale production of antibodies, while they have not proven to substantially increase purity of the final product when compared with simple precipitation methods, such as ammonium sulfate precipitation.

Filtration methods, such as ultrafiltration[52,70], have also been used as IgY extraction methods.

As reported, a combination of the aforementioned methods, *e.g.*, a combination of PEG precipitation with affinity chromatography[22] or ammonium sulfate precipitation with ion exchange chromatography[59], can further increase the purity of the IgY preparation. Moreover, sequential precipitation with 31% ammonium sulfate and 12% PEG resulted in IgY antibodies of more than 95% purity without any loss in immunoreactivity[64].

Despite the numerous protocols described in the literature, the most popular isolation strategy of IgYs from immune eggs involves a de-lipidation step, in which IgY is extracted in the supernatant after treating the egg yolk with 10 volumes of acidic water and a subsequent precipitation step, in which IgY precipitates with ammonium sulfate or PEG, at suitable concentrations[30].

Storage

According to the literature, after their isolation, IgYs can be stored for long periods (from a few months to a few years), preferably at -20 °C[22,71], since they are considered reasonably stable biomolecules, like mammalian IgGs[72]. IgY is stable at pH 4-9 and up to 65 °C in aqueous solutions. The addition of stabilizing reagents or high concentrations of salts can further increase resistance of the IgY molecule; *e.g.*, heat stability could be increased up to 70 °C by the addition of sugars, such as 30% sucrose, trehalose or lactose[3]. Useful information concerning earlier findings on the stability and storage conditions of IgYs has appeared in recent review articles[73]. Freeze-drying has been used to facilitate long storage of IgYs[74], though some researchers have reported that freeze-drying may lead to some loss of antigen-binding activity of IgY[45]. Lyophilization of proteins, including IgYs, induces freezing and dehydration stresses, which may result in protein structural changes or even unfolding [75]. Therefore, the addition of cryoprotectants and lyoprotectants has been recommended to protect IgYs during lyophilization[45]. Our team has recently evaluated IgYs that were developed against a KLH-conjugate of the polypeptide prothymosin alpha many years ago and kept as lyophilized powder at -30 °C. As revealed, the IgYs have kept immunoreactivity and were successfully applied to a specific enzyme-linked immunosorbent assay (ELISA) for prothymosin alpha[76].

Evaluation of egg yolk IgYs

Protein concentration: Determination of protein concentration in IgY extracts is usually performed before proceeding to further IgY evaluation. Total protein concentration in IgY extracts has been determined mainly with the Bradford method (indicative references[30,34,35,38,42]), the Lowry method[58] and the bicinchoninic acid protein assay[77]. In addition, protein concentration was assessed with ultraviolet absorption at 280 nm, according to the Lambert-Beer law (indicative references[29,32,33,57,76]).

Purity: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is considered the gold standard technique and has been widely used to assess the purity of the egg yolk-isolated IgYs (indicative references[30,33,34,62,78]). SDS-PAGE

separation under non-reducing or reducing conditions would reveal one or two protein bands, the latter corresponding to heavy and light IgY chains.

Western blotting has been used complementarily with SDS-PAGE to confirm the presence and assess purity of IgYs isolated from immune egg yolks (indicative references[31,33,62,78]). Visualization of the specific protein bands is performed mainly through a color or chemiluminescence development.

In a few cases, additional analytical methods such as high-performance liquid chromatography[57] have also been used to evaluate the purity of IgYs.

Immunoreactivity: The immunoreactivity of egg yolk-derived IgYs is evaluated with well-established immunochemical methods such as dot-blot and ELISA. Dot-blot can be actually considered as a simplified form of ELISA offering mostly qualitative results. Nevertheless, it is a fast, easy, and low-cost technique that may provide useful information and has, therefore, been used by several researchers to evaluate immunoreactivity of IgYs[32,34,36,39,76]. In most cases, however, evaluation of IgY immunoreactivity involves determination of titer against the target antigen through non-competitive ELISAs (indicative references[32,35,45,76,78]). Moreover, other immunochemical characteristics of the isolated IgYs are assessed, such as putative cross-reactivity with various substances through competitive ELISAs (indicative references[31,36,39]). It should be noted that till now and despite the numerous new technologies introduced in the field, ELISA remains the gold standard method for evaluating the basic immunological characteristics of any antibody developed, independently of the antibody class or the production method.

IN VITRO APPLICATION OF EGG YOLK-DERIVED POLYCLONAL IGYS TO THE DETECTION OF BIOMOLECULES

IgY is considered an excellent tool especially for developing *in vitro* methods to detect biomolecules of interest in biological specimens for a series of reasons. First, the evolutionary distance between mammals and birds may facilitate generation of specific IgYs against conserved mammalian proteins, since avian organisms possess a different antibody repertoire than that of mammals and the epitope spectrum of avian antibodies is potentially larger/different than that of mammalian IgGs including novel specificities[19,64]. Second, IgY does not activate the mammalian (including human) complement system and does not react with mammalian Fc receptors; this feature has rendered IgYs an ideal *in vitro* reagent, especially for immunoassays designed to detect biomolecules in human blood serum[64]. Third, substantial amounts of IgY can be isolated from egg yolks; as already mentioned (isolation of IgYs from the egg yolks), one hen can produce 20-40 g of IgY in 1 year, 1%-10% of which is antigen-specific. This advantage of egg yolk IgY is accompanied by other practical superiorities, such as low animal care cost, ease of isolation of antibodies from the egg yolk with simple biochemical methods and overall low production cost[73]. These advantages along with the large-scale facilities currently available render production of egg yolk-derived IgYs, a technically feasible and efficient procedure at industrial level. Some other positive characteristics of IgYs have been reported in the literature, *e.g.*, they can be developed even when hens are immunized with very small amounts of the corresponding antigens[64,71] or that they show higher specificity, binding affinity, and avidity for their targets in comparison with mammalian IgGs[38,73], although other reports have shown controversial data[3]. Last but not least, in the list of IgY advantages is that use of egg yolk IgYs is especially desirable from an ethical aspect of view, concerning refinement of animal experimentation, as already mentioned. Some recent indicative applications of IgYs to the *in vitro* detection of biomolecules (as well as whole viruses/microorganisms) have been summarized and presented in Table 1. Lately, specific IgYs have been developed and used for the immunodiagnosis of pandemic coronavirus disease-2019 (COVID-19)[79], while non-specific IgY has been used to form/visualize the "control line" in point-of-care *in vitro* tests that detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigens[80].

Table 1 *In vitro* applications of polyclonal IgYs

Target biomolecule(s)	*In vitro* immunochemical technique	Proposed field of application	Ref.
Major surface antigen of *Toxoplasma gondii* (SAG1)	Latex agglutination assay	Diagnosis of Toxoplasmosis	Cakir-Koc *et al* [132], 2020
Protein A of *Staphylococcus aureus*	Immunocapture PCR assay	Detection of *Staphylococcus aureus* in food samples, skin and nasal swabs	Kota *et al*[133], 2020
Peptides/proteins present in detoxified western Russell's viper venom	Paper-based microfluidic immunochromatographic test	Differential diagnosis of Russell's viper envenomation	Lin *et al*[134], 2020
SARS-CoV-2 antigen	Fluorescence immunochromatographic rapid-antigen test	Diagnosis of COVID-19	Porte *et al*[79], 2020
Antigens present in total saline extract of *Taenia crassiceps* metacestodes	ELISA	Detection of neurocysticercosis	daSilva *et al* [32], 2020
Antigens present in total saline extract of *Ancylostoma ceylanicum*	ELISA	Diagnosis of Hookworm infection	Souza *et al*[135], 2020
Non-glycosylated synthetic oligopeptides of *Dermatophagoides* group I allergens	Immuno-dot blot assay (with the use of IgY-colloidal gold nanoparticles conjugates)	Detection of indoor dust mite allergens	Egea *et al*[136], 2019
Antigens present in whole bacterial suspension of formalin- and heat-inactivated*Salmonella typhimurium* and *Salmonella enteritidis*	*In vitro* immunochemicaltechniques	Diagnosis of infection with *Salmonella typhimurium* and *Salmonella enteritidis*	Esmailnejad *et al*[26], 2019
Antigenic extracts of *Strongyloides venezuelensis* infectious filariform larvae and parthenogenetic females	ELISA	Diagnosis of human strongyloidiasis	deFaria *et al* [33], 2019
Antigens present in total saline extract of *Ascaris suum* adult life forms	Tissue indirect immunofluorescence assay & ELISA	Diagnosis of human ascariasis	Lopes *et al*[31], 2019
Free prostate specific antigen	ELISA	Diagnosis of human prostate cancer	Łupicka-Słowic *et al*[137], 2019
Antigens (capsid proteins VP2 & VP3) present in beta-propiolactone-inactivated enterovirus 71	Fluorescence sensor assay	Diagnosis of hand-foot-and-mouth disease caused by enterovirus 71 infection	Nie *et al*[138], 2019
Fusarium verticillioides 97K exoantigen	ELISA	Detection of *Fusarium verticillioides* (and prediction of fumonisin contamination) in poultry feed	Omori *et al* [139], 2019
Recombinant purified catalytic domain of Karilysin	ELISA	Evaluation of karilysin (*i.e.* an enzyme secreted by the periontopathogen *Tannerella forsythia*) as a biomarker for the diagnosis of periodontitis	Skottrup *et al* [34], 2019
Fumonisin B1	Lateral flow immunoassay	Detection of fumonisin B1 and fumonisin B2 in maize	Tran *et al*[140], 2019
Synthetic extracellular peptide of matrix-2 protein of influenza A virus, conserved in all strains	Latex agglutination assay	Diagnosis of infection with Influenza A virus	Budama-Kilinc *et al*[141], 2018
Sulfamethazine (SMZ)	ELISA, FPIA	Detection of veterinary drug residues (SMZ) in milk	Liang *et al*[142], 2018
Native calf adenosine deaminase (ADA)	ELISA	Evaluation of ADA as a cancer biomarker	Łupicka-Słowic *et al*[143], 2018
Nucleoprotein of influenza A virus	Immunocytochemistry, Immunohistochemistry	Diagnosis of infection with influenza A virus	da Silva *et al* [144], 2018

ADA: Adenosine deaminase; COVID-19: Coronavirus disease-2019; ELISA: Enzyme-linked immunosorbent assay; FPIA: Fluorescence polarization immunoassay; PCR: Polymerase chain reaction; SAG1: Surface antigen 1 of *Toxoplasma gondii*; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; SMZ: Sulfamethazine.

RELEVANT APPROACHES AND FUTURE PROSPECTS

Monoclonal IgYs

Since the late 1980s many efforts have been directed toward development and use of avian monoclonal antibodies (mAbs) for research, diagnostic, and therapeutic purposes, because avian mAbs may combine the advantages of avian immuno-

globulins with those of monoclonality, *i.e.* precise characterization and continuous production. Initially, several technical difficulties have emerged; even after technical problems have been addressed and avian mAbs have been produced by hybridomas [81,82], the hybridoma technology has not gained wide application, because it is considered a complex, time-consuming and low-yield process by many researchers. By contrast, antibody-engineering methods proved to be the most frequently techniques used for the production of chicken mAbs. Actually, chicken provides an ideal basis for generating large immune antibody fragment libraries as compared to most mammalian species. In chickens, the large and diverse antibody repertoire is generated by gene conversion, in which segments from non-functional V pseudogenes located upstream are inserted into the rearranged gene, and somatic hypermutation. Since gene conversion has not been observed at the 5′- and 3′-ends of the rearranged gene, it is possible to perform real-time reverse transcription polymerase chain reaction (PCR) of the V-region repertoire with a single pair of primers[20,72]. Of the various recombinant antibody fragments, the full-length single-chain variable fragment (scFv) is the most commonly used. For construction of the scFv antibody library, total RNA is isolated from the spleen cells of immunized or non-immunized chicken and reverse-transcribed into cDNA. Then the variable heavy and light chain domain genes of immunoglobulin antibody cDNA are amplified by PCR and properly assembled to form the full-length scFv fragments, which resemble a functional Fv region. Then the scFv genes are cloned into suitable vectors to construct an antibody-expressing library [83]. Currently, phage display systems are the most often applied recombinant methods for generation and isolation of chicken mAbs[83,84]. In phage display methods, genetically-engineered phages that are capable of displaying recombinant fragments of antibodies on their coat surface can undergo several rounds of biopanning and re-propagation in *Escherichia coli* to enrich for clones exhibiting specific binding. Many IgY-scFv were produced with the phage display method combined with *in vitro* selection technologies, either by research groups[84-87] or companies that provide custom services for the development of monoclonal antibodies Y[88,89]. Among recent technologies reported for producing and isolating monoclonal IgYs is the gel encapsulated microenvironment assay, which is capable of "cross-examining" the entire population of splenic B cells from immunized chickens[90]. In an effort to produce mAbs suitable for *in vivo* administration in immunotherapy, the highly immunogenic constant region of chicken IgYs has been replaced with that of human to generate chicken-human chimeric antibodies[91]. Moreover, humanization of chicken scFvs has been successfully performed using the complementarity-determining region (CDR)-grafting strategy, which replaces human CDRs with chicken CDRs while retaining the human framework region residues, and followed by further optimization when necessary[92,93]. On the other hand, chimeric chicken-mouse or mouse-chicken recombinant mAbs have been produced and their characteristics have been studied[94,95].

Antibodies produced by genetically modified chickens (transgenic chickens)

Over the last decades, significant progress was made in generating recombinant proteins, including mAbs for therapeutic applications, in genetically modified chickens[21,96]. Difficulties in generating modified chickens are mainly attributed to the complex structure of the chicken zygote and the different organization of the chick embryo in comparison to mammals. To successfully generate genetically modified chickens, different methods have been used to achieve stable genomic integration of transgenes and the highest efficiency of germline transmission[97], including direct DNA microinjection into the chicken zygote[98] and use of viral vectors for gene transfer, which is the first applied and considered one of the most successful methods. Thus, the first genetically modified chicken was generated by the insertion of retroviral foreign DNA delivered by avian leukosis virus successfully integrated to the germline[99]. Since then, various viral vectors have been used to generate transgenic chickens for the production of recombinant proteins[100-102] including mAbs[103]. Among these, lentiviral vectors have been reported to offer specific advantages, including ability to transduce dividing and non-dividing cells, a relatively large transgene capacity and the apparent resistance of transduced cells to gene silencing [104]. Lentiviral vectors have been used to introduce transgene constructs comprising suitable sequences from the ovalbumin gene to direct synthesis of associated proteins to oviduct[105]. Despite the fact that the use of viral vectors improves germline transmission, the size limitation of the transgene and the lack of possibility of precise edits remain as drawbacks. One of the most effective approaches to produce transgenic chickens is the *in vitro* transfection of avian cell lines, such as primordial germ cells (PGCs) and embryonic stem cells (ES), the clonal selection and reinsertion

into the embryo leading to fully transgenic progeny in the next generation[106-108]. Following this approach, production of human mAbs in the egg white of chimeric transgenic chickens with the use of genetically modified ES cells carrying ovalbumin expression vectors was successfully performed for the first time; however, although a high amount of functional mAb was produced in the egg white, no transgenic offspring were initially obtained[107]. Heritable transgenic chickens capable of producing mAbs in their egg whites were generated using transfected PGCs with a gene construct designed to express the mAb in chicken oviduct magnum[108]. Specific gene editing of PGCs could be improved using genome-editing tools, such as transcriptional activator-like effector nucleases[109] and the clustered regularly interspaced short palindromic repeats–associated protein 9 system (CRISPR/Cas9 system)[110,111]. CRISPR/Cas9 has been used to generate transgenic chickens for the production of recombinant proteins in the white egg[111], including mAbs[112], or exhibiting resistance to pathogens[113]. Another recent promising approach is the replacement of the chicken immunoglobulin variable regions by human V regions and use of synthetic pseudogene arrays in order to produce affinity matured antibodies in transgenic chickens, called OmniChickens; OmniChicken can thus generate antibodies of basically human sequence, which retain the epitope repertoire of chicken immunoglobulins[114].

DISCUSSION

IgY technology has produced a great number of valuable immunochemical tools for biotechnology and medicine since 1990's. Various parameters that are associated with and can affect the results of the IgY technology have been reported in the literature, such as the immunization procedure. One of the most important parameters is the extraction/purification protocol used for isolating the IgYs from the egg yolk. Several methods of isolation and purification of IgYs from "immune" egg yolks have been reported, as already mentioned; the choice of a specific method depends on several criteria, such as desired yield, purity and final application of the IgYs along with cost and scale of extraction. The most popular isolation strategy consists in a de-lipidation step, in which IgY is extracted in the supernatant after treating the egg yolk with 10 volumes of acidic water, and a subsequent precipitation step, in which IgY precipitates with ammonium sulfate or with PEG, at suitable concentrations[30].

Our team have used the acidified water dilution method followed by precipitation with 19% sodium sulfate[39] or with 8.5% and 12% PEG 6000[27] for the isolation of IgYs from immune egg yolks. SDS-PAGE and western blot analysis of IgYs isolated with sodium sulfate precipitation has revealed a protein impurity with MW of ~35 kDa, which underwent liquid chromatography tandem mass spectrometry analysis and was proposed to be identical with the C-terminal fragment of vitellogenin II precursor protein[39]. The same impurity was also observed by other researchers, who had followed a different isolation protocol involving precipitation with PEG 6000[22]. As later shown[115,116], IgY from hen egg yolk occurs as a complex with peptides, named yolkin, which exhibit immunoregulatory and other biological activity. Yolkin contains several peptides with an apparent molecular weight ranging between 1 and 35 kDa. As reported, purified yolkin constituents are homologous with some fragments of the C-terminal region of vitellogenin II; more specifically, yolkin fractions of MW > 16 kDa are glycoproteins corresponding to the amino acid sequence of vitellogenin II starting at position 1572 aa[12,117]. In our hands, presence of the above impurity did not seem to interfere with the efficiency of IgYs as specific *in vitro* immune reagents.

As already mentioned, egg yolk IgYs have been thought to be superior to mammalian IgGs for *in vitro* applications. The *in vitro* efficiency of IgYs may be questioned only under rare conditions, *e.g.*, due to the putative presence of anti-hen antibodies in biological samples of specific individuals who have been sensitized to hen egg yolk[72]; however, to what extent IgY-specific antibodies may occur in human individuals remains to be clarified. Exempt from the aforementioned few concerns, IgYs are considered ideal and are being continuously developed and used as invaluable *in vitro* laboratory tools up to now (Table 1).

One of the great advantages of the IgY technology is the enhanced probability of generating specific IgYs against conserved mammalian proteins, since hens may exhibit a different antibody repertoire than that of mammalian organisms. With this in mind, our team has immunized hens against the poorly immunogenic, highly conserved polypeptide prothymosin alpha (ProTα, MW: approximately 12 kDa,

isolated from bovine thymus). The anti-ProTα antibodies Y were isolated from the egg yolk and evaluated through dot-blot and ELISA experiments in parallel with antibodies G isolated from the antiserum of rabbits immunized against the same immunogen. As revealed, not only antibodies G, but also antibodies Y showed hardly detectable titer/affinity for ProTα[39]. The above negative result may be attributed to the fact that ProTα is thought to be highly conserved during evolution and ProTα-homologues have been reported in non-mammalian organisms as well[76,118]. Similarly, hens were immunized against the highly conserved polypeptide thymosin beta4 (Tβ4, MW: ~5 kDa, synthetic), either conjugated to KLH (Tβ4/KLH) or non-conjugated, leading to IgYs of either relatively high titer or, on the contrary, not-detectable titer, respectively[41]. Interestingly, antibodies Y that we developed against a KLH-conjugate of ProTα (anti-ProTα/KLH IgYs) showed high titer and practically no cross-reactivity with a series of ProTα-fragments, including the N-terminal fragment ProTα[1-28] (also known as Tα1), being therefore highly specific for whole-length ProTα, while the corresponding anti-ProTα/KLH rabbit IgGs did cross-react with Tα1[76]. Moreover, when various synthetic fragments of ProTα or Tβ4 were conjugated to KLH and used for immunizing hens and rabbits, the results revealed that specific antibodies Y of hardly detectable titer were obtained; on the contrary, rabbit immunization with the same immunogens led to high-titer antibodies G, specific for ProTα or Tβ4, respectively[39,41]. The above results support the assumption that novel antibody specificities may emerge *via* the avian immune system and can be obtained through the IgY technology.

Although IgYs for research applications are mainly produced in hens, other birds have also served this purpose, as already mentioned, including duck[23,119], goose [24], quail[26] and ostrich[25], following immunization and isolation protocols similar to those used for hens[18]. Quail, ostrich and other avian species may provide further advantages in the field of IgY technology, such as convenient housing and breeding conditions (quail[26]) or exceptionally high amounts of IgYs obtained (ostrich[25]). Previously, our team has isolated immunoglobulins Y from the egg yolk of several avian species, including ostrich (Struthio camelus) and quail (Coturnix japonica); the isolation protocol has been developed in-house and based on the acidified water dilution and the PEG precipitation method. Ostrich and quail immunoglobulins Y were characterized in terms of their molecular weight (SDS-PAGE and western-blotting) and their ability to recognize and bind to a commercially available horseradish peroxidase (HRP)-labeled rabbit anti-hen IgY antibody in an ELISA system[120]. As revealed, the ostrich IgYs could be hardly recognized by the HRP-labeled anti-hen antibody we used, though other researchers reported successful use of commercially available secondary anti-hen antibodies to assess the immuno-chemical efficiency of specific ostrich IgYs[121]. On the other hand, HRP-labeled secondary anti-ostrich-IgY antibodies have been specially developed and used to evaluate ostrich IgYs with ELISA[25]. According to experimental results of ours[120] and others'[26,122], the quail IgYs could be recognized by the HRP-labeled secondary anti-hen antibody, which indicates that quail and hen IgYs may share at least some homology in immunochemically important structural features[123,124]. Wide availability of secondary antibodies for IgYs originated from avian species other than hens will support further expansion of the IgY technology.

In addition to their unequivocal usefulness as in *vitro* immunodetection reagents, IgYs have been proposed as promising *in vivo* therapeutics, *e.g.*, as an alternative to antibiotics treatment against multi-drug resistant or difficult-to-treat pathogens, since they exhibit *in vivo* pathogen-neutralizing activity, especially in mouth, throat, the respiratory tract and lungs[73]. Moreover, since IgYs are not absorbed by the gastro-intestinal tube, they have been proposed as perorally administered immunotherapeutics against various viral, bacterial, and fungal infections of the gastro-intestinal tract, especially in veterinary medicine and fish-cultivation[3]; a limitation in wide therapeutic application of perorally administered IgYs is their reduced stability at low pH[72] and several efforts have been made to address this shortcoming. IgYs have been also proposed as locally administered immunotherapeutics for treating skin and other local infections[3]. Lately, specific IgYs have been developed and used for treatment of the pandemic COVID-19[35,64,66,125]. Overall, despite the new promising technologies emerged, literature on the IgY technology continues to expand, encompassing various applications ranging from *in vitro* immunodetection of biomolecules and *in vitro* immunodiagnostics to *in vivo* immunotherapeutics[18,126].

Though development of monoclonal IgYs cannot be considered as a part of the original "IgY technology", it seems very attractive and will probably be the next big step in the area, since it combines the advantages of mAbs with those of avian IgYs. At the initial phase, production of chicken mAbs had to overcome several technical

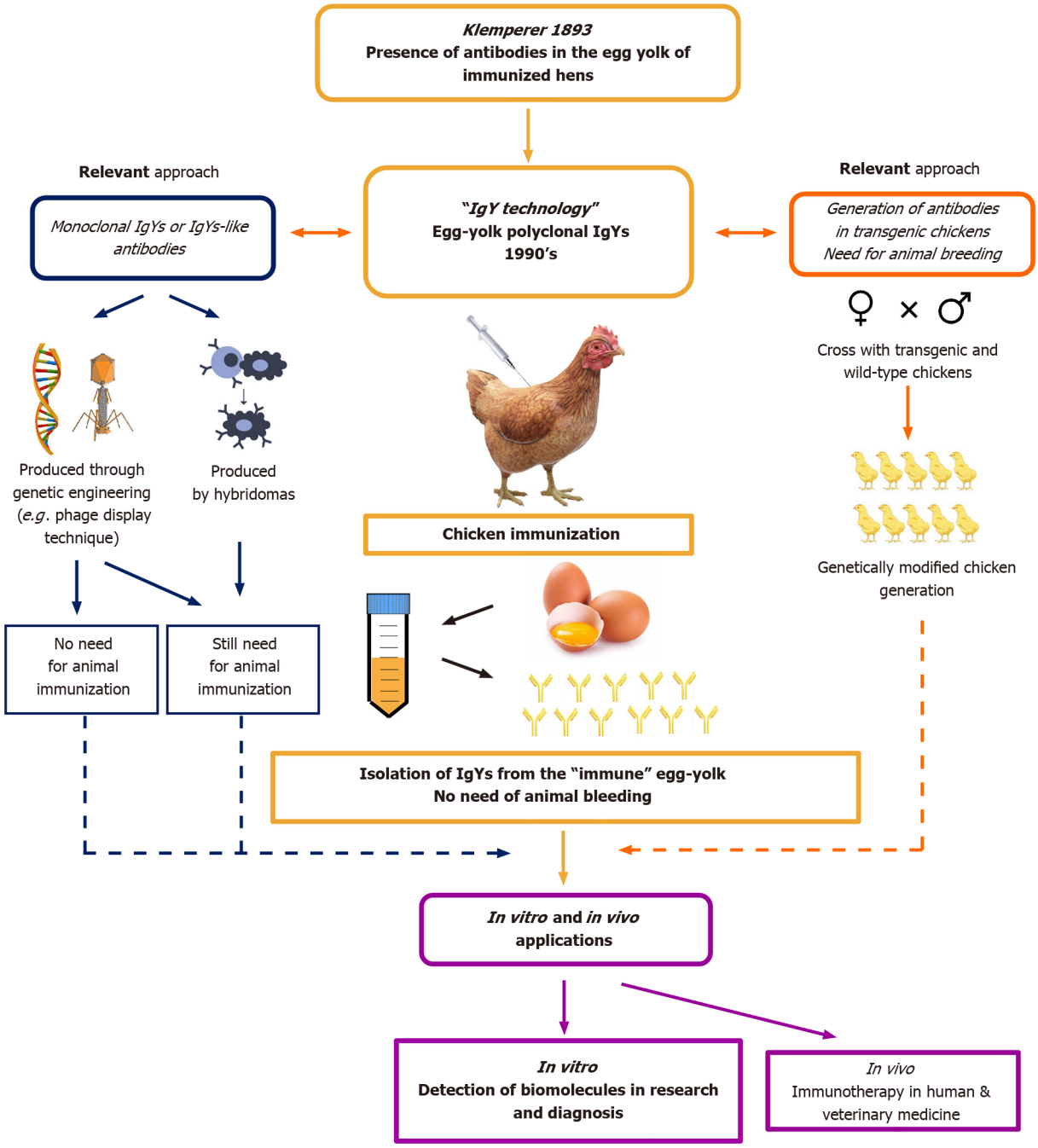

Figure 1 Schematic representation of the main parts comprising the original immunoglobulin Y technology (central axis); promising relevant approaches are also shown (periphery, left and right).

difficulties, including lack of appropriate fusion partners and loss of antibody secreting ability by the hybridoma cells over time[81]; this has been successfully addressed when monoclonal IgYs were generated through combinatorial antibody libraries *via* the phage display methodology[127]. Thus, over the past years, avian libraries have been constructed and several reports on the isolation of avian-derived antibody fragments have been published[20]. The different spectrum of epitopes recognized by the avian immune system may facilitate the development of novel diagnostics, *e.g.*, through targeting highly conserved mammalian proteins, while monoclonality may especially facilitate the development of novel therapeutics for human use, provided that the technology of chimeric avian/human fusions could be fully exploited. One should also keep in mind that recombinant technologies can lead to the generation of monoclonal IgY or IgY-like antibodies circumventing the need for animal immunization[72,83], which is desirable from an ethical point of view concerning the animal welfare.

It is important to remind that the IgY technology was introduced in 1990's as an alternative that could at least partly fulfil the ethics requirements set by the 3Rs principle[1,3]. Recently, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) has recommended that "animals should not be used for the development and production of antibodies for research, regulatory, diagnostic and therapeutic applications any longer", taking into account the Opinion of the EURL ECVAM Scientific Advisory Committee (ESAC) on the scientific validity of replacements for animal-derived antibodies[128]. As referred to by the ESAC, the 2018 Nobel Prize in Chemistry was awarded "for the phage display of peptides and antibodies"[129,130], which, according to the Committee, proves maturity and supports wide application and full exploitation of the phage display technology in the area of antibody production. The EURL ECVAM recommendation may accelerate transformation/switch of the original IgY technology toward development of monoclonal IgYs through phage display techniques that totally avoid the animal immunization step. Total avoidance of animal immunization will further minimize the risk of zoonotic diseases, which is very low but still present when antibodies are produced in chickens, both wild and transgenic.

HIGHLIGHTS

The avian polyclonal antibodies/IgYs have unique and highly desirable functional features.

The term "IgY technology" describes the procedure involving immunization of avian species, consequent isolation of the polyclonal IgYs from the "immune" egg yolk (thus avoiding bleeding and animal stress) and application of the IgYs to various areas of medicine and biotechnology.

During the last decades the IgY technology has been widely used, especially as a means for the efficient *in vitro* immunodetection of biomolecules in many fields of research and disease diagnosis.

Despite the very promising relevant new approaches, there is still space for further exploiting the original IgY technology, due to specific functional, practical and ethical reasons and/or advantages.

CONCLUSION

Until now, development of polyclonal IgYs through the IgY technology has been widely used as a low cost and highly efficient tool, offering a lot of advantages and thus gaining wide application mainly in the *in vitro* immunodetection of biomolecules in biological specimens. Since polyclonal antibodies exhibit some unique functional qualities[131], there is still space for performing research to improve different aspects of the IgY technology. On the other hand, the original IgY technology may "merge" with relevant highly promising approaches, eventually leading, *e.g.*, to worldwide application of non-animal-derived recombinant IgYs or IgY-like immunoglobulins, which, among other benefits, will fulfil strict ethical requirements concerning animal welfare (Figure 1). However, until the practical problems associated with the above-mentioned approaches, *e.g.*, high-cost and/or limited availability of necessary reagents and protocols, have been fully addressed, the original IgY technology still remains a feasible, well-established procedure, in particular for low- and middle-income countries and research laboratories and especially in the field of *in vitro* immunodetection of biomolecules.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 September 20; 11(5): 263-277

DOI: 10.5662/wjm.v11.i5.263

ISSN 2222-0682 (online)

SYSTEMATIC REVIEWS

Evaluation of the red reflex: An overview for the pediatrician

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Conflict-of-interest statement: All the authors declare that they have no competing interests.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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Abstract

BACKGROUND

Red reflex test (RRT) is a simple, non-invasive method that can be performed easily by pediatricians during the clinical examination in neonatal period, infancy and childhood. Abnormal reflexes can lead to prompt diagnosis of several ocular disorders, with potentially severe consequences on patient's vision, cognitive function and even life.

AIM

To underline the contribution of pediatricians to early detection of vision and life threatening diseases by using RRT effectively.

METHODS

For the present systematic review, PubMed searches were performed using the key words "red reflex and newborn"; "red reflex and neonate"; "red reflex and complications"; "red reflex and necessity"; "red reflex and retinoblastoma"; "red reflex and congenital cataract"; "red reflex and glaucoma"; "red reflex and prematurity"; "red reflex and leukocoria"; "red reflex and blindness"; "red reflex sensitivity and specificity"; "red reflex and differential diagnosis"; "red reflex and guidelines". The relevant articles were selected without language restrictions. When a full-text publication was not available, their English abstracts were used. In some cases, studies from the reference lists of the selected articles provided useful information. The research took place in September 2020, in the Ophthalmology Department of University Hospital of Alexandroupolis.

RESULTS

A total of 45 articles were selected according to the used key words. After reviewing data from these articles, it is supported that red reflex remains an effective tool of undeniable importance for early detection of severe eye conditions, such as cataract, retinoblastoma, retinopathy of prematurity and glaucoma. Although literature reports some limitations of RRT, including a notable percentage of false positive tests, the inability to detect small, peripheral retinoblastomas and the lower sensitivity for posterior segment pathology, it is widely accepted that the benefits from the regular evaluation of the test on public

Manuscript source: Invited manuscript

Specialty type: Pediatrics

Country/Territory of origin: Greece

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 27, 2021
Peer-review started: January 27, 2021
First decision: May 6, 2021
Revised: May 19, 2021
Accepted: August 3, 2021
Article in press: August 3, 2021
Published online: September 20, 2021

P-Reviewer: Marickar F
S-Editor: Ma YJ
L-Editor: Filipodia
P-Editor: Guo X

health are significant. Therefore, RRT has been established by international guidelines and should be an essential component of pediatricians clinical practice. Red reflex implementation should be incorporated in pediatricians educational programs, so that they would be able to provide quality services and safe diagnoses.

CONCLUSION
The implementation of RRT should be encouraged in all neonatal/pediatric departments. Prompt education of pediatricians should be empowered in order to achieve careful vision screening, according to current guidelines.

Key Words: Red eye reflex; Leukocoria; Visual screening; Newborn; Prevention; Pediatric examination

Core Tip: Red reflex test (RRT) is an easy, non-invasive examination that enables detection of vision- and life-threatening eye disorders. Various studies have dealt with the effectiveness, sensitivity/specificity and abnormalities of the RRT. The aim of the present review is to emphasize the advantages of RRT implementation from neonatal period to childhood and to underline the pediatricians' role in early diagnosis and treatment of the aforementioned diseases. This study presents a practical guide for the evaluation of the RRT, based on literature data. With appropriate education and compliance to the vision screening protocols, the pediatric society could reduce the incidence of preventable pediatric blindness.

Citation: Toli A, Perente A, Labiris G. Evaluation of the red reflex: An overview for the pediatrician. *World J Methodol* 2021; 11(5): 263-277
URL: https://www.wjgnet.com/2222-0682/full/v11/i5/263.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i5.263

INTRODUCTION

The first few weeks of life are of paramount importance for the development of visual function, thus the assessment of newborns' visual system should be part of the routine clinical examination. Numerous eye disorders of the neonatal and childhood period can lead to permanent visual impairment, even to blindness and loss of life[1].

It is worth mentioning that about 75% of blindness cases are preventable[2], a fact that emphasizes the need for early detection of the sight threatening conditions. Congenital infections, metabolic and chromosomal disorders and inheritance are among the most common causes of childhood blindness[3]. Therefore, eye examination should begin in newborn infancy and be continued as a part of the routine pediatric examination in order to achieve early diagnosis, prompt treatment and better prognosis.

The red reflex test (RRT), which was firstly introduced by Bruckner in 1962[3], is an effective, non-invasive examination that contributes to the diagnosis of various eye diseases, such as cataract, glaucoma, retinoblastoma and retinal disorders[3,4]. The RRT is easily performed in a darkened room by holding a direct ophthalmoscope, focused on the patient's pupil, with a lens power at ''0'', from a distance of approximately 30-45 cm (12-18 in)[4,5]. The normal RRT should be symmetric in both eyes, round, bright reddish-yellow or light grey in darkly colored eyes. Any asymmetry or lack of a red reflex, white reflexes or dark spots are abnormal and require referral to ophthalmologists[4,6]. The main causes of red reflex abnormality include congenital cataract, opacity of the cornea, iris abnormalities, vitreous opacities, tumors or chorioretinal malformations[6].

According to the latest suggestions of the American Academy of Pediatrics (AAP) and the American Association for Pediatric Ophthalmology and Strabismus (2016) the RRT should be performed at every well-baby visit from newborn to 6 mo age, afterwards, at 12 mo, 1-3 years, 4-5 years and 6 years and older[7,8].

The primary objective of this article is to provide an updated review on RRT published literature and highlight its importance in the detection of potentially sight threatening or even life threatening ocular diseases.

MATERIALS AND METHODS

Study design and selection criteria

This systematic review met the statements checklist of the Preferred Reporting Items for Systematic Reviews (PRISMA)[9]. The selection criteria were defined by applying the Problem/Population, Intervention, Comparison and Outcome framework. Articles were screened by title and abstract, according to the following inclusion criteria: Articles focused on the contribution of the RRT in diagnosing pathologic conditions, articles on RRT sensitivity and specificity, articles on guidelines about RRT and articles focused on compliance with RRT recommendations. Articles referring to adult patients were excluded. All of the eligible articles provided valuable information about the usage of the RRT in pediatric clinical practice.

Literature research strategy

A systematic search on PubMed databases was performed by two reviewers (A.T. and A.P.) in September 2020. Search terms used in this review are presented in Table 1. The initial search was performed without search filters and language restrictions. When the eligible articles were not available in full text in English, abstracts were used as a source of information. The date of publication was not an exclusion criterion. Additionally, the reference lists of the eligible articles were checked, and articles that met the inclusion criteria and provided useful information were also selected.

Study selection and quality assessment

A total of 45 articles that were relevant to the topic of interest and exclusively referring to the pediatric population were finally selected. Afterwards, the eligible articles were scanned diligently and independently by the two reviewers and the following data were extracted: Correct evaluation of RRT, correlation between RRT and certain ocular diseases (such as retinoblastoma and congenital cataract) and compliance and limitations of RRT. Any conflict was dissolved by a third reviewer (G.L.). Risk of bias of the eligible articles was conducted with "Quality Assessment Tool for Quantitative Studies" by Effective Public Health Practices[10]. Again, the same two individual reviewers assessed the articles, blinded to each other's decisions, and a third reviewer resolved any conflict. The results are demonstrated in Table 2.

RESULTS

Literature review returned 45 articles that met our inclusion criteria. They covered the whole spectrum of the topic in interest. The final selection of the eligible papers are presented in Table 1. Detailed data on the articles are shown in Table 3.

Diagnostic procedure of RRT

The evaluation of RRT is an easy, low-cost method that can be performed by pediatricians and other first care physicians in order to provide early diagnosis of severe pathologies. A necessary precondition that allows valid diagnoses is the appropriate training of medical students and trainee pediatricians during the years of specialty. For ideal performance of the test, it is essential to keep the room completely darkened (to maximize pupil dilation) and the direct ophthalmoscope fully charged. The lens power should be set at "0", unless there is a refractive error of the clinician's eyes. In this case, she/he could examine without wearing spectacles, by holding the ophthalmoscope closely to the examiner's eye and dialing the spectacle corrective power into the instrument. A practical way is to look through the peephole and dial the lenses till a pure image is viewed. The clinician should sit at a distance of approximately 0.5 m, but it could be increased in case of a nervous or uncooperative child. The co-axial position of the examiner is appropriate for estimating the RRT, however, it may not reveal ocular pathologies of small dimensions in peripheral areas. Therefore, the pediatrician needs to perform the RRT by using different angles along the horizontal meridian of the retina in order to assess the nasal and temporal retina by oblique viewing[4,5,11]. It is quite easy to perform, even in younger patients, as after

Table 1 Search terms

RRT and neonate/newborn	RRT congenital cataract
RRT and complications	RRT retinoblastoma
RRT and necessity	RRT and glaucoma
RRT and sensitivity and specificity	RRT and blindness
RRT and differential diagnosis	RRT and leukocoria
RRT guidelines	RRT and prematurity

RRT: Red reflex test

evaluating the red reflex from a co-axial position, the pediatrician should make the patient look in different directions. It may be helpful for the examiner to make the child focus behind the examiner's back by using a light or a toy. In case of infants or newborns, parents should hold the baby with a "chair hold" manner. This may keep the baby calm and able to focus the gaze straight forward. In this way, the examiner could perform the RRT without opening the baby's eyes with her/his hands. Taking into account that in the first days of life it is hard for the neonates to fixate or even to open their eyes, pediatricians should have plenty of patience and time in order to evaluate RRT safely.

A normal RRT consists of symmetrical bright red reflexes of both eyes, indicating that the ocular media (cornea, aqueous humor, lens, vitreous body) are transparent. A reduced or absent red reflex indicates an obstacle to the anatomical path to and from the retina (Table 4)[12].

Despite its name, the "red" reflex is often normally yellow, orange, red or any combination of these colors. In some patients with darker complexion, an increased pigmentation of the eye could be the cause for less bright reflex. Therefore, many variations among different racial or ethnic groups may be observed. Thus, examination of the parents would set the normal baseline[4,12].

In any case of atypical coloration of the red reflex, pediatricians should take into account many parameters and risk factors, such as gestational age, birth weight, use of oxygen therapy, phototherapy, blood transfusion and conjunctivitis, that could significantly affect the development of vision problems and subsequently the result of RRT[2]. Black reflex, which is suggestive of corneal scar, cataract or intraocular hemorrhage, and asymmetrical or non-homogenous reflexes require further investigation[13]. Asymmetry in the refractive power of the eye may cause asymmetrical red reflexes and should be checked, because any delay could lead to amblyopia and loss of vision[7]. Refractive errors may also give a yellow-white edge to a red reflex[14].

A white pupillary reflex is characterized as leukocoria, from the Greek words "leucos" (white) and "kóre" (pupil). White pupils are often noted by parents and described as something white, shiny, jello-like or a discoloration of the eye. This finding is pathological. Therefore, its presence is always concerning and requires urgent referral to an ophthalmologist. The most common cause of leukocoria in newborns is congenital cataract[15], while the most ominous pathology is retinoblastoma[16]. Other causes of leukocoria are presented in Table 3[15].

Pharmaceutical pupil dilation before performing RRT remains a controversial issue. Some infants and young children may have small pupils and restricted fixation, making the ophthalmoscopy a difficult examination. Moreover, patients with risk factors, such as family history of retinoblastoma or cataract, need a thorough evaluation of RRT in order to exclude any possibility of pathological lesions. In these cases, dilation of pupils could enhance the evaluation of RRT. In a survey by Ozkurt *et al*[17], RRT without pupillary dilation presented a positive predictive value of 70%; as without dilation, 2.2% of newborns presented an abnormal RRT. After dilation, ocular pathology that caused an abnormal RRT was detected in 1.5% of these neonates. For many years, pupil dilation has been used by pediatric ophthalmologists on infants over 2 wk on a routine basis. However, the pharmaceutical agents used for dilation (phenylephrine, anticholinergic agents such as cyclopentolate hydrochloride, tropicamide) were occasionally associated with significant complications. The reported adverse effects include elevated blood pressure and heart rate, urticaria, cardiac arrhythmias, and contact dermatitis. It is worth noting that extra caution is needed in cases of preterm infants, as they presented increased sensitivity to the aforementioned dilating eye drops[4]. Thus, the last policy statement of the AAP in 2016 clarified that if

Table 2 Quality assessment

Ref.	Year	Selection bias	Study design	Confounders	Blinding	Data collection methods	Withdrawals and drop-outs	Global rating
Nye[1]	2014	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
De Aguiar *et al*[2]	2011	Strong	Weak	Strong	Moderate	Strong	NA	Moderate
Eventov-Friedman *et al*[3]	2010	Moderate	Weak	Strong	Moderate	Strong	NA	Moderate
AAP[4]	2008	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Litmanovitz *et al*[5]	2010	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Cagini *et al*[6]	2017	Strong	Weak	Strong	Moderate	Strong	NA	Moderate
Loh *et al*[7]	2018	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Donahue *et al*[8]	2016	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Li *et al*[11]	2010	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Gurney *et al*[12]	2018	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Levin[13]	2015	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Sloot *et al*[14]	2015	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Wan *et al*[15]	2014	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Tuli *et al*[16]	2011	Moderate	Weak	Weak	Moderate	Moderate	NA	Weak
Ozkurt *et al*[17]	2018	Strong	Weak	Strong	Moderate	Strong	NA	Moderate
Bell *et al*[19]	2014	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Balmer *et al*[20]	2007	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Mansoor *et al*[21]	2016	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Popoola *et al*[22]	2019	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Abramson *et al*[23]	2003	Moderate	Weak	Weak	Moderate	Strong	NA	Weak
AAP *et al*[24]	2002	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Li *et al*[25]	2013	Strong	Weak	Strong	Weak	Strong	NA	Weak
Butros *et al*[26]	2002	Moderate	Weak	Weak	Weak	Weak	NA	Weak
Sun *et al*[28]	2016	Strong	Weak	Strong	Weak	strong	NA	Weak
DerKinderen *et al*[29]	1989	Moderate	Weak	Strong	Weak	Strong	NA	Weak
Goddard *et al*[30]	1999	Moderate	Weak	Strong	Weak	Strong	NA	Weak
Bhatti *et al*[31]	2003	Moderate	Weak	Weak	Moderate	Moderate	NA	Weak
Rajavi *et al*[32]	2016	Moderate	Weak	Weak	Moderate	Weak	NA	Weak
Gogate *et al*[33]	2011	Weak	Weak	Weak	Moderate	Weak	NA	Weak
Haargaard *et al*[34]	2015	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Atiq *et al*[35]	2004	Weak	Weak	Strong	Moderate	Weak	NA	Weak
Meier *et al*[36]	2006	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Donahue *et al*[37]	2016	Moderate	Weak	Weak	Moderate	Strong	NA	Weak
Mndeme *et al*[38]	2010	Moderate	Weak	Strong	Moderate	Strong	NA	Moderate
Magnusson *et al*[39]	2013	Moderate	Weak	Strong	Moderate	Strong	NA	Moderate
Özkurt *et al*[40]	2019	Moderate	Weak	Weak	Moderate	Strong	NA	Weak
Ulanovsky *et al*[41]	2015	Strong	Weak	Strong	Moderate	Moderate	NA	Moderate
Raoof *et al*[42]	2016	Weak	Weak	Weak	Moderate	Strong	NA	Weak
Wall *et al*[44]	2002	Weak	Weak	Weak	Moderate	Strong	NA	Weak

Gupta *et al*[45]	2019	Weak	Weak	Strong	Moderate	Weak	NA	Weak
Munson *et al*[46]	2019	Moderate	Weak	Weak	Moderate	Moderate	NA	Weak
Chen *et al*[52]	2019	Moderate	Weak	Weak	Moderate	Strong	NA	Weak
AAP[53]	2003	Moderate	Weak	Strong	Moderate	Weak	NA	Weak
Anderson[54]	2019	Moderate	Weak	Weak	Moderate	Weak	NA	Weak

NA: Not applicable.

Table 3 Data extracted from the eligible articles

	Total number of articles	Ref.
Emphasis on the importance of RRT	44	[1-8,11-17,19-26,28-42,44,46,52-54]
Retinoblastoma	20	[3,6,11,12,15-17,19-23,26,28-30,36,38,40,54]
Congenital cataract	16	[3,5,6,12,15-17,19,21,31-35,38-40]
Retinopathy of prematurity	4	[2,3,16,20]
Specificity and/or Sensitivity of RRT	10	[3,5,6,11,12,22,25,28,32,38]
Compliance of health care providers with current screening protocols	12	[2,3,5,6,14,22,23,32,39,40,42,44]
Limitations of RRT	9	[1,4,6,11,12,23,26,28,41]
Comparison of RRT with other techniques	7	[17,25,28,34,38,45,46]

RRT: Red reflex test.

Table 4 Anatomical approach for an abnormal red reflex test[4,12]

Tear film	Mucus or other foreign bodies
Cornea	Dysgenesis of the anterior segment (Peters anomaly), congenital glaucoma, birth trauma
Lens	Cataract
Vitreous	Persistent fetal vasculature, vitreous hemorrhage or inflammation
Retina	Retinoblastoma, retinal detachment, Coat's disease, chorioretinalcoloboma, toxocariasis
Other	Anisometropia, strabismus

the pediatrician provides conditions of a fully darkened room, further pharmaceutical dilation is not necessary. Abnormal findings in RRT including dark spots, absent or significantly reduced reflex, leukocoria, or any asymmetry of the reflexes are indications for referral to an ophthalmologist with experience in children for thorough dilated fundus examination[8].

Retinoblastoma

Retinoblastoma is a neuroblastic tumor of the retina, with an incidence of approximately 1:20000 live births per year, which can lead to blindness, metastatic disease and loss of life[18,19]. The onset of the disease may occur *in utero* and up to 4 years of age[20]. It was estimated to be responsible for 17% of all neonatal cancers[15,20]. Sixty percent of retinoblastomas are non-heritable, usually unilateral[21,22]. Studies recorded positive family history in only 15%-25% of patients[19,22]. Typically, heritable retinoblastomas are bilateral, usually presented within the first year of life, with better visual potential in the eye with the smaller tumor size. According to literature data, the most common reason that concerned the family was the presence of leukocoria (initial sign in 50%-60%), often observed on flash photographs. Other manifestations included strabismus (initial sign in 20%-25%), inflammatory signs (initial sign in 6%-10%), *e.g.*, painful/red eye, tearing, heterochromia and hyphema[15,18,20,23]. In 50% of the cases worldwide there were extraocular signs and symptoms, which were associated with poorer survival rate (0%-50% *vs* 95%)[23].

Currently, the RRT is the main screening tool used by primary care physicians for the detection of retinoblastoma[24]. Li *et al*[25] reported a case of a 3 d old newborn with retinoblastoma, as the youngest patient with the disease. Notably, the median age of retinoblastoma diagnosis is 24 mo[8,26]. Therefore, the RRT should be performed by the pediatrician on a regular basis at every age, from birth to childhood. During the clinical examination, leukocoria seen on RRT is the primary sign that sets the suspicion for retinoblastoma[20]. A normal RRT is not equal with the absence of retinoblastoma [27]. According to some studies[26,28], peripheral or small tumors could give falsely normal RRT, while larger tumors were generally detected by RRT. The studies underlined that early diagnosis *via* RRT implementation and prompt treatment of retinoblastoma were associated with better prognosis and higher cure rate (95%)[18, 19].

Delayed detection increases the possibility for larger tumors and metastases, rendering the treatment significantly more aggressive and costly, with no certain outcome[20]. More specifically, an 8-wk-delay after the onset of signs and symptoms led to elevated risk of local invasion[29] and a 6-mo-delay highly increased the extraocular extension risk[30]. Untreated retinoblastomas are fatal[18,19]. A recent study reported that in most cases (80%), the parents firstly noticed the presenting signs of retinoblastoma and not pediatricians (8%) or ophthalmologists (10%)[23]. Possible explanations for these findings included (1) The difficulty to evaluate peripheral tumors with RRT performed by co-axial position *vs* more opportunities for the family members to view the eye from multiple angles; (2) Underutilization of well-child care visits; (3) A not dark enough pediatric office during RRT; (4) Uncooperative child; (5) Miotic pupils; (6) Inappropriate RRT technique, lack of education; and (7) Low clinical suspicion[23]. Another study mentioned delayed referral from the primary care physicians to specialists in ⅓ of the cases, due to justification of the presenting signs as normal findings or as part of other diagnosis. An additional reason for delayed therapy was the time spent by parents seeking treatment. The family unwittingly contributed to the delay in 77% of patients[26].

It is also emphasized that the pediatricians must have the education and skills to identify and refer to specialists a patient with the suspicion of retinoblastoma, in prompt time[26]. It has to be clear to all pediatricians that a positive family history of retinoblastoma, regardless of the RRT result, is an absolute indication for ophthalmologic examination in newborn nursery. Afterwards, regular ophthalmologist evaluations must be arranged, with aggressive surveillance and communication between the supervisor pediatrician and ophthalmologist until at least 28 mo of life. In case of revealed tumor, the follow-up should be continued until at least the age of 7 years. Additionally, the observation of eye abnormalities (leukocoria, strabismus) by the family, at any age, regardless of the RRT results, requires similar investigation to rule out any possibility for malignancy[23,26].

Congenital cataract

Congenital cataract is the opacification of the crystalline lens, which can be present at birth or develop within the first 3 mo of life. According to published literature[6,21,31-33], the incidence of congenital cataract ranges between 0.6 to 15 per 10000 live births, while it was estimated to be responsible for approximately 10% of childhood blindness [3]. In most cases, the etiology remains unknown. Inheritance is involved in 25% of congenital cataracts (autosomal dominant pattern). Other causes include chromosomal abnormalities (trisomy 21, trisomy 18), metabolic disorders (*e.g.*, galactosaemia) and congenital infection syndrome (toxoplasmosis, cytomegalovirus, syphilis, rubella, herpes simplex virus, varicella zoster virus)[21]. It is worth mentioning that most unilateral cataracts are isolated anomalies, however, 20% of cataracts attributed to congenital rubella are unilateral. Bilateral cataracts that are not correlated with genetic mutations need further investigation to exclude systemic disorders[15].

Early detection and treatment of congenital cataract have become a priority of the Global Vision 2020 initiatives of the World Health Organization[6]. The RRT is a highly sensitive screening test for congenital cataract. Cagini *et al*[6] performed RRT screening on neonates up to 3 d old, over a period of 3 years, indicating a congenital cataract rate of 0.009%. Haargaard *et al*[34] indicated the superior sensitivity of RRT compared to other diagnostic techniques in the detection of congenital cataract. The absence of red reflex during the routine neonatal eye screening could reveal early diagnosis of congenital cataract. This finding requires a thorough systemic clinical examination and the appropriate investigation to rule out every common or rare condition causing congenital cataract. Atiq *et al*[35] presented a case report of a 5-mo infant with bilateral congenital cataract, which at birth was investigated only for rubella and galactosemia. In the months that followed, the progressive clinical status

(including delayed motor milestones, irritability, sweating during feeding, generalized hypotonia, supraventricular tachycardia) and the family history of neonatal deaths, in combination with detected lactic acidosis and hypertrophic cardiomyopathy led to the diagnosis of Senger's disease. Other clinical findings that would empower the suspicion for cataract include nystagmus, absence of interest for surroundings and inability to fix and follow[21].

The time of detection of congenital cataract is crucial for the visual outcome following surgery, since early therapeutic intervention before the age of 6 wk for unilateral cases and 8 wk for bilateral cataracts was associated with best visual outcome[6,32]. Any delay on the detection and treatment of congenital cataract could give rise to severe consequences in visual evolution, even blindness or amblyopia, which has considerable impact on the neurobiological development of the children. In Bhatti *et al*[31] study, more than half of the infants with isolated cataract were diagnosed during the first 6 wk of life, but 38% of them were detected later, with a percentage of 15% after the age of 5 mo. The findings above underlined the importance of the method in detecting the cases of congenital cataract.

Retinopathy of prematurity

Retinopathy of prematurity (ROP) is a vasoproliferative disorder that affects premature babies, especially those with low weight of birth[2]. In Meier *et al*[36] study, ROP was responsible for 12% of leukocoria cases. Gestational age of less than 30 wk, birth weight less than 1500 g, history of oxygen therapy, septicemia and blood transfusion are among the most prevalent risk factors for the development of this potentially vision threatening disease[2,16]. The first clinical finding that could be observed in these newborns is the demarcation line, (Stage 1) which indicates the difference between avascular and vascularized retina. As the disease progresses, capillary growth begins at the edges of the demarcation line leading to the formation of ''ridge'' (Stage 2); the development of fibrovascular proliferation from the ridge into the vitreous body constitutes the third stage of ROP. From this stage, fibrovascular membrane may be grown posteriorly causing tractions and thus partial (Stage 4) or total retinal detachment (Stage 5)[20]. It becomes clear that advanced stages are equal with severe and permanent visual impairment. Therefore, careful examination of all preterm infants from pediatric ophthalmologists is of great clinical importance and has to be emphasized and encouraged from pediatricians[37]. It is worth noting that leukocoria may be detected after Stage 3 of ROP, however, the RRT performed by pediatricians or primary care physicians and the early referral of all suspicious cases may save the vision of a neonate as early implementation of laser or cryotherapy on peripheral retina protects the central retina from damage[16].

Sensitivity and specificity of RRT

Several studies have dealt with the sensitivity and specificity of the RRT. The estimated rates of sensitivity and specificity of RRT without pupillary dilation immediately after birth were 85% and 38.5% respectively for the diagnosis of any congenital ocular disorder[32]. Concerning the early diagnosis of congenital cataract and retinoblastoma, the RRT was proved to be a useful tool, with high rates of estimated respective sensitivity (100%) and specificity (97.9%). However, researchers identified a remarkable percentage of false positive tests (2%) and a positive predictive value of 0.7%[6]. On the contrary to these findings, another study revealed a much smaller percentage of false positives (0.0006%), with a positive predictive value of 42%[3]. Furthermore, Sun *et al*[28] evaluated the sensitivity and specificity of RRT in the detection of anterior (cornea, iris, aqueous humor or lens) and posterior (vitreous body, retina, choroid, optic nerve) ocular disorders. They considered the RRT as a useful diagnostic tool for diseases located on the anterior segment of the eye, since it detected 99.6% of the anterior pole anomalies (*vs* 4.1% of the posterior pole diseases). Therefore, RRT presented high rates of overall specificity (95%), but low overall sensitivity (13.9%). The rates of RRT sensitivity and specificity may range depending on the circumstances. Rajavi *et al*[32] found a significant increase of false positive RRT when newborns were examined by pediatricians in the first hours of life, under non-standard conditions (at the delivery room, beside mother and without pupil dilation), in comparison with the examination that took place by ophthalmologists, on the third day of life, under standard conditions (dark room and dilated eyes). In this study, the RRT sensitivity was 85% and specificity 38.5%, when performed under non-standard conditions. The results could be explained by the lack of experience of the pediatricians and by the ocular and tear film problems that usually disappear within a few days after birth. However, the researchers underlined that better conditions could make the evaluation of RRT more accurate.

Importance of the RRT

All of the eligible articles of the present review unanimously pointed out the value of the red reflex examination as an efficient tool for early diagnosis of pediatric ocular diseases, achieving prompt treatment with better outcomes. At this point, the literature highlights the unique role of pediatricians, who own the advantage not only to contribute to the improvement of the course of eye diseases and of the quality of patients' lives but also to save lives of children with severe diseases, such as retinoblastoma. The low incidence of retinoblastoma might reassure the general pediatrician. Nevertheless, the fact that plenty national newborn screening programs include tests for rare diseases, such as phenylketonuria (1/18000) and galactosemia (1/57000), indicates that early detection of severe diseases could prevent grave consequences for the economies, the health care systems and also for the quality and even the existence of the patient's life[26]. Similarly, applying RRT correctly could prevent the bad outcomes of delayed detection of retinoblastoma.

The performance of the RRT, from infancy and at every well child visit, following current pediatric guidelines could prevent childhood blindness[3,38]. Literature data correlated the RRT with early detection of congenital cataract. In Sweden[39] RRT seemed to be performed at the maternity ward at high rates (90%), a fact that was connected with an increase in the percentage of early detection and treatment of patients with congenital cataract. More specifically, 75% of the children who underwent surgery before the age of 1 year were cases with diagnosis and treatment before the sixth week of life. Another study[34] dealt with the 5-year experience of the different eye screening protocols of Sweden and Denmark. The results revealed a significantly higher rate of prompt diagnosis of pediatric cataract, when RRT was performed at maternity wards, in comparison with the absence of RRT. A survey in Tanzania concluded the same results[38]. The need for implementation of red reflex screening is even stronger in Turkey, the Middle East, Asia and Africa. Countries with high rates of consanguineous marriages showed significant correlation with red reflex abnormality (Turkey: 70.6% of neonates with abnormal RRT with a history of parental intermarriages *vs* 29.4% among normal reflexes), which was related with higher prevalence of genetic causes of common pediatric ocular diseases[17]. In a Turkish study[40], 72% of the pediatricians questioned considered that they should add RRT to follow-up charts, as they do for somatometric data. After all, pediatricians should be aware and well educated on including RRT in their routine clinical practice, as the importance of this method is to enable them to assess the quality of the transparent media of the eye, in an easy, non-invasive, low-cost manner[2].

Limitations of RRT

Although RRT remains a useful method for detecting severe pediatric ocular diseases, it has to be noticed that it also has some drawbacks. It has been emphasized that abnormalities of RRT have to be referred to ophthalmologists. However, a normal RRT does not exclude ocular pathology. First of all, the RRT enables the evaluation of only a small area and not the whole retina[11]. Thus, abnormal lesions, including retinoblastoma, could be missed when they are of small size or situated peripherally[12,26,28]. It is worth mentioning, that the detection of such cases was significantly improved while using additionally oblique viewing of the fundus and even more with pupil dilation[11]. Nevertheless, some tumors could still be missed. So, patients at high risk of retinoblastoma or other eye disorders leading to leukocoria (*e.g.*, family history of retinoblastoma, infantile or juvenile cataracts, retinal dysplasia, glaucoma) should be evaluated by specialists, regardless of the RRT result[4,11]. Moreover, RRT cannot diagnose some disorders of the retina or the optic nerve, which cause visual impairment, such as retinal dystrophy or optic atrophy. As a result, cases with impaired vision demand ophthalmologist's investigation, despite a normal RRT[12]. In another survey[28], RRT was unable to detect some fundus abnormalities, such as pigment, vascular, hemorrhage and subretinal exudative changes. Furthermore, the researchers proved that the accuracy of RRT in diagnosing disorders of the posterior pole of the eye was significantly lower than those of the anterior pole[12,28]. Literature data also recorded a limitation of the RRT in the neonatal period, enforcing the recommendations for repetition of the test routinely. Sun *et al*[28] reported some newborns with normal RRT in the first days of life who were diagnosed with familial exudative vitreoretinopathy after abnormal RRT or presence of nystagmus at the age of 1-6 mo. Performing RRT on a newborn or an infant's eyes could be a difficult challenge for inexperienced pediatricians. An infant's pupils are small and difficult to assess, so that physicians' complaints usually include "infant is uncooperative", "eyelids are closed tightly" or "unable to evaluate red reflex"[1]. However, these

statements are unacceptable and dangerous and underline the need for education to provide the RRT procedure effectively in order to avoid undesirable consequences. Ulanovsky *et al*[41] performed a retrospective observational study including 18872 neonates born from 2008 to 2011. During the years that RRT was performed, the researchers found a significantly higher incidence of clinical conjunctivitis with positive bacterial culture. This result was correlated with direct contact of the examiner's hands with newborns' eyes, hence the avoidance of direct contact with neonates during the RRT should be the general rule.

Despite the reported high levels of sensitivity for certain ocular diseases, false positive results (reduced red reflexes on eyes without abnormalities) were not unusual in the literature. Cagini *et al*[6] noticed high rates of false positives (only 3 of 461 patients with a positive or equivocal test were diagnosed with a congenital disease). On the contrary, another study recorded a much lower percentage of false positives (1 of 1643 tests was false positive)[6]. Inexperienced examiners, inappropriate equipment, small pupils, strongly pigmented fundus and conditions during the diagnostic procedure could explain the high rates of false positives[12,32]. However, the researchers considered the RRT essential part of the neonatal eye screening, as it provided early detections with high sensitivity rates[6].

Compliance with RRT guidelines

Although the necessity and effectiveness of RRT have been supported by international guidelines, data from the literature revealed insufficient implementation of the examination. Raoof *et al*[42] assessed the performance of RRT from health care professionals (general practitioners, midwives, pediatricians) *via* questionnaire. They found that 10% of responders admitted implementation of the test only when they had the time or when the parents were worried. Despite the fact that New Zealand's guidelines clearly define the appropriate time of red reflex examination, the majority of professionals (50.1%) seemed to perform the RRT at 6 wk age. Only 17.3% of the responders had received formal training for RRT, while 16.6% declared to feel underconfident during the examination[42]. Moreover, 46.1% of the neonatal units in Israel performed RRT during the years 2007-2008, while in Sao Paulo Brazil, the relative percentage was 81% during 2004[3]. Another study measured the lowest number of RRT evaluations at the high-risk neonatal units, and this finding was attributed to the unstable health status of the neonates[43]. Wall and colleagues[44] investigated the compliance of the pediatricians with vision screening guidelines. They found that a significant part of the pediatricians did not examine red reflexes beyond 6 and 24 mo of age (23% and 44%, respectively). The reasons for non-compliance seemed to be multifactorial and included the lack of time, patience, education, skills and also some worries about adequate reimbursement for vision screening[44]. Additionally, the limited staff and the perception of the pediatricians that the RRT was not their responsibility were also among the reported causes[3]. Moreover, several environmental factors could affect physician and patient compliance on RRT implementation. Nowadays, cataract remains one of the most usual reasons of preventable blindness in middle-low income countries, with very poor post-surgery visual outcomes. In these countries, late presentation of patients was very common[38]. A survey in East Turkey mentioned that 19% of pediatricians did not have an ophthalmoscope or did not know how to use it[40].

RRT and other techniques

Nowadays, RRT is a part of visual screening, supported by official guidelines for the pediatric community. However, the last recommendations published by the World Health Organization (WHO) did not include RRT but the pencil light examination. Literature provided controversial data regarding this method. Concerning the torchlight, Mndeme *et al*[38] strongly recommended that WHO guidelines should replace torchlight examination with RRT using direct ophthalmoscope due to the very low sensitivity rates (7.5%) of torchlight. Moreover, a Turkish study[17] emphasized that pencil light illumination should not be used by pediatricians and general practitioners, as this method missed most of the cases with congenital cataract. On the other hand, the researchers made it clear that RRT should become an essential part of the national pediatric eye screening protocol. Haargaard *et al*[34] studied the different protocols for congenital cataract screening that were used in Sweden and Denmark during 2008-2012. In 2011, Denmark introduced the pencil light as the screening tool for detection of congenital cataract at 5 wk of life, without any change in the age of diagnosis to be noted, in comparison with the absence of screening previously. On the other hand, the disease was detected significantly earlier in Sweden, where the RRT was a part of routine newborn examination.

Mndeme *et al*[38] dealt with three alternatives to the standard direct ophthalmoscopy for the evaluation of red reflex and also compared the standard RRT with the torchlight examination. The first tool was ArchLight, an easy and cheap device that uses a light emitting diode (LED). The second device was Peek Retina, an adaptor for smartphones, with prisms and LED that allows examination of the retina snaps and differentiation of normal and abnormal red reflexes *via* the coaxial light source. Thirdly, CatCam device, a prototype comprising a modified smartphone with a coaxial infra-red LED and infrared sensitive camera, provides evaluation of fundus reflex without causing miosis. All three methods had very high sensitivity (over 90%) and specificity (86.7%-100%), with the CatCam performing the best, followed by the Archlight and then the Peek Retina. The CatCam could improve the accuracy of pediatric cataract diagnosis; however, it still remains expensive and commercially unavailable. On the other hand, the Archlight is easily performed on infants, with much lower cost. These new technologies could strengthen the accuracy of RRT and make it easier and more feasible to the medical community.

Digital images analysis could provide an opportunity for telemedicine as well. However, it was underlined that more investigation of their efficacy on detecting pediatric ocular disorders is required. Another study[25] included advanced digital fundus imaging using RetCam during the eye screening of newborns. The digital examination revealed a significant number of well-being neonates with abnormal ocular findings, which RRT was unable to detect. The most common among them was retinal hemorrhages, a usually benign and transient condition after birth trauma. However, more studies need to be performed to investigate any correlation between early fundus findings and final visual impairment. Smartphone photography for the detection of amblyogenic conditions in children 5-8 years of age, through snaps of pupillary red reflexes, was the objective of Gupta *et al*[45] trial. All high refractive errors were detected with success, however, moderate errors revealed false negative results. The sensitivity of the photographs for all other ocular diseases was 100%. The usage of smartphones should be further investigated in all ages and for more eye disorders in order to provide a low-cost, effective screening tool for developing countries. It has been reported[23]for more than 90% of leukocoria cases that the family firstly noticed the presence of white pupils in photographs. Munson *et al*[46] dealt with a tool based on red reflex of the pupils that could provide to parents the detection of leukocoria earlier. They presented a novel application for smartphones, the CRADLE, which can be downloaded for free and allows early detection of leukocoria. More specifically, the application creates a private storage of photographs and after digital analysis, it can detect cases of leukocoria and automatically alert the parents for further investigation. In this study, the CRADLE detected leukocoria in the snaps captured before diagnosis by 1.3 years, for the majority of cases with ocular diseases (80%). The estimated sensitivity of the application was increased and the specificity was decreased with age. The CRADLE could provide to parents a useful tool for early detection of leukocoria. However, it was not able to differentiate the pathological for the physiological leukocoria due to specific conditions during photo shooting, and it definitely could not replace clinical evaluation with RRT and further examination. The researchers underlined that although pathological white pupils are usually signs of refractive errors or amblyopia and physiological leukocoria is a typical artifact of off-axis photography; recurrent white pupils in many photos are red flags. However, they made it clear that all cases of leukocoria in photographs should be investigated. More studies on these new technologies could empower the contribution of the RRT in diagnosing certain ocular pathologies of the pediatric population in the future.

DISCUSSION

RRT is a non-invasive, low-cost, essential diagnostic method, highly effective for the detection of several sight and life threatening ocular diseases. Its importance has been highlighted in 2016 by the AAP, American Association for Pediatric Ophthalmology and Strabismus, American Academy of Ophthalmology and American Association of Certified Orthoptists who published the revised policy statement on "Visual System Assessment in Infants, Children, and Young Adults by Pediatricians"[37]. The policy recommended that RRT should be performed at every pediatrician visit for newborns and infants 0-6 mo and again at 6 mo, 12 mo, 1-3 years, 4-5 years and 6 years. Furthermore, it has been emphasized that cases with abnormal findings or with history of prematurity or family history of congenital cataracts, retinoblastoma,

metabolic disease or systemic diseases with suspicion of severe ocular disorders must be referred to the ophthalmologist immediately[37].

Despite its clinical importance, literature research revealed that there is heterogeneity worldwide, regarding current recommendations for RRT implementation. Indicatively, some of the different strategies of European countries are hereby presented. In the United Kingdom, healthy infants should be examined for red reflexes during the first 3 d of life and again between 6-8 wk[47]. In Germany, the guidelines require repeated RRT at the ages of 4, 6, 12 and 24 mo. In Sweden, RRT is performed at birth and again at 4-6 wk[48]. In Greece, RRT is recommended at birth, at the age of 1-2 wk, 2 mo, 3-36 mo, and 36 mo-7 years[49]. Denmark and the recommendations by WHO do not include RRT in the pediatric vision screening but rather pencil light examination[38,50]. Remarkably, data from literature supported that RRT could never be replaced by pencil light examination, as the second tool seemed to be able to detect only advanced cases of cataract and retinoblastoma[17,38]. In Australia, each state and territory health department provides separate guidelines for pediatric vision screening. Most of the better quality guidelines recommended red reflex examination during newborn to 6 mo of age and later in the following years (from 18 mo to 3.5 years of age)[51]. The literature provides a little information about RRT recommendations in Asia and Africa. However, many countries, such as China and middle-low income countries, do not include RRT in the vision screening programs. The main reason may be the increased cost of eye examination from infancy[52].

Despite its clinical value, the RRT is not implemented by the entire pediatric community appropriately, as it is recommended. In order to improve the pediatricians' awareness and compliance with the current guidelines, prompt emphasis should be placed on the educational procedure of the physicians starting from university programs and during the years of pediatrics training. In this context, some researchers even suggested education videos on the internet[23,39]. The fact that many relevant articles are published only in ophthalmologic and not pediatric journals could be an obstacle for the pediatricians' awareness on RRT. Indicatively, 20 of the eligible articles for this review were available in pediatric journals, while the rest of them were published in non-pediatric journals.

The majority of the eligible articles of this review highlighted the importance of the RRT technique in the detection of certain pathologies (Table 3). On the other hand, Munson *et al*[46] considered the RRT as "dying art", since they focused on the limitations of the technique that have been described previously. However, the contribution of RRT in pediatric visual screening could be more promising in the future. New technologies based on red reflex may improve RRT's weak points[38,45,53]. Further investigation of these new devices and development of appropriate software is required in order to improve RRT accuracy on pediatric ocular diagnosis.

CONCLUSION

The present paper attempts to provide an updated review of the red reflex examination for the pediatrician. Published research indicates that the RRT should begin at birth and afterwards it should be an essential part of the routine clinical examination by pediatricians. Its simplicity and high sensitivity and specificity suggest that RRT should be part of the educational curriculum for every new pediatrician, since it will contribute to prompt diagnosis of many sight and life threatening conditions.

ARTICLE HIGHLIGHTS

Research background

Red eye reflex test (RRT) is a widely known examination that has been used by clinicians for the diagnosis of several ocular disorders. However, its implementation by pediatricians in the clinical practice still remains controversial. This study aims to highlight the importance of RRT and to provide a practical guide for its usage for pediatricians.

Research motivation

The literature data show insufficient implementation of the RRT. This result is in accordance with clinical observation in our country. Therefore, the present study could

contribute to raise pediatricians' awareness on this diagnostic tool.

Research objectives

The main objectives of the present study were the assessment of RRT value in specific disorders (such as retinoblastoma and congenital cataract) and the compliance of the clinicians with current guidelines. Moreover, this article investigated reported limitations of this diagnostic technique and motivated future research for the improvement of this method through new technological achievements.

Research methods

A thorough search on PubMed databases took place by two independent reviewers.

Research results

Eligible articles highlighted the significance of the RRT in the diagnosis of sight threatening or even life threatening eye pathologies. The implementation rates seemed to present a wide range among the countries. This fact, underlines the need for appropriate education and official guidelines from health systems.

Research conclusions

This study demonstrates why pediatricians should include the RRT in their clinical practice and provides a practical guide for the prompt implementation of this diagnostic examination. Further investigations are in progress in order to overcome the main limitations of the traditional red reflex examination.

Research perspectives

The rapid progress of technology achievements should improve the usage of the traditional red reflex method, making it easier and more efficient.

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Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
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Help Desk: https://www.f6publishing.com/helpdesk
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ISSN 2222-0682 (online)

World Journal of *Methodology*

World J Methodol 2021 November 20; 11(6): 278-301

Published by Baishideng Publishing Group Inc

World Journal of Methodology

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INDEXING/ABSTRACTING

The *WJM* is now abstracted and indexed in PubMed, PubMed Central, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Methodology

ISSN
ISSN 2222-0682 (online)

LAUNCH DATE
September 26, 2011

FREQUENCY
Bimonthly

EDITORS-IN-CHIEF
Gerhard Litscher

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/2222-0682/editorialboard.htm

PUBLICATION DATE
November 20, 2021

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World J Methodol 2021 November 20; 11(6): 278-293

DOI: 10.5662/wjm.v11.i6.278

ISSN 2222-0682 (online)

MINIREVIEWS

Preterm nutrition and neurodevelopmental outcomes

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Author contributions: Both authors contributed to the writing of the manuscript; Narchi H designed the figure.

Conflict-of-interest statement: No conflict of interest.

Provenance and peer review: Invited article; Externally peer reviewed

Specialty type: Medical laboratory technology

Country/Territory of origin: United Kingdom

Peer-review report's scientific quality classification

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Abstract

Survival of preterm infants has been steadily improving in recent years because of many recent advances in perinatal and neonatal medicine. Despite these advances, the growth of survivors does not reach the ideal target level of the normal fetus of the same gestational age. Postnatal weight gain is often not achieved because extrauterine growth has higher energy requirements than intrauterine growth, due to the intensive care environment, illness and inadequate nutrition. Although many other factors influence infant brain development, including family socioeconomic and educational background, the role of nutrition is considerable and fortunately, amenable to intervention. In the preterm neonate, the brain is the most metabolically demanding organ, consuming the largest proportions of energy and nutrient intake for its function and programmed growth and maturation. Weight gain, linear and head circumference growth are all markers of nutritional status and are independently associated with long-term neurodevelopment. Brain development is not only the result of nutrients intake, but in addition, of the interaction with growth factors which depend on adequate nutrient supply and overall health status. This explains why conditions such as sepsis, necrotizing enterocolitis and chronic lung disease alter the distribution and accretion of nutrients thereby suppressing growth factor synthesis. In this review, we will focus on the direct role of nutrition on neurodevelopment, emphasizing why it should be started without delay. The nutritional requirements of the preterm infant will be discussed, followed by the effects of general nutritional interventions and specific nutrients, as well as the role of nutritional supplements on neurodevelopment. The primordial role of human breast milk, breast milk fortifiers and human milk oligosaccharides will be discussed in detail. We will also examine the role of nutrition in preventing neonatal complications which can affect neurodevelopment in their own right.

Key Words: Brain; Nutrition; Preterm infants; Neurodevelopmental outcomes; Newborn;

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 28, 2021
Peer-review started: March 28, 2021
First decision: June 17, 2021
Revised: July 13, 2021
Accepted: September 27, 2021
Article in press: September 27, 2021
Published online: November 20, 2021

P-Reviewer: Zeng H
S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

Breast milk

Core Tip: Survival of preterm infants has been steadily improving because of the many recent advances in perinatal and neonatal medicine. However, neither the growth of survivors reaches the ideal target level of the normal foetus of the same gestational age, nor is the optimum postnatal weight gain often achieved. In the preterm neonate, the brain is the most metabolically demanding organ. Growth is a marker of nutritional status and is also independently associated with long-term neurodevelopment. In this review, we will discuss the direct role of nutrition and the effect of general and specific nutritional interventions on neurodevelopment.

Citation: Skinner AM, Narchi H. Preterm nutrition and neurodevelopmental outcomes. *World J Methodol* 2021; 11(6): 278-293
URL: https://www.wjgnet.com/2222-0682/full/v11/i6/278.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i6.278

INTRODUCTION

Survival of preterm infants has been steadily improving in recent years because of the many advances in perinatal and neonatal medicine, such as antenatal corticosteroids administration, surfactant therapy and novel mechanical ventilation modalities. Despite these advances, the growth of preterm infants, as indicated by anthropometric indices and body composition, does not reach the target level of the normal foetus of the same gestational age. Often optimal postnatal weight gain is not achieved because extrauterine growth has higher energy requirements than intrauterine growth, due to the intensive care environment, illnesses and inadequate provision of nutrition. The persisting suboptimal nutrition in survivors remains an issue and is associated with adverse outcomes, such as chronic lung disease (CLD), necrotising enterocolitis (NEC), sepsis and, more worrying, neurodevelopmental impairment, especially in the cognitive domains, which is increasing in prevalence[1]. These adverse developmental outcomes can be secondary to abnormal brain development, postnatal brain injury and suboptimal nutrition occurring during hospital admission. Therefore, preterm birth can be considered a nutritional emergency.

Improved neurodevelopmental outcomes in extremely low birth weight (ELBW) infants are associated with a reduction in the incidence of CLD, apnea, intraventricular hemorrhage (IVH) and any associated nutritional deficiency[2]. Although many other factors influence infant brain development, not least family socioeconomic and educational background, the role of nutrition is considerable and fortunately, amenable to intervention[3].

In the preterm neonate, the brain is the most metabolically demanding organ and consumes the largest amount of energy and nutrients for its function and programmed growth and maturation[4]. Starting in the prenatal period and continuing until the second year of life, the development of the cerebral white and gray matter structures of the central nervous system (CNS) involves many processes, especially between 22 and 42 wk post-conception. Cell replication, neurogenesis, neuronal differentiation, cell migration, myelination, synaptogenesis, progression and regression of specific structures in different periods of development and neuroplasticity, are some of these processes[4]. The number of cell replication cycles in the CNS is decreased in malnutrition, thereby reducing total brain DNA and leading to a restriction in dendritic arborization and a reduction in the connections between neurons[5]. These cellular mechanisms affect the pre-oligodendrocyte, the widely disseminated activated microglia and the reactive astrocytes. As they occur over a relatively long time period, neurorestorative interventions, such as better nutrition can improve brain development[6]. Also, alterations in dietary precursors may affect neurotransmitter levels, for example, serotonin, norepinephrine, dopamine and acetylcholine in specific brain regions. In addition, essential and nonessential lipid components play an important role in the structural composition of the brain and of myelin sheaths. The

resulting CNS impairment, caused by these nutrient deficiencies, involves motor and cognitive development and social abilities. They are also associated with alterations in the sleep-wake cycle organization, neurovegetative activities during sleep, waking electroencephalographic (EEG) activity and visual and auditory evoked responses.

The neurodevelopment of premature babies is very sensitive to nutrition in the first few weeks of life, and these effects may be long lasting until the age of eight years[7]. Cerebellar development is most affected by nutritional deprivation around the time of birth, and also, synaptic connectivity occurring before the third year of life. Nutrition is positively associated with weight gain, increased brain volumes and white matter maturation on magnetic resonance imaging at term equivalent age and neurodevelopment in infancy[7-9].

Adequate early nutrition may also attenuate the adverse effects of neonatal illness. Studies of EEG maturational age (EMA) during periods of quiet sleep which analysed spectral power, continuity and interhemispheric synchrony, have shown that a lower intake of calories and carbohydrate is associated with a greater reduction of spectral amplitude in the delta band. A lower protein intake was associated with higher discontinuity. In contrast, both higher proteins and lipid intake were associated with better developmental increase in interhemispheric synchrony and with brain activity maturation [10]. Increased caloric and protein intakes in preterm infants are associated with a higher fat free mass (a key marker for organ growth) and better neurodevelopment outcomes[11]. Protein and energy deficits in preterm nutrition are commonly associated with other nutritional deficiencies and also with psychosocial deprivation. Such interactions impact on the brain's structural development, disrupting neurodevelopment and adversely affecting cognitive performance[12].

Weight gain, linear and head circumference growth are all markers of nutritional status and are independently associated with long-term neurodevelopment. Brain development is not only a result of nutrients intake, but also of the interaction with growth factors, which depend on adequate nutrient status and overall health status. Sepsis, inflammation (occurring in NEC, CLD) and corticosteroids administration, alter the distribution and accretion of nutrients and suppress growth factor synthesis. Therefore, in addition to provision of adequate nutrients, strategies to reduce the incidence of these conditions are also required to optimise brain growth and development[4].

In this review, we will focus on the direct role of nutrition on neurodevelopment. First, we will discuss the nutritional requirements of the preterm infant, followed by the effects of general nutritional interventions, specific nutrients and nutritional supplements on neurodevelopment[9]. We will then examine the role of nutrition in preventing neonatal complications, such as sepsis, NEC and CLD, which can also affect neurodevelopment in their own right (Figure 1)[4,13].

NUTRITIONAL REQUIREMENTS

Macronutrients refer to carbohydrates, fats and proteins while micronutrients include trace elements, electrolytes, and vitamins. Nutritional requirements, based on the estimated average requirement of a specific population group, are defined as the type and amount of nutrients needed to support normal health, growth and development. However, as preterm infants do not constitute a homogeneous population, their requirements must be individualised based on their clinical condition and developmental stage[14].

The high growth rate of premature infants requires a proportionally very high intake of all nutrients. With the general assumption that adequate postnatal growth should approximate the in-utero growth of a normal foetus, nutrient requirements were initially derived from postmortem measurement of the size and body composition of fetuses at varying gestational ages. The resulting nutritional intakes were estimated as the amounts required to replicate intrauterine rates of growth and nutrient accretion. The traditional method for estimating protein requirement is the factorial method, based on the body composition of the foetus with an estimate of the inevitable urinary nitrogen losses (occurring even in the absence of nitrogen intake) and of the amount deposited in-utero for accretion of tissue, corrected for efficiency of absorption and deposition[15]. Improvements to this model now take into account, not only the estimation of intrauterine accretion rate, organ development and factorial estimates of requirements, but also nutrient interactions, supplemental feeding and long-term developmental outcomes[16].

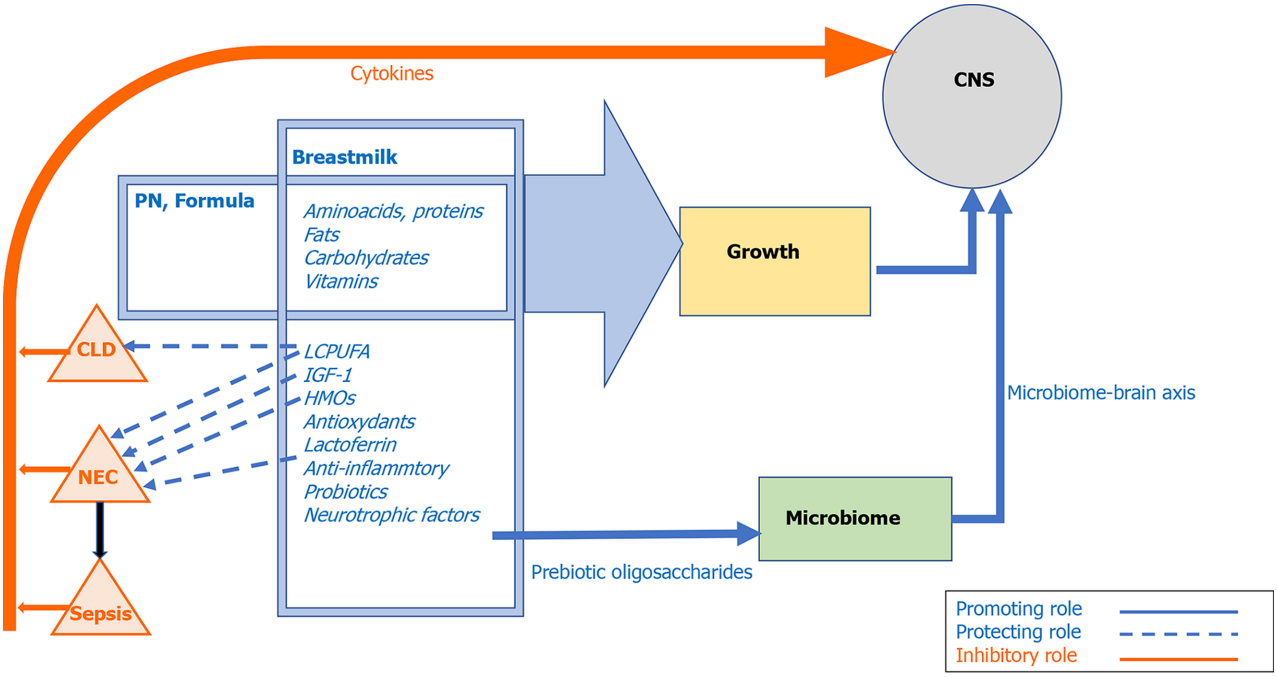

Figure 1 Direct effect of nutrients on brain development of the preterm infant and indirect impacts in preventing some neonatal complications, which can affect neurodevelopment in their own right. PN: Parenteral nutrition; LCPUFA: Long chain polyunsaturated fatty acids; IGF-1: Insulin-like growth factor 1; HMOs: Human milk oligosaccharides; CLD: Chronic lung disease; NEC: Necrotising enterocolitis.

Energy, stored in the body in the form of adenosine triphosphate (ATP), is required for several cellular and organ functions. Preterm infants have low energy reserves because they are born before adequate accumulation of fat and glycogen has occurred. In healthy premature infants, a recommended daily energy intake of 110-130 kcal/kg/day allows a growth rate similar to that of the intrauterine growth rate. For the neonate, carbohydrates are the main energy source (4 kcal/gram), in the form of glucose. Glucose synthetic rates in preterm infants are much higher at 6-8 mg/kg/min compared to 3-5 mg/kg/min in term infants. Because of their high energy-density (9 kcal/gram), fats or lipids are a very good source of energy. In addition, they also provide essential fatty acids indispensable for brain and visual development. Proteins are essential for normal growth and development (see below). After the initial free water diuresis, 2-3 mmol/kg/day of sodium and potassium (as a minimum) will be required to maintain serum levels in the normal range. Preterm infants often develop low sodium levels in the second week of life due to renal immaturity resulting in sodium loss. A normal serum sodium level is a requirement for adequate growth. Calcium, magnesium and phosphorus are essential for tissue structure and function. Vitamins are important for growth and development facilitating many reactions in intermediary metabolism. Preterm infants have low body stores of fat-soluble vitamins at birth due to limited transfer of lipid-soluble substrate across the placenta and, therefore, provision of the latter should be started as soon as lipid containing parenteral nutrition is initiated. A summary of the high nutrient requirements of the preterm infant is shown in Table 1. Human milk alone cannot provide the nutritional needs (especially protein) of preterm infants and requires fortification.

Providing preterm infants with adequate nutritional support presents unique challenges. Failure to provide adequate nutrient intakes at all stages of development places them at risk of impaired neurodevelopment. Therefore, every effort must thus be made to meet their complete nutritional needs from birth[15]. The main and most important reasons for growth failure in premature infants are the physiological limitations preventing the provision of nutrients by enteral feeding. This, in turn is compounded by the very high nutritional needs. Provision of nutrients has to take into account the immaturity of the intestinal tract, a major risk factor for the development of NEC. To overcome this, parenteral nutrition is used during the early days and often weeks of life. Although there are problems associated with parenteral nutrition, especially that of infection, failure to provide nutrients parenterally would place these infants at high risk of impaired neurodevelopment and host defenses. Providing early nutrient intakes by the parenteral route or with breast milk when possible, are therefore crucial to optimise long-term health outcomes[3].

Table 1 Nutritional requirements[1]

Body weight	500-1000 g	1000-1500 g	1500-2200 g	2200-3000g
Foetal weight gain (g/kg/d)	19.0	17.4	16.4	13.4
Protein(g/kg/d)	4.0	3.9	3.7	3.4
Energy (kcal/kg/d)	106	115	123	130
Protein/energy (g/100 kcal)	3.8	3.4	3.0	2.6
Glucose	6-8 mg/kg/min			
Lipids	2.5-3.5 g/kg/d			
Calcium	60-90 mg/kg/d			
Phosphorus	40-70 mg/kg/d			

[1]Estimated by factorial and empirical methods, derived from several references in this article[14-16].

INDIVIDUAL NUTRIENTS DIRECTLY AFFECTING NEURODEVELOPMENT IN PRETERM INFANTS

Caloric intake

Caloric intake in a large population of extremely premature infants (born < 28 wk' gestation) was associated with the development of retinopathy of prematurity (ROP) [17]. In preterm infants, caloric deprivation is associated with a significantly low composite score on Bayley-III scales performance at 18-24 mo corrected age[13]. In the same study, late onset sepsis, NEC, and CLD were also found to be risk factors for impaired neurodevelopment. Improving nutritional support, specifically total caloric intake is therefore necessary to prevent neurodevelopment impairment.

Protein

Adequate protein intake is crucial for satisfactory growth and neurodevelopment in preterm infants. It has been recommended that a high protein intake starts on day 1 of life to limit extrauterine growth restriction, which in itself negatively affects neurodevelopment. Brain magnetic resonance imaging (MRI) studies in the first month of life in extremely preterm infants, who had received early nutrition, demonstrated larger brain volumes in those who had received a higher protein and caloric intake than controls[18]. In very low birth weight (VLBW) newborns, serial cranial ultrasound measurements of the size of several brain structures in the first month of life showed a positive correlation between enteral protein intake and brain volume[19]. Supplemental enteral proteins were also shown to reduce postnatal growth restriction and improve neurological outcomes in preterm infants < 1000 g and in small for gestational age (SGA) infants (birth weight < 10th percentile)[20]. Similarly, parenteral amino acid intake within the first two weeks of life was correlated with neurodevelopmental outcomes at 2 years, with higher language and motor scores on the 2-year Bayley Scales of Infant and Toddler Development (Bayley III)[21].

However, not all studies have reported a consistently positive relationship between early protein intake and neurocognitive outcomes. Protein supplementation in very low birth weight infants (VLBW) (birth weight < 1500 g) showed a significant positive correlation with neurological development in communication, auditory, verbal language, cognitive function, social connection and gross motor development, but not with fine motor, problem solving or personal-social relationships in the third year of life[22]. However, this study involved small population numbers and protein supplementation was stopped when the infants weighed 1500 g. In preterm infants born before 32 wk' gestation or with birth weight < 1500 g, addition of fortifier to expressed human milk to increase protein intake, was associated with better head growth and weight gain at 40 wk' postmenstrual age. However, this intervention had no benefits on long-term growth and neurodevelopment at 12 to 18 mo corrected age[23]. Likewise, early aggressive parenteral nutrition with high-dose amino acids and mixed fat emulsions did not improve neurodevelopmental scores of VLBW infants, nor decrease the incidence of major disabilities[24]. Again this study included a small study population divided into 5 groups with different parenteral feeding regimes.

Fats and long-chain polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids (LCPUFA), including docosahexaenoic acid (DHA) and arachidonic acid (ARA), are essential for neurodevelopment and normal vision. In-utero DHA accretion occurs primarily in the last trimester of pregnancy supporting rapid growth and brain development. Premature infants, born before the completion of this process are therefore relatively deficient in these essential fatty acids. This deficiency persists for a long period after birth because of ineffective conversion from precursor fatty acids, lower fat stores, and limited nutritional provision of DHA and ARA. Besides long-term visual and neurodevelopmental risks, very preterm infants have significant morbidity and mortality from diseases specific to premature birth, including CLD, NEC and ROP. There is increasing evidence that DHA has protective benefits against these complications[25]. The ratio of n-3 to n-6 LCPUFAs, which generates eicosanoids, is important in moderating the effects of hypoxia, inflammation, infection, thrombosis and oxidative damage of key organs such as the lungs, brain and retina[26]. These actions improve the prognosis for ELBW and VLBW infants. By providing LCPUFAs, including DHA, breastfeeding improves cognitive development, especially in infants with a proven lower activity of DHA synthesis[27].

The outcomes of supplementation with PUFAs are still contested. Although early PUFAs supplementation in preterm infants had a positive impact on early childhood psychomotor development and visual acuity, they had no significant effects on global intelligence quotient (IQ) in childhood[28]. Similarly, a metanalysis of LCPUFAs supplementation in preterm infants showed a significant improvement in their neurodevelopment (assessed by the Mental Development Index of the Bayley Scales at one to three years of age). Supplementation during lactation accelerated neurodevelopment but their final developmental outcome remained unchanged[29]. Other studies have shown no beneficial effect of early high-dose mixed fat emulsions on neurodevelopment of VLBW infants[24,30]. Studies of formula milk supplemented with LCPUFAs showed mixed results, some reporting better psychomotor outcomes [31] while others showed no long-term benefits on visual or psychomotor development[32]. Infants enrolled in the supplemented formula study were mature in general and the quality of evidence was low for both visual and neurodevelopmental outcomes. Current recommendations for follow-on formula suggest that they should contain omega-3 DHA equal to the mean content of human milk, and also AHA[33].

Iodine supplementation

Iodide supplementation in babies born before 31 wk' gestational age provided no benefit to neurodevelopment measured at 2 years of age[34]. Similarly, a large high-quality study showed no beneficial effects of iodine supplementation for preterm infants[35].

Iron supplementation

In infants born ≥ 34 wk gestational age, latent in-utero iron deficiency (serum ferritin < 75 ng/mL at birth) was associated with abnormal auditory neural maturation[36]. The age at which to begin iron supplementation remains controversial. It is often given from day 28 of life onwards, assuming until then, the infant has sufficient iron stores from the breakdown of fetal hemoglobin. As breast milk does not provide sufficient iron to sustain a premature infant's growth and normal haemopoietic function, breast-fed infants require iron supplementation. Formula-fed babies do not need iron supplements as milk formulas are iron fortified.

BREAST MILK

Fresh maternal milk contains factors involved in antioxidant and anti-inflammatory defense[35], gut microbiome establishment and the maturation of immune defenses [37,38], feed tolerability and metabolism[39] and also protects infants against NEC [16]. For these and other reasons, human milk is the preferred choice for feeding premature infants. It reduces the risk of morbidities and promotes growth through elevated serum levels of insulin-like growth factor 1 (IGF-1), an essential intrauterine hormonal mediator of growth[40].

Human milk contains critical nutrients and other neurotrophic factors that benefit the brain of preterm infants[41]. A systematic review of neurodevelopment outcomes of ex-preterm children found better test scores in those who had been fed breast milk compared with formula feeds, when evaluated in the first three years of life and also,

between the age of five to eleven years[42]. In a prospective study of 316 VLBW infants, correcting for gestational age (GA), being born SGA, and for complications at birth and during hospital admission, better neurodevelopment at 24 mo corrected age was associated with breast milk feeds[43]. VLBW infants fed fortified human milk had increased head circumference velocity and decreased regional white matter diffusivity when assessed by magnetic resonance diffusion tensor imaging suggesting improved maturation of the cerebral connective tracts[44]. They also had improved visual perception as judged by their response to global motion[45]. Brain MRI studies at term equivalent age, using network-based statistics, tract-based spatial statistics and volumetric analysis of white matter water diffusion parameters, reported that feeding with breast milk in the weeks after preterm birth was associated with improved structural connectivity of developing networks and greater fractional anisotropy (FA)-weighted connectome in major white matter fasciculi[46]. There were also better outcomes observed in infants fed breast milk for more than 90% of their hospital admission duration compared with those who received breast milk for 75% of the same period.

When a mother expressed breast milk is unavailable, donor breast milk is used widely to feed premature babies. The methods used to process donor milk reduce the levels of protective and growth promoting factors found in fresh breast milk. However, donor milk still offers protection against NEC but remains inferior to fresh human milk in promoting the growth and development of very preterm infants[47].

Breast milk fortification

Human milk, although the preferred feeding for premature infants because of its protective effects, does not provide adequate amounts of nutrients to support the rapid growth and development of the premature baby and therefore, must be fortified. Failure to fortify mother's milk places the baby at risk of neurodevelopmental impairment. As mentioned earlier, an enhanced nutrient supply with breast milk fortification to VLBW infants was associated with increased head circumference growth and decreased regional white matter mean diffusivity seen on magnetic resonance diffusion tensor imaging, suggesting improved maturation of cerebral connective tracts[44]. A recent study of long-term outcomes of preterm infants fed an exclusive human milk-based diet using a donor human milk-based fortifier reported an absence of severe cognitive developmental delay using a cutoff score of 70 (Bayley Scales of Infant and Toddler Development III) measured at 18-22 mo corrected gestational age (GA)[48].

Some controversies still exist. An earlier study showed no significant differences at 18 mo corrected age in mean Bayley-III cognitive composite score, language composite score, or motor composite score in VLBW infants fed fortified donor milk when compared with formula fed babies[49]. Similarly, a study of the balance of risks and benefits of feeding formula *vs* donor breast milk for preterm or LBW infants found no evidence of an effect on long-term neurodevelopment, although the level of evidence was moderate[50]. This may be explained by the effects of the pasteurisation process on donor milk causing reduced levels of the non-nutritive content. Even fewer data are available for growth and developmental outcomes assessed beyond infancy and many do not show consistent effects. Feeding with a nutrient enriched formula had no effect on Bayley Mental Development Index scores at 18 mo post term or on cerebral palsy, and there were no effects on long-term development[51].

Human milk oligosaccharides

Human breast milk has two types of carbohydrates, lactose and oligosaccharides. HMOs are indigestible unconjugated complex carbohydrates forming the third most abundant component of breast milk, after lactose and lipids. They are a complex mixture of over 200 non-digestible and non-nutritional carbohydrates. There are three main HMO categories based on their building blocks. The first category comprises non-fucosylated neutral (core) HMOs, which are the foundations upon which other HMOs are built, lacto-N-tetraose isomer (LNT) being the most abundant representative in this category. The fucosylated neutral HMOs form the second category, with 2'FL the most abundant fucosylated HMO and DFL (LDFT) among the 10 most abundant representatives in this group. The third category is composed of the sialyated acidic HMOs, 6'SL is the leading representative of this group followed by 3'SL.

In animal studies, using a genome-wide profiling of the intestinal epithelial transcriptome in response to HMOs, the latter were found to protect against NEC in part by altering the differentiation of the crypt-villus axis host transcriptome in 225 target genes involved in cell proliferation and differentiation, including upregulation

of the stem cell differentiation marker HMGCS2. HMOs also directly induced a series of biological processes to protect the intestine[52]. In another experiment, milk supplementation with 2'-FL and 6'-SL, but not lactose, prevented NEC in mice and piglet models and attenuated NEC inflammation in the ileum, in part through TLR4 inhibition with reduced apoptosis and inflammation[53].

In neonates, HMOs have been shown to decrease the incidence of late-onset sepsis and NEC, both conditions being associated with neurodevelopment impairment. Reducing the risk of these complications leads to less disruption of feeds which is also beneficial for normal neurodevelopment. In the premature baby's gut, HMOs and the microbiome of breast milk act as immunomodulatory agents that provide intestinal homeostasis through regulation of the microbiome and protection of the intestinal barrier, thereby protecting the premature baby at risk of NEC[54]. The concentrations of different HMOs found in neonates vary according to the lactation period and maternal secretory phenotype status[55]. Fucosyl--N-hexose (FDSLNH) was associated with a reduced risk of late-onset sepsis in VLBW infants[55].

An animal study examined the effect of HMOs on cognition, novel object recognition task, brain development, and hippocampal gene expression using magnetic resonance imaging procedures to assess structural brain development and hippocampal tissue analysis for mRNA expression. The results showed improved recognition memory and better absolute and relative volumes of the cortical and subcortical brain regions. Hippocampal mRNA expression of GABRB2, SLC1A7, CHRM3, and GLRA4 were also affected by HMOs[56].

Relative abundances of several individual HMOs were associated with normal growth and neurodevelopment in infants, mainly motor development at 12 mo, including ability to stand or walk alone. They were also associated with language skills, socioemotional development, executive function and working memory at 18 mo, mostly among secretors[57].

Microbiota and probiotics

While the newborn term microbiome is normally dominated by Bifidobacterium species, the microbial composition and functions in preterm babies has a higher initial percentage of Lactobacillaceae[58]. It has been established that intestinal dysbiosis, (abnormal microbial colonisation), can occur as a result of exogenous factors such as mode of delivery, formula feeding, and exposure to antibiotics. These factors predispose preterm infants to sepsis and NEC in association with an already existing impaired intestinal barrier and immature immunity. The prolonged use of antibiotics and parenteral nutrition were reported to have significant adverse effects on the Lactobacillus and Bifidobacterium levels in the gut of preterm infants[59].

Inflammation and perinatal infection play a crucial role in the pathogenesis of white matter injury in preterm infants. Therefore, nutritional components with immunomodulatory and/or anti-inflammatory effects may serve as neuroprotective agents. There is growing evidence of the existence of a microbiome-gut-brain axis in which the microbiome interacts with the brain through immunological, endocrine, and neural pathways. Consequently, nutritional components that influence gut microbiota may also exert beneficial effects on the developing brain. Based on these properties, probiotics, prebiotic oligosaccharides, and certain amino acids may offer neuroprotection. Certain nutritional components may also have a neuroprotective role against white matter injury through modulation of inflammation and infection and may influence the microbiome-gut-brain axis[60].

As stated above, the normal term newborn microbiome is dominated by Bifidobacterium species but the establishment of abnormal microbial colonisation (intestinal dysbiosis) can be influenced by several factors, thereby increasing the risk of developing sepsis and NEC. Probiotics have been shown to reduce rates of necrotising enterocolitis (NEC), sepsis, and mortality[61]. However, research in this area has been complicated by studying different bacterial strains, administration of different doses of probiotics and for different time periods of administration. Provided all safety issues are met, it is currently recommended that either Lactobacillus rhamnosus GG ATCC53103 or the combination of Bifidobacterium infantis Bb-02, Bifidobacterium lactis Bb-12, and Streptococcus thermophilus TH-4 would help reduce the rates of NEC[62]. It is essential that probiotic strains should be devoid of transferable antibiotic resistance genes.

MEDIUM AND LONG-TERM EFFECTS OF CONDITIONS INFLUENCED BY NUTRITION WHICH HAVE AN IMPACT ON NEURODEVELOPMENT

Growth

The long-term effect of inadequate early postnatal growth (EPG) is a growing concern. Poor head growth during hospital admission and post discharge is associated with delayed motor and cognitive development between 16 and 36 mo of age[63]. A systematic review of the relationship between EPG before 3 years of age and neurodevelopmental outcome, showed that EPG was positively associated with good neurodevelopmental outcome in a non-linear association, with a plateau attained with higher weight gain, suggesting a possible ceiling effect[64]. Similarly, post-discharge failure to thrive was found to be significantly correlated with poor neurodevelopmental outcomes. There was an association between lower body weight (< 3rd percentile) at corrected ages of 6, 12, and 24 mo and poor neurodevelopmental outcomes among 30% of VLBW premature infants[65]. Extra uterine growth retardation (EUGR) of VLBW infants is significantly associated with low mental development index (MDI) at a corrected age of 24 mo[66].

Studies examining the relationship between body composition and neurodevelopment in VLBW infants reported higher growth, fat mass and fat-free mass were associated with larger cerebellar volumes at term[67]. Also, greater fat-free mass (measured by air displacement plethysmography) gained by VLBW premature infants as inpatients, was associated with improved cognitive and motor scores at 12 months corrected age (CA)[68]. Increased provision of protein and calories during the first week of life is positively associated with fat-free mass gains, but not fat mass gains. Frondas-Chauty *et al*[69] reported a deficit of fat-free mass in VLBW infants at discharge was associated with neurological impairment at 24 mo (corrected age), independent of sex, GA and BW.

Necrotising enterocolitis, intestinal failure, and sepsis

NEC often occurs in premature infants at a time of rapid brain growth and development and is associated with poor neurodevelopmental outcomes. The pathogenesis of neurodevelopmental impairment following NEC is likely multi-factorial, involving both nutritional and non-nutritional factors[70]. A variety of circulating cytokines are known to be associated with white matter injury. There is also increased risk of secondary blood stream infections and subsequent nutritional compromise[71]. Breast milk, probiotics and lactoferrin have all been shown to reduce the incidence of NEC and late-onset sepsis[72].

NEC is also a major cause of intestinal failure (IF), which in turn is associated with developmental delay in multiple domains. While gross motor skills appear to be most significantly affected, cognitive deficits become more apparent at 26 months[73]. In children with IF assessed before 15 mo corrected age, 88% had abnormal general movements and over 50% had suspect or abnormal gross motor scores. Factors significantly associated with poorer outcomes at 12-15 mo included prematurity, low birth weight, CNS co-morbidity, longer neonatal intensive care admission, NEC, number of operations and conjugated hyperbilirubinemia. Thus, multiple risk factors contribute to early developmental delay in children with IF and close developmental follow-up is essential in these patients[73].

GENERAL DIETARY INTERVENTION

Early feeding

As mentioned earlier, the preterm brain is very vulnerable to undernutrition in the first few weeks of life, even over a period as short as 4 wk, and these effects persist up to the age of 8 years[74]. Suboptimal nutrient intakes permanently affect later cognitive attainment and therefore, early nutrient intakes and breast milk provision are essential[3]. A positive association between several components of early nutrition, growth, brain volumes, white matter maturation, and neurodevelopment in infancy has been shown[7,75]. Immediate breast milk feeding results in elevated insulin-like growth factor 1 (IGF-1), an essential intrauterine hormonal mediator of growth, measured at term, in the serum of preterm infants[40]. Early administration of optimal postnatal parenteral and enteral nutrients helps to prevent neurodevelopmental impairment caused by extrauterine growth restriction, NEC, sepsis, CLD dysplasia, and ROP[72].

Early introduction of enteral feedings to stimulate or prime the gut even in the acutely ill preterm infant, is well tolerated[76,77]. Several metanalyses have shown the safety of early trophic feeding with breast milk within 48 h after birth and progressing the volume of enteral feeding before four days of life at a rate of 24 mL/kg/d in clinically stable very preterm and VLBW infants. These measures did not lead to higher mortality or an increased incidence of NEC[77,78].

However, despite the introduction of more aggressive early feeding guidelines and improved energy and nutrient intakes, many preterm infants do not achieve their recommended nutrition intake in the first week of life, especially those with morbidities[79].

FEEDING IN PRACTICE

During the immediate postnatal period, the aim of nutritional support is twofold: i) to provide an early and uninterrupted flow of nutrients to ensure the anabolic state that existed in-utero continues with minimal or no interruption and ii) to stimulate the immature gastrointestinal tract to undergo maturation. However, as described above, early provision of the preterm infant with the recommended nutritional intakes is fraught with difficulties. The latter include comorbidities which preclude normal enteral feeding such as respiratory distress, haemodynamic instability and sepsis. Co-morbidities also indirectly affect the delivery of adequate nutrition because a significant proportion of the total daily fluids may be used to deliver several medications, thereby limiting the amount of actual nutrition given to the baby. This problem can be overcome to some extent by concentrating the constituents of TPN in a smaller volume. In addition, gut immaturity limits the amount of enteral feed the baby will tolerate and is also a major risk factor for NEC, which will further impair enteral feeding. Furthermore, the high nutritional requirements cannot be fully provided immediately after birth and thus need progressive and cautious escalation. All these factors contribute to the risk of undernutrition with its resulting outcomes.

Strategy for nutritional support

Nutritional support of preterm infants occurs in four distinct phases, each with its own risks and challenges. During the early phase, nutrients are almost exclusively provided *via* the parenteral route, while small enteral feedings (gastrointestinal priming or trophic feeds) are used to stimulate the intestinal tract towards maturation. During the subsequent transition phase, enteral feeding is slowly advanced as the intestinal tract shows evidence of maturation, and parenteral nutrition is gradually phased out. During the late phase, infants are exclusively enteral fed and are expected to grow normally. If provided with the necessary nutrients, preterm infants may also show catch-up growth, making up for lost time during the early phase. Preterm infants continue to have special nutritional needs after discharge from hospital.

The importance of providing early nutrition has led to the use of parenteral nutrition immediately after birth with the knowledge that it differs from enteral nutrition in nutrient composition and carries the risk of infection. Nevertheless, the use of total parenteral nutrition (TPN) has contributed to improving survival rates and at its most fundamental level, it helps the newborn premature infant maintain a neutral nitrogen balance.

While nutrients are provided parenterally immediately after birth, starting trophic feeds with breast milk from day 1 of life, with volumes as low as 2 mL every 4 to 6 h, is the safest and most effective method of stimulating the intestinal tract and accelerating its maturation.[18]

When breast milk is not available, pasteurised donor milk (free of viruses such as HIV and cytomegalovirus) can be used for gastrointestinal priming. Although pasteurisation diminishes some of the protective and trophic factors of human milk, donor milk still remains protective against NEC and sepsis while maintaining trophic effects. Using gastrointestinal priming has been shown to lead to earlier establishment of full feedings and to earlier hospital discharge without an increase in morbidities. Moreover, earlier achievement of full feedings has been shown to decrease the risk of sepsis.

Increasing enteral feed volumes

Once intestinal tract maturation occurs, enteral nutrients can be progressively increased while parenteral nutrition is proportionally reduced before being discontinued. Gastrointestinal motility is a marker of gut maturation and is monitored by the

assessment of gastric residuals. Improving gastric emptying (facilitated by breast milk) reflects the improving ability to digest and absorb nutrients. Feeding volumes can be gradually increased by 20-25 mL/kg each day as gastric residuals permit. The absence of significant gastric residuals should be ascertained before feeding volume is further increased. Although more rapid increases in feed volumes have been achieved safely, intestinal maturation requires time and therefore more rapid increases are not necessary. Parenteral nutrition is usually maintained until enteral feedings reach 120 mL/kg/day after which it may be discontinued, provided the baby is tolerating enteral feeds.

Breast milk fortification

When feeding volumes of 80-100 mL/kg/day are tolerated, fortification of breast milk is usually initiated, although in some neonatal units, fortification is started at an earlier stage. Half-strength fortification may be tried before proceeding to full strength.

Once full feed volumes (≥ 150 mL/kg/day) are established, the aim is to facilitate a growth rate similar to intrauterine growth. If the growth rate is not satisfactory, enteral intake may be increased by 10%-20%. The best nutrition is fortified human mother's milk or, when not available, fortified donor milk or special formulas with higher protein content (3.3-3.6 g/100 kcal). Babies should also be given multivitamins and, if being exclusively fed with expressed breast milk, iron supplements from day 28 onwards. It is important to measure serum sodium and phosphate levels regularly (minimum once weekly). Serum sodium levels should be kept in the normal range to facilitate the action of IGF1 at cellular level. Serum phosphate levels should be at least 2.0 mmol/L as the premature baby's requirement for phosphate is high, not only for bone development but also for intermediary metabolism.

After discharge from hospital, preterm infants continue to have high nutrient requirements in addition to potential accrued deficits in bone mineral content. There is therefore a need for continued fortification of human milk and mineral and vitamin supplementation or to feed with an enriched post-discharge formula.

DIRECTIONS FOR FUTURE RESEARCH

Despite the major improvements already made in preterm infant nutrition, several unknowns remain and should direct future research efforts. Many studies report historical data in small populations.

There is a lack of a standardised approach to report nutritional intake data and measures of outcomes. Also, there is substantial variation in methods used to estimate and calculate nutritional intakes, making comparison amongst studies difficult and meta-analysis unreliable. Future research should focus on the development of minimal reporting sets of standardised nutritional interventions, and an agreed checklist for standardised reporting of neonatal nutrition and core outcomes.

More research is needed to determine the optimal nutrition, growth rates and body composition in preterm infants that are associated with the best neurocognitive benefits while minimising the long-term risk of chronic diseases.

Adequately powered randomised controlled trials on the late commencement of parenteral nutrition in term and late preterm infants who have IF as well as trials of intensified initial parenteral nutrition are required. Also, large-scale adequately powered randomised controlled trials should determine the optimal intake of amino acids and the effects of caloric balance in parenteral nutrition on the brain and neurodevelopment. The comparison of different types and amounts of protein in multi-component fortifiers on long-term neurodevelopment merits further investigation.

Further studies investigating the interactions between maternal HMOs and the intestinal microbiome, and the identification of specific pathways by which individual HMO structures exert protective actions are needed. More information on the development of a simple, high-throughput method to allow full characterization of HMOs is required and also, to determine if a causal relation exists between HMOs and neurodevelopmental outcome.

That LCPUFAs, particularly DHA, provide beneficial effects on preterm infants needs further exploration. These potential effects include better neurological outcomes at 2 years and reduction in the incidence and severity of neonatal morbidities such as CLD, NEC, ROP and possibly sepsis. Knowing how LCPUFAs affect different steps of the immune and anti-inflammatory response would contribute to determining the optimal LCPUFA requirements for good neurodevelopmental outcomes.

CONCLUSION

Despite the many difficulties encountered in nutrition research, several studies to date have demonstrated a positive relationship between aspects of nutrition when optimised and growth and neurocognitive outcomes for premature babies. Survival rates of premature infants, including those born at the extremes of viability, have improved enormously but future research needs to address how better growth and neurodevelopmental outcomes may be achieved. As more knowledge becomes available, neonatologists and all those involved in neonatal care, can significantly improve nutritional management, particularly in terms of quality, thereby making a major contribution to the lives of individuals, families and society.

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World Journal of Methodology

Submit a Manuscript: https://www.f6publishing.com

World J Methodol 2021 November 20; 11(6): 294-301

DOI: 10.5662/wjm.v11.i6.294

ISSN 2222-0682 (online)

ORIGINAL ARTICLE

Retrospective Study

Comparison of indirect immunofluorescence and western blot method in the diagnosis of hantavirus infections

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Author contributions: Vilibic-Cavlek T made contributions to the conception and design of the study and was involved in drafting and revising the manuscript; Stevanovic V, Savic V, Mrzljak A, and Bogdanic M were involved in collecting data and drafting and writing the manuscript; Barbic L and Tabain I revised the manuscript critically; All authors read and approved the final manuscript.

Institutional review board statement: The study was approved by the Ethics Committee of the Croatian Institute of Public Health, No. 030-02/17-10/1.

Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: All

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Abstract

BACKGROUND

Serologic cross-reactivity between hantaviruses often complicates the interpretation of the results.

AIM

To analyze the diagnostic value of indirect immunofluorescence assay (IFA) and western blot (WB) in the diagnosis of hantavirus infections.

METHODS

One hundred eighty-eight serum samples from Puumala (PUUV) and Dobrava (DOBV) orthohantavirus infected patients were analyzed. Serology was performed using commercial tests (Euroimmun, Lübeck, Germany).

RESULTS

Using IFA, 49.5% of acute-phase samples showed a monotypic response to PUUV, while 50.5% cross-reacted with other hantaviruses. The overall cross-reactivity was higher for immunoglobulin G (IgG) (50.0%) than for immunoglobulin M (IgM) (25.5%). PUUV IgM/IgG antibodies showed low/moderate reactivity with orthohantaviruses Hantaan (12.3%/31.5%), Seoul (7.5%/17.8%), DOBV (5.4%/

authors declare no conflict of interest.

Data sharing statement: No sharing data.

Provenance and peer review: Invited article; Externally peer reviewed

Specialty type: Medical laboratory technology

Country/Territory of origin: Croatia

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: January 2, 2021
Peer-review started: January 2, 2021
First decision: July 8, 2021
Revised: July 16, 2021
Accepted: August 31, 2021
Article in press: August 31, 2021
Published online: November 20, 2021

P-Reviewer: Khan S
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Guo X

28.1%), and Saaremaa (4.8%/15.7%). Both DOBV IgM and IgG antibodies were broadly reactive with Hantaan (76.2%/95.2%), Saaremaa (80.9%/83.3%), and Seoul (78.6%/85.7%) and moderate with PUUV (28.5%/38.1%). Using a WB, serotyping was successful in most cross-reactive samples (89.5%).

CONCLUSION

The presented results indicate that WB is more specific than IFA in the diagnosis of hantavirus infections, confirming serotype in most IFA cross-reactive samples.

Key Words: Hantaviruses; Serology; Cross-reactivity; Indirect immunofluorescence; Western blot

Core Tip: Serologic cross-reactivity among hantaviruses often complicates the interpretation of the results. The overall cross-reactivity is generally higher for immunoglobulin G antibodies than for immunoglobulin M antibodies. Western blot seems to be a more specific serology method than indirect immunofluorescence assay in the diagnosis of hantavirus infections, confirming serotype in the majority of cross-reactive samples detected by indirect immunofluorescence assay.

Citation: Vilibic-Cavlek T, Barbic L, Stevanovic V, Savic V, Mrzljak A, Bogdanic M, Tabain I. Comparison of indirect immunofluorescence and western blot method in the diagnosis of hantavirus infections. *World J Methodol* 2021; 11(6): 294-301
URL: https://www.wjgnet.com/2222-0682/full/v11/i6/294.htm
DOI: https://dx.doi.org/10.5662/wjm.v11.i6.294

INTRODUCTION

Hantaviruses represent a group of serologically related rodent-borne RNA viruses that belong to the genus *Orthohantavirus* of the family *Hantaviridae*. Two different diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), are caused by hantaviruses in humans[1]. Orthohantaviruses Hantaan (HTNV), Dobrava (DOBV), Puumala (PUUV), Seoul (SEOV), and Saaremaa (SAAV) cause HFRS with varying degrees of severity. While HTNV and DOBV cause a severe form of HFRS in Asia and Europe, SEOV causes less severe disease worldwide[2,3]. SAAV is also found to be responsible for a relatively mild human disease in Europe[4]. PUUV is a causative agent of nephropathia epidemica, the mildest form of the disease, endemic in Western Europe and Scandinavia[2].

Diagnostic methods for hantavirus infections include serology, reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry, and virus isolation [5].

Vero E6 cell culture has been used to isolate hantaviruses causing HFRS and HPS. Hantaviruses usually are not cytopathic in cultured cells; therefore, the detection of infection is confirmed using an immunofluorescence antibody test for viral antigen. Virus isolation is not performed as part of routine hantavirus diagnostics, since it is laborious and time-consuming and requires biosafety level 3 and 4 laboratories[6].

Serology is the main method for the diagnosis due to the hazardous nature of hantaviruses and a short-term viremia in infected humans[7,8]. Enzyme-linked immunosorbent assay and indirect immunofluorescence assay (IFA) are broadly used serologic tests used for detection of hantavirus immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies[9]. Immunoblot tests [western blot (WB) and line immunoassay] are also used in some laboratories[10].

Hantavirus nucleocapsid (N) protein is the major antigen in early humoral response in patients with hantavirus infection[11,12]. N protein is highly cross-reactive between different hantaviruses due to its conserved nature[11,13]. Overall, serologic cross-reactivity within the genus *Orthohantavirus* is the highest among viruses associated with (phylo)genetically closely related rodent species. DOBV is genetically and antigenetically related to other orthohantaviruses transmitted by *Murinae* rodents (Old

World mice and rats) such as HTNV, SEOV, and SAAV. PUUV is more distantly related to this group since its reservoirs belong to the *Arvicollinae* rodents (voles and lemmings)[14-16]. The interpretation of serology results is often complicated by the cross-reactivity, especially in areas where different hantaviruses co-circulate. Virus neutralization test is still the gold standard serologic test. Since this test has to be performed in biosafety level 3 laboratory, it is confined mainly to the reference laboratories[17].

Molecular diagnostic methods, including classic and real-time RT-PCR, are also widely used for the diagnosis of hantaviruses. Hantavirus RNA is detectable in blood early after the onset of symptoms; therefore, RT-PCR is a sensitive method for detecting hantavirus infections before the appearance of IgM antibodies. Primers specific for the hantavirus S and M segments have been used in different studies. The advantage of the molecular methods is that the RT-PCR product may be sequenced to identify the virus and perform phylogenetic analysis[5,18].

In Croatia, PUUV and DOBV have been demonstrated in humans[19-23], while SAAV and Tula orthohantavirus were also documented in rodents[24,25]. This study aimed to analyze the diagnostic value of IFA and WB methods in the diagnosis of hantavirus infections.

MATERIALS AND METHODS

A total of 188 serum samples from patients with serologically confirmed acute hantavirus infection (2015-2019) tested at the National Reference Laboratory for Arboviruses and Hantaviruses, Croatian Institute of Public Health were included in the study. Serologic tests were performed using a commercial IFA (Hantavirus mosaic; Euroimmun, Lübeck, Germany) to detect IgM/IgG antibodies of the most common hantaviruses: PUUV, DOBV, HTNV, SEOV, and SAAV. A fluorescence occurring as fine droplets in the cytoplasm of infected cells in a dilution 1:100 was considered a positive result.

Cross-reactive samples were further tested for hantavirus IgG antibodies using a WB (Euroline Hantavirus profile, Euroimmun). WB test strips were coated with nucleocapsid PUUV; DOBV and HTNV antigens. Band signal intensity at least as of IgG control was considered a positive result. According to the band intensity, results were interpreted as follows: strong positive-very strong band (+++); positive-medium to strong band (+/++); borderline-very weak band (+/-).

The study was approved by the Ethics Committee of the Croatian Institute of Public Health (Decision number: 030-02/17-10/1). Informed consent was obtained from all subjects included in the study.

RESULTS

PUUV was confirmed in 146 (77.6%) and DOBV in 42 (32.4%). Using IFA, 93 (49.5%) of 188 acute-phase serum samples reacted only with the homologous PUUV antigen, while in 95 (50.5%) samples, cross-reactive IgM and/or IgG antibodies were found. The overall cross-reactivity was higher for IgG antibodies (94/188; 50.0%) than for IgM antibodies (48/188; 25.5%). Among 95 cross-reactive samples, 55 (57.9%) were confirmed as PUUV and 30 (31.6%) samples as DOBV using a WB.

Cross-reactive patterns to different hantavirus antigens in PUUV- and DOBV-infected patients detected using IFA are presented in Figures 1 and 2. Among PUUV positive samples, a low/very low IgM reactivity was observed with HTNV (18/146; 12.3%), SEOV (11/146; 7.5%), DOBV (8/146; 5.4%), and SAAV (7/146; 4.8%). PUUV IgG antibodies showed a moderate reactivity with HTNV (46/146; 31.5%) and DOBV (41/146; 28.1%), while reactivity with SEOV and SAAV was low (26/146; 17.8% and 23/146; 15.7%, respectively).

In DOBV positive samples, both IgM and IgG antibodies showed a high degree of cross-reactivity. Among IgM positive samples, the highest cross-reactivity was observed with SAAV (34/42; 80.9%), 33/42 (78.6%) with SEOV, and 32/42 (76.2%) with HTNV. In 12 samples (28.5%), cross-reactive antibodies with PUUV were found. DOBV IgG antibodies showed the highest reactivity with HTNV (40/42; 95.2%). Almost equally high reactivity was found with SEOV and SAAV (36/42; 85.7% and 35/42, 83.3%, respectively), and moderate reactivity was found with PUUV (16/42; 38.1%). The majority of DOBV-positive samples (IgM 24/42, 57.1%; IgG 35/42; 83.3%) showed reactivity with all three hantavirus antigens (HTNV + SEOV + SAAV).

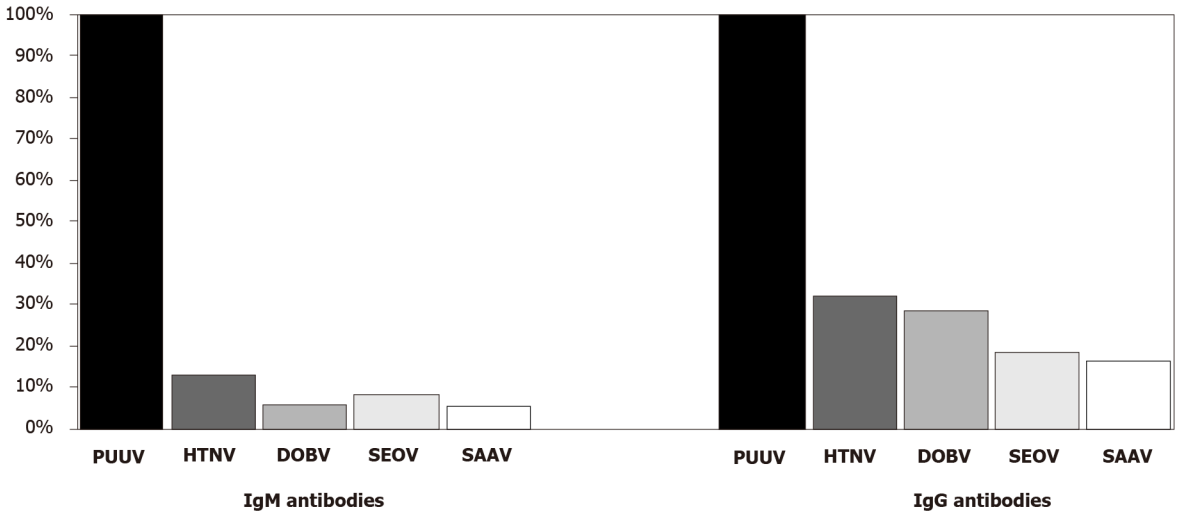

Figure 1 Cross-reactive patterns of hantavirus immunoglobulin M and immunoglobulin G antibodies in Puumala-infected patients by indirect immunofluorescence assay. PUUV: Puumala; DOBV: Dobrava; HTNV: Hantaan; SEOV: Seoul; SAAV: Saaremaa; Ig: Immunoglobulin.

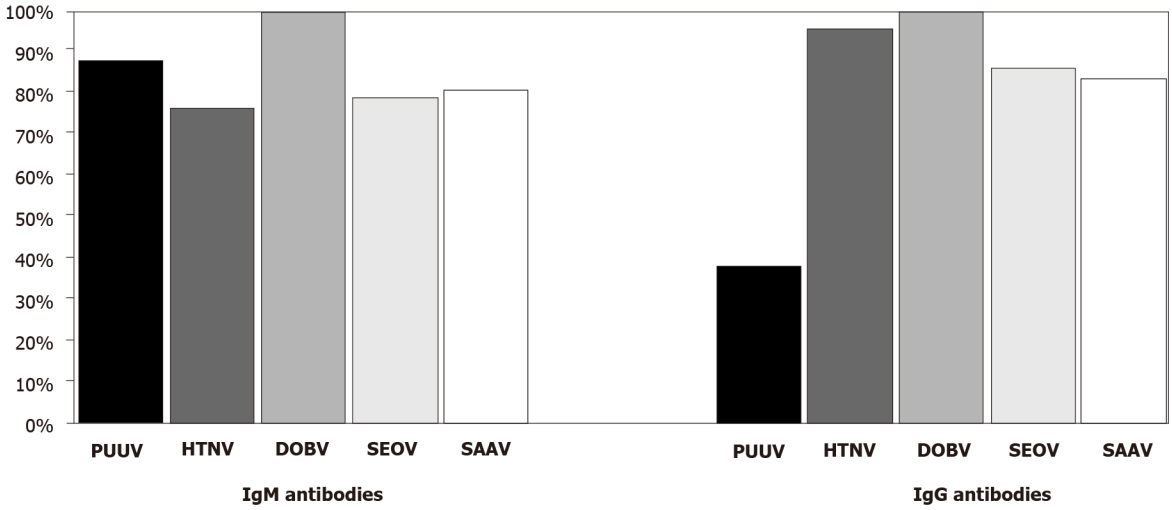

Figure 2 Cross-reactive patterns of hantavirus immunoglobulin M and immunoglobulin G antibodies in Dobrava-infected patients by indirect immunofluorescence assay. PUUV: Puumala; DOBV: Dobrava; HTNV: Hantaan; SEOV: Seoul; SAAV: Saaremaa; Ig: Immunoglobulin.

Forty-six of 172 (24.5%) IgG-positive samples cross-reacted with other hantaviruses by WB. However, based on signal intensity, a very strong band to the homologous viral antigen was observed in most cross-reactive samples compared to a weak/medium band of the related hantavirus antigens (Figure 3). Among PUUV positive samples, 8 (5.5%) tested borderline to HTNV and 10 (6.8%) to DOBV. Among DOBV positive samples, 19 (45.2%) tested positive/borderline to HTNV and 5 (9.5%) to PUUV. Only 8 PUUV positive samples (5.5%) showed a very strong band to PUUV and DOBV antigens. Additionally, two DOBV positive samples (4.7%) showed a very strong band to both DOBV and HTNV antigens (Table 1). The detection of PUUV and DOBV IgM antibodies by IFA in these samples indicated acute PUUV and DOBV infection, respectively.

DISCUSSION

Results of this study indicated broadly cross-reactive patterns of hantaviruses detected by IFA, which were found to be much higher for DOBV compared to PUUV. One published multicenter study on the simultaneous detection of hantaviruses showed a high cross-reactivity of serum samples from DOBV-infected patients with SAAV, HTNV, and SEOV (60%-100%), while cross-reactivity with PUUV was moderate (up to

Table 1 Cross-reactive patterns of hantavirus immunoglobulin G antibodies by western blot

Band intensity	PUUV	HTNV	DOBV
PUUV-infected patients (n = 146)			
Strong positive (+++)[1]	-	0 (0%)	8 (5.5%)
Positive (+, ++)[2]	-	0 (0%)	0 (0%)
Borderline (+/-)[3]	-	8 (5.5%)	10 (6.8%)
DOBV-infected patients (n = 42)			
Strong positive (+++)[1]	0 (0%)	2 (4.7%)	-
Positive (+, ++)[2]	1 (2.4%)	8 (19.0%)	-
Borderline (+/-)[3]	4 (9.5%)	11 (26.2%)	-

[1]Very strong band.
[2]Medium to strong band.
[3]Very weak band.
PUUV: Puumala; DOBV: Dobrava; HTNV: Hantaan.

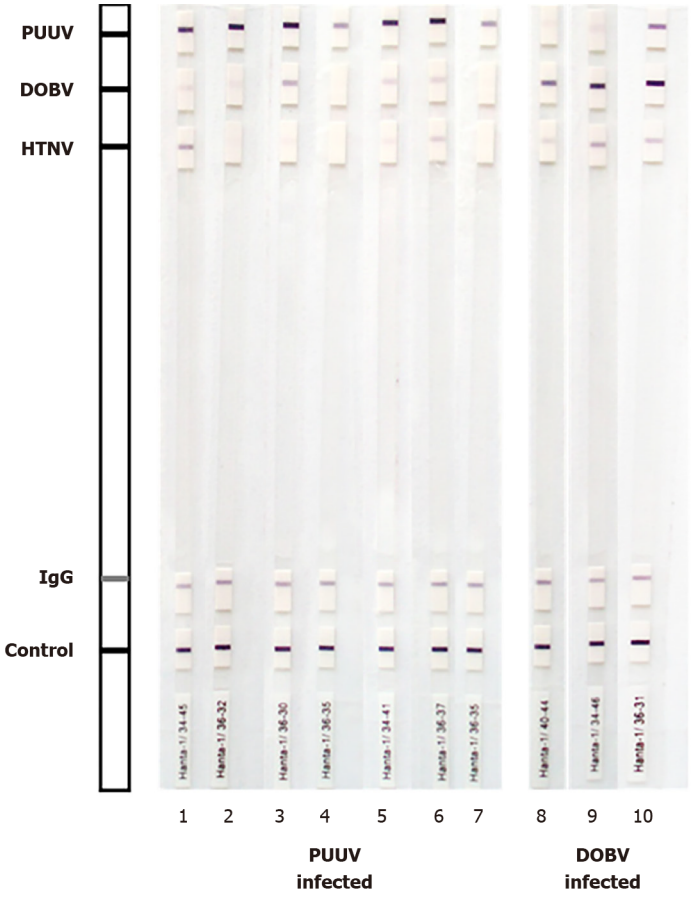

Figure 3 Western blot analysis of Puumala and Dobrava- infected patients. The test strips were coated with the affinity purified nucleocapsid Puumala (PUUV); Dobrava (DOBV) and Hantaan (HTNV) antigen. A correctly performed test for immunoglobulin (Ig)G antibodies against hantavirus antigens is indicated by a positive reaction of the control band and the IgG band. Some samples (strips 1, 3, 5, 6, 8-10) cross-reacted with other hantaviruses, however, based on signal intensity, a very strong band to the homologous virus antigen was detected compared to a very weak/weak band of the related hantavirus antigens.

43%) using IFA[26]. This study observed a remarkably high cross-reactivity for both DOBV IgM/IgG antibodies with SAAV, HTNV, and SEOV antigens (IgM 76.2%-80.9%, IgG 83.3%-95.2%). In addition, 57.1% IgM and 83.3% IgG positive samples cross-reacted with all three hantavirus antigens. These results are in accordance with the phylogenetic relatedness of hantaviruses. However, a substantial cross-reactivity was also found with PUUV (IgM 28.5%, IgG 38.1%), although PUUV is phylogenetically

distantly from DOBV.

IgM/IgG antibodies of PUUV-infected Croatian patients reacted moderately with HTNV (12.3%/31.5%). In a study by Lederer *et al*[26], even higher cross-reactivity between PUUV and HTNV IgM/IgG was found (49%/79%), while the reactivity to other tested hantaviruses was low, similar to our results.

In this study, a lower degree of cross-reactivity was also found by WB (24.5%). However, in all but 8 samples, differentiation of hantavirus serotype was possible based on powerful signal intensity to homologous antigen compared to weak/medium signal intensity to heterologous antigens. Some other studies which used WB for result confirmation showed similar results[27,28].

Since the clinical course and prognosis differ in PUUV and DOBV infection, the determination of hantavirus serotype is important for diagnosing acute HFRS cases. In addition, due to specific rodent hosts, identification of currently circulating hantavirus serotype is also useful for planning rodent control programs. Using IFA, serotype identification in seroepidemiological studies is often difficult because of extensive cross-reactivity among IgG antibodies. In DOBV infected individuals, considerable cross-reactivity was also observed between IgM antibodies. Using WB, differentiation of hantavirus serotype was possible in most cases by comparing the signal intensity in most IFA cross-reactive samples.

CONCLUSION

Although cross-reactivity among hantaviruses was detected in both IFA and WB, the results of this study showed that WB seems to be more specific than IFA, confirming hantavirus serotype in 89.5% of cross-reactive samples detected by IFA.

ARTICLE HIGHLIGHTS

Research background

The cross-reactivity among hantaviruses often complicates the interpretation of serology results, especially in areas where different hantaviruses co-circulate.

Research motivation

Data on the comparison of different serologic methods in the diagnosis of hantaviruses are scarce.

Research objectives

This study aimed to analyze the diagnostic value of indirect immunofluorescence (IFA) and western blot (WB) methods in diagnosing hantavirus infections.

Research methods

A commercial IFA was used to detect immunoglobulin M (IgM)/immunoglobulin G (IgG) antibodies to the most common orthohantaviruses: Puumala (PUUV), Dobrava (DOBV), Hantaan (HTNV), Seoul (SEOV), and Saaremaa (SAAV). Cross-reactive samples were additionally tested by a commercial WB using PUUV, DOBV, and HTNV antigens.

Research results

Using IFA, 49.5% of acute-phase serum samples reacted only with the homologous PUUV antigen, while in 50.5% samples, cross-reactive IgM and/or IgG antibodies were found. PUUV IgM/IgG antibodies cross-reacted with HTNV (12.3%/31.5%), SEOV (7.5%/17.8%), DOBV (5.4%/28.1%), and SAAV (4.8%/15.7%). Both DOBV IgM and IgG antibodies were broadly reactive with HTNV (76.2%/95.2%), SAAV (80.9%/83.3%), and SEOV (78.6%/85.7%) and moderate with PUUV (28.5%/38.1%). Using a WB, serotyping was successful in 89.5% cross-reactive samples.

Research conclusions

WB seems to be more specific than IFA, confirming hantavirus serotype in the majority of cross-reactive samples detected by IFA.

Research perspectives

Further studies on a large sample caused by different hantavirus serotypes are needed.

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