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EVIDENCE REVIEW

# Human papillomavirus infection and gastric cancer risk: A meta-epidemiological review

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## Abstract

Gastric cancer (GC) is a multifactorial disease, and several modifiable risk factors have been reported. This review summarizes and interprets two previous quantitative systematic reviews evaluating the association between human papillomavirus (HPV) infection and GC risk. The results of two systematic reviews evaluating the same hypothesis showed a statistically significant difference in summary odds ratios and their 95% confidence intervals. Thus, it is necessary to conduct a subgroup analysis of Chinese and non-Chinese studies. Additional meta-analyses that control for heterogeneity are required. Reanalysis showed that all the Chinese studies had statistical significance, whereas the non-national studies did not. The funnel plot asymmetry and Egger's test confirmed publication bias in the Chinese studies. In addition, the proportion of HPV-positive cases in Chinese studies was 1.43 times higher than that in non-Chinese studies and 2.81 times lower in controls. Therefore, the deduced evidence is currently insufficient to conclude that HPV infection is associated with GC risk.

**Key Words:** Papillomavirus; Stomach neoplasm; Case-control studies; Meta-analysis; Systematic review; Risk factors



**Core Tip:** Chinese studies showed that human papillomavirus infections increased the risk of gastric cancer; however, non-Chinese studies showed no statistical significance. Therefore, the deduced evidence is currently inadequate to conclude that human papillomavirus infection is associated with gastric cancer risk.

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## INTRODUCTION

Gastric cancer (GC) is the fifth most common incident cancer according to Global Cancer Statistics 2018[1] and ranks third in absolute years of life lost[2]. GC is a multifactorial disease, and several modifiable risk factors have been reported[3,4].

Infection with *Helicobacter pylori* or oncogenic viruses has important implications for preventing and managing GC[5]. *Helicobacter pylori* eradication is one of the reasons behind the steady decline in global GC incidence[6]. Therefore, human papillomavirus (HPV), which is among potential oncoviruses posing GC risk reviewed by Niedźwiedzka-Rystwej *et al*[7], should be considered to control GC occurrence because HPV vaccines have been used to prevent uterine cervix cancer[8,9].

However, the International Agency for Research on Cancer did not suggest an association between HPV infection and GC risk in a monograph published in 2007 [10]. This review summarizes and interprets previous quantitative systematic reviews evaluating the association between HPV infection and GC risk.

## PREVIOUS SYSTEMATIC REVIEWS

A PubMed (https://pubmed.ncbi.nlm.nih.gov) search, using "papillomavirus infection" and "stomach neoplasms" as the keywords of the hypothesis, identified two systematic reviews as of December 31, 2020[5,11]. Both selected case-control studies and their results are summarized in Table 1.

Zeng *et al*[11] reported that in 2016, a total of 15 case-control studies, including 12 studies on Chinese patients, and a meta-analysis showed that HPV infection increased the risk of GC by 7.39 times [95% confidence interval (CI) of summary odds ratio (sOR): 3.88–14.1]. Further, a study by Wang *et al*[5] published in 2020 selected a total of 14 case-control studies, including five studies on Chinese patients, and the sOR was 1.53 (95%CI: 1.00–2.33).

The results of two systematic reviews evaluating the same hypothesis showed a statistically significant difference in sORs and their 95%CI. These findings can be inferred from the following three reasons. First, there was a difference in selection criteria. Wang *et al*[5] included three serological studies, in addition to tissue tests. Therefore, it is necessary to limit future research to tissue studies and conduct a meta-analysis again. Second, there was a difference in search databases between the two systematic reviews. Zeng *et al*[11] and Wang *et al*[5] selected 12 and five Chinese studies, respectively. Whereas Zeng *et al*[11] did not report a subgroup analysis, Wang *et al*[5] showed different subgroup analysis results between Chinese and non-Chinese studies. Therefore, it is necessary to conduct subgroup analyses of Chinese and non-Chinese studies in all selected articles. Finally, potential bias is possible due to heterogeneity. Wang *et al*[5] found no statistical significance in subgroups with less than 50% of the I-squared value, such as non-Chinese studies, serum studies, and HPV-18 studies (Table 1). Therefore, additional meta-analyses that control for heterogeneity are required.

## RE-ANALYSIS OF META-ANALYSIS

Both systematic reviews selected a total of 25 articles. After excluding three serological studies[12-14], three studies had no information on the control group[15-17], and one showed zero HPV positivity in both the case and control groups[18]; hence, 18 articles were selected for reanalysis[19-35].

Table 2 illustrates the information extracted for the reanalysis of each study. Xu *et al*[25] extracted the results for cardia as well as those for the entire region for use in subgroup analysis by GC site.

Figure 1 displays a forest plot showing the results of the reanalysis. The sOR for 18 studies was 5.80 (95%CI: 3.27–10.31), showing statistical significance. While the I-squared value was reduced from 60% in all studies to 0% in 12 Chinese studies, their sOR remained statistically significant at 7.86 (95%CI: 5.19–11.89). However, the sOR

**Table 1 The summary odds ratio with its 95%CI from two systematic reviews**

| Ref. | Search to | Subgroup | Case-control studies | sOR (95%CI) | $I^2$ (%) |
|---|---|---|---|---|---|
| Zeng *et al*[11], 2016 | Jun 2016 | All | 15 | 7.39 (3.88-14.1) | 56.7 |
| Wang *et al*[5], 2020 | Apr 2020 | All | 14 | 1.53 (1.00-2.33) | 59.8 |
| | | Chinese | 5 | 1.98 (1.04-3.75) | 73.7 |
| | | Non-Chinese | 9 | 1.17 (0.68-2.02) | 33.4 |
| | | Tissue | 11 | 2.24 (1.13-4.43) | 66.5 |
| | | Serum | 3 | 1.04 (0.75-1.44) | 0.0 |
| | | HPV-16 | 8 | 2.42 (1.00-5.83) | 67.5 |
| | | HPV-18 | 3 | 1.08 (0.59-1.99) | 0.0 |

HPV: Human papillomavirus; sOR: Summary odds ratio.

**Table 2 Extracted information of the 18 selected case-control studies**

| Ref. | Year | Nation | Site | Test | Sample | PCa | NCa | PCo | NCo |
|---|---|---|---|---|---|---|---|---|---|
| Sha *et al*[19] | 1998 | China | Gastric | PCR | FFPE | 27 | 38 | 4 | 61 |
| Dong *et al*[20] | 1999 | China | Gastric | PCR | Other | 10 | 27 | 0 | 20 |
| Yu *et al*[21] | 1999 | China | Gastric | PCR | FFPE | 30 | 102 | 3 | 101 |
| Zhou *et al*[22] | 1999 | China | Gastric | PCR | FFPE | 19 | 31 | 0 | 20 |
| Zhu *et al*[23] | 2000 | China | Gastric | PCR | FF | 11 | 31 | 0 | 42 |
| Liao *et al*[24] | 2001 | China | Gastric | ISH | Other | 26 | 24 | 2 | 28 |
| Xu *et al*[25] | 2003 | China | Cardia | ISH | FFPE | 50 | 24 | 10 | 40 |
| Xu *et al*[25] | 2003 | China | Gastric | ISH | FFPE | 111 | 125 | 10 | 40 |
| Ma *et al*[26] | 2007 | China | Gastric | PCR | FFPE | 15 | 25 | 2 | 38 |
| Ma *et al*[27] | 2007 | China | Cardia | PCR | FFPE | 32 | 61 | 0 | 21 |
| Rong *et al*[28] | 2007 | China | Cardia | PCR | FFPE | 16 | 5 | 2 | 19 |
| Wang *et al*[29] | 2013 | China | Gastric | PCR | FFPE | 20 | 72 | 4 | 82 |
| Su *et al*[15] | 2015 | China | Gastric | PCR | Other | 1 | 14 | 0 | 15 |
| Anwar *et al*[30] | 1995 | Japan | Gastric | PCR | FFPE | 23 | 28 | 2 | 10 |
| Erol *et al*[31] | 2009 | Turkey | Gastric | PCR | FFPE | 17 | 21 | 33 | 73 |
| Cândido *et al*[32] | 2013 | Brazil | Gastric | PCR | FFPE | 4 | 36 | 10 | 30 |
| Türkay *et al*[33] | 2015 | Turkey | Cardia | PCR | FFPE | 2 | 17 | 0 | 8 |
| Bozdayi *et al*[34] | 2019 | Turkey | Gastric | PCR | Other | 20 | 33 | 5 | 21 |
| Leon *et al*[35] | 2019 | Ethiopia | Cardia | PCR | FF | 11 | 51 | 0 | 56 |

FF: Fresh frozen tissue; FFPE: Formalin-fixed paraffin-embedded tissue; ISH: *In situ* hybridization; NCa: Negative in cases; NCo: Negative in controls; PCa: Positive in cases; PCo: Positive in controls; PCR: Polymerase chain reaction.

for six non-Chinese studies was 1.97 (95%CI: 0.79–4.89), which was not statistically significant. In other words, all Chinese studies showed statistical significance; however, the non-national studies did not. This finding was the same in the subgroup analysis by cardiac tissue, formalin-fixed paraffin-embedded tissue, fresh frozen tissue, and polymerase chain reaction (Table 3).

Twelve Chinese studies were examined for publication bias. The asymmetry of the funnel plot (Figure 2) and Egger's test ($P$ = 0.013) confirmed publication bias. The trimming sOR from trim-and-fill analysis[36] was 6.78 (95%CI: 4.40–10.45).

**Table 3 Subgroup analysis by nationality**

| | | All | Chinese studies | Non-Chinese studies |
|---|---|---|---|---|
| All | | 5.80 (3.27-10.31) [60.0] <18> | 7.86 (5.19-11.89) [0.0] <12> | 1.97 (0.79-4.89) [56.8] <6> |
| Area | | | | |
| | Gastric | 4.83 (2.64-8.83) [62.4] <14> | 7.08 (4.60-10.89) [0.0] <10> | 1.54 (0.60-3.92) [62.6] <4> |
| | Cardia | 10.88 (5.42-21,8) [0.0] <5> | 11.17 (5.34-23.35) [0.0] <3> | 8.62 (0.88-84.8) [14.2] <2> |
| Sample | | | | |
| | FFPE | 5.13 (2.55-10.34) [68.4] <12> | 8.02 (4.74-13.6) [19.6] <8> | 1.38 (0.45-4.16) [58.5] <4> |
| | FF | 27.9 (3.70-211.7) <2> | 31.0 (1.76-546.6) <1> | 25.2 (1.45-439.1) <1> |
| Methods | | | | |
| | PCR | 5.88 (3.00-11.52) [62.2] <16> | 10.93 (6.44-18.5) [0.0] <10> | 1.97 (0.79-4.98) [56.8] <6> |
| | ISH | 6.23 (1.56-24.9) [64.0] <2> | 6.23 (1.56-24.9) [64.0] <2> | - |

Study: Summary odds ratio (95% confidence interval) [$I^2$ value (%)] <Number of selected studies>; FF: Fresh frozen tissue; FFPE: Formalin-fixed paraffin-embedded tissue; ISH: *In situ* hybridization; PCR: Polymerase chain reaction.

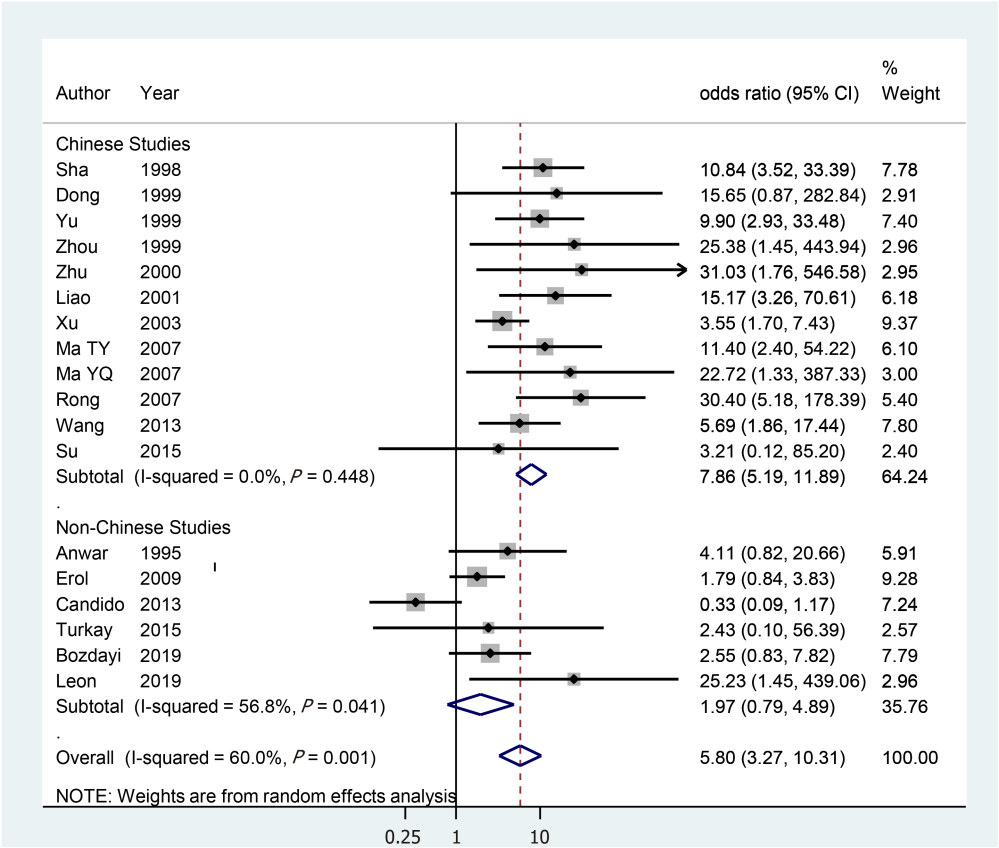


**Figure 1 Forest plot for estimating summary odds ratio.** CI: Confidence interval.

## CONCLUSION

To summarize the above reanalysis results, Chinese studies demonstrated that HPV infections increased the risk of GC; nonetheless, non-Chinese studies showed no statistical significance. Therefore, the deduced evidence is currently insufficient to conclude that HPV infection is associated with GC risk.

The following interpretations and suggestions may be made based on the significant associations observed only in Chinese studies. First, there is a possibility that publication bias was involved in the selection of Chinese studies. After checking for

**Table 4 Proportion of human papillomavirus positivity (%) by nationality**

| | | Chinese studies | Non-Chinese studies |
|---|---|---|---|
| Total | | | |
| | Positive/Observe | 335/1225 | 127/511 |
| | PP (95%CI) | 27.3 (24.9-29.9) | 24.9 (21.2-28.8) |
| Case | | | |
| | Positive/Observe | 298/711 | 77/263 |
| | PP (95%CI) | 41.9 (38.2-45.6) | 29.3 (23.8-35.2) |
| Control | | | |
| | Positive/Observe | 37/514 | 50/248 |
| | PP (95%CI) | 7.2 (5.1-9.8) | 20.2 (15.4-25.7) |

PP: Human papillomavirus positivity.

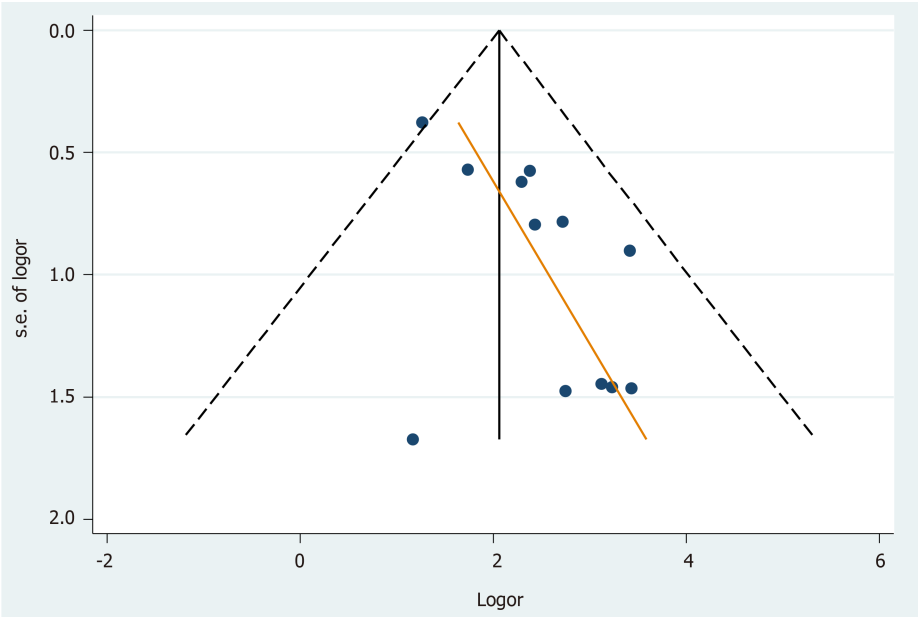


**Figure 2 Funnel plot in 12 Chinese studies (*P* value of Egger test = 0.013).**

publication bias using the funnel plot (Figure 2) and Egger's test, trim-and-fill analysis was performed. However, the trimming sOR in Chinese studies showed that HPV infections persistently increased the risk of GC. This mandated an alternative interpretation. The author attempted to infer that HPV positivity might have been different between Chinese and non-Chinese studies.

Using the information in Table 2, the proportion (%) of HPV positivity (PP) was obtained from both Chinese and non-Chinese studies (Table 4). On combining both the case and control groups, the PPs in Chinese and non-Chinese studies were 27.3% (95%CI: 24.9–29.9) and 24.9% (95%CI: 21.2–28.8), respectively. Their 95%CIs overlapped, showing no statistically significant differences. However, the case-group PP in Chinese studies was 41.9% (95%CI: 38.2–45.6), higher than that in non-Chinese studies (29.3%;95%CI: 23.8–35.2), and their 95%CIs did not overlap, showing a statistically significant difference. In contrast, the control-group PP in Chinese studies was 7.2 % (95%CI: 5.1–9.8), lower than the 20.2 % (95%CI: 15.4–25.7) in non-Chinese studies, and their 95%CIs did not overlap. In other words, the case PP in Chinese studies was 1.43 times (= 41.9/29.3) higher than that in non-Chinese studies and 2.81 times (= 20.2/7.2) lower in controls. This indicates a potentially significant relationship between HPV infection and GC risk in Chinese studies.

Given that the PP in the control group of the Chinese studies was significantly lower, descriptive epidemiological studies on HPV infection in the Chinese population are warranted. It is also necessary to conduct follow-up studies on whether the GC incidence rate due to HPV infection will change in the future due to the HPV vaccination project currently targeted at the Chinese population.

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*EVIDENCE REVIEW*

# Scientific evidence in the COVID-19 treatment: A comprehensive review

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## Abstract

In December 2019, cases of unknown origin pneumonia appeared in Wuhan, China; the causal agent of this pneumonia was a new virus of the coronaviridae family called severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). According to the clinical severity, symptoms and response to the different treatments, the evolution of the disease is divided in three phases. We analysed the most used treatments for coronavirus disease 2019 and the phase in which they are supposed to be effective. In the viral phase, remdesivir has demonstrated reduction in recovery time but no mortality reduction. Other drugs proposed for viral phase such as convalescent plasma and lopinavir/ritonavir did not demonstrate to be effective. In the inflammatory phase, corticosteroids demonstrated reduction of 28-d mortality in patients who needed oxygen, establishing that a corticosteroid regimen should be part of the standard treatment of critically ill patients. There are other immunosuppressive and immunomodulatory treatments such as anakinra, sarilumab, tocilizumab, colchicine or baricitinib that are being studied. Other treatments that were proposed at the beginning, like hydroxichloroquine or azithromycin, demonstrated no efficacy and increased mortality when combined.

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**Core Tip:** Severe acute respiratory syndrome coronavirus-2 is responsible for the unknown pneumonia that appeared in Wuhan, China, in December 2019. Lots of known drugs have been proved for coronavirus disease 2019. Corticosteroids demonstrated reduction of 28-d mortality in patients who needed oxygen and remdesivir proved to be effective reducing recovery time. Other drugs need more evaluation before establishing their effectiveness.

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## INTRODUCTION

In December 2019, cases of unknown origin pneumonia appeared in Wuhan, a province of China. It was determined that the causal agent of pneumonia was a new virus of the coronaviridae family called severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)[1,2]. The spread of this virus was so fast that resulted in a pandemic in a few months, causing more than 2.5 million deaths worldwide as of the writing of this paper.

It has become a priority to establish a treatment that reduces mortality, the time of illness and the severity of the virus. For that reason, a wide variety of trials and studies have been developed to evaluate the effectiveness of different already known drugs. Boregowda *et al*[3] published a review of experimental treatments in coronavirus disease 2019 (COVID-19) in October 2020 concluding that the best method of dealing with the pandemic is to reduce the community spread. A lot of investigation has occurred since then, so we have reviewed the updated literature with focus on articles published in high impact journals.

### *Pathogeny*

Siddiqi *et al*[4] proposed a three-phase classification of the evolution of COVID-19, according to the clinical severity, symptoms and response to the different treatments (Figure 1): (1) Viral phase or early infection: onset of infection and viral replication. The virus enters host cells through the angiotensin-converting angina 2 receptor, which is highly present in lung cells[5-7]. This phase includes the first seven days of symptoms; symptoms such as fever, myalgias and digestive inconveniences predominate. The polymerase chain reaction (PCR) of the virus is positive and there may be lymphopenia on laboratory tests and pulmonary infiltrates visible by computerized tomography; (2) Pulmonary phase: the virus continues to replicate and the host's humoral response develops. It appears approximately 7-14 d after the initial symptoms. It is technically divided into two sub-phases depending on whether the patient has respiratory failure (IIB) or not (IIA). The cytokine cascade is activated causing a severe inflammatory reaction in the lung tissue that can lead to respiratory distress. The most common manifestations are viral pneumonia, hypoxemia, cough and fever; and (3) Hyperinflammatory phase: it is the most severe phase and it is characterized by systemic inflammation with elevated blood levels of acute phase reactants and inflammatory cytokines[8]. It usually occurs 10-14 d after the initial symptoms. It can cause myocardial damage, shock, respiratory failure, *etc*. Only a few patients have this severe form of the disease. In this phase, treatment with immunomodulatory drugs or intravenous immunoglobulins may be useful.

### *Objective*

The objective of this article is to do a brief review of the drugs that have been used the most to treat the disease since the beginning of the pandemic until today[9].

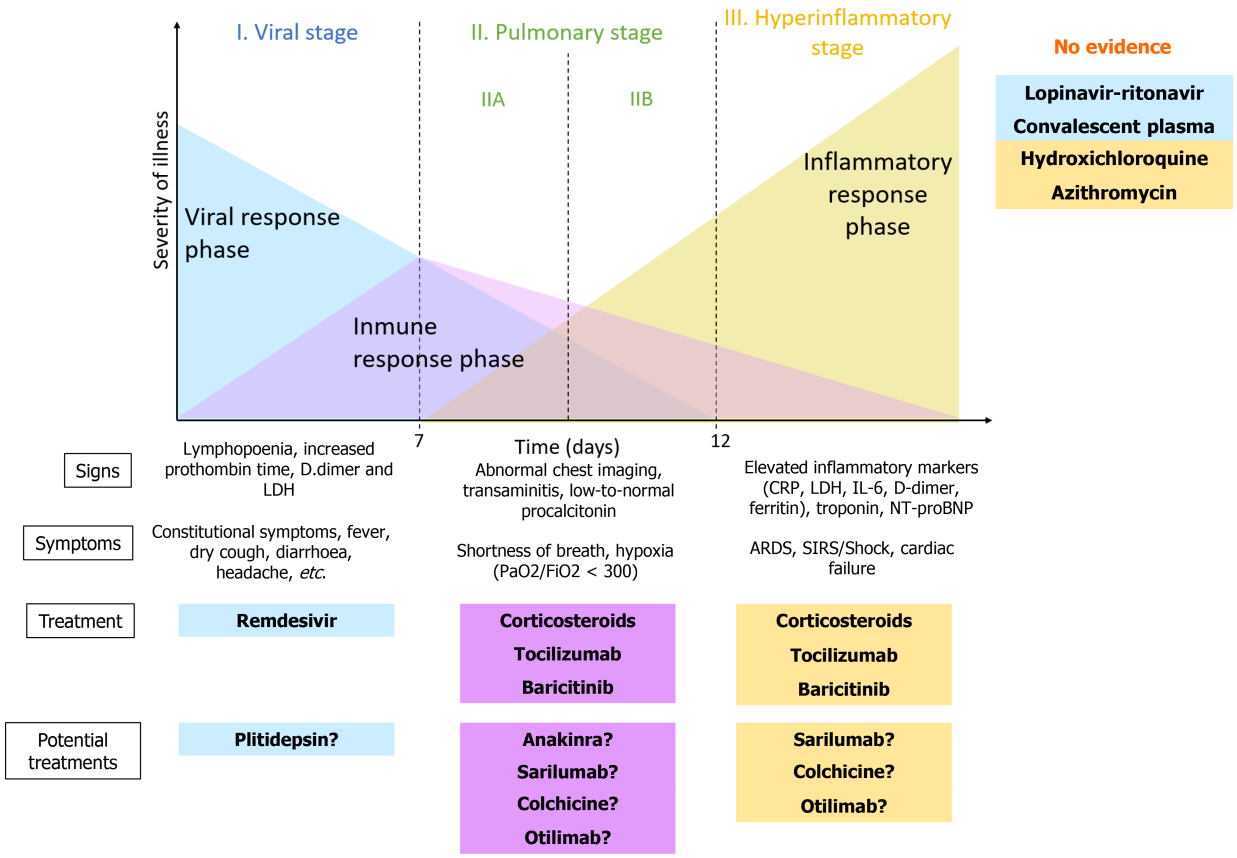


**Figure 1 Classification of coronavirus disease 2019 states and potential therapeutic targets.** Adaptation from Siddiqi *et al*[4]. LDH: Lactate dehydrogenase; CRP: C-reactive protein.

## LITERATURE SEARCH

We performed a search in PubMed with the keywords "COVID-19" and the most frequent drugs (Corticosteroid, Hydroxychloroquine, Remdesivir, *etc.*) as well as "COVID-19 + TREATMENT". The most relevant articles have been selected in order of mention and by scientific relevance, prioritizing those published in journals with the highest impact factor.

## VIRAL PHASE TREATMENTS

### *Remdesivir*

This RNA inhibitor drug has been studied since an early stage of the pandemic for its inhibitory effect on the viral replication of SARS-CoV-1 and Middle East respiratory syndrome coronavirus (MERS-CoV), demonstrating *in vitro* activity against SARS-CoV-2[10].

Since then, multiple studies and clinical trials have been conducted in order to prove its efficacy against COVID-19 infection. We highlight two of the largest: the Solidarity study and the Adaptive COVID-19 Treatment Trial (ACTT-1).

In November 2020, the final report of the clinical trial conducted by ACTT-1 group about the use of remdesivir for COVID-19 was published. In this clinical trial, 1062 patients with SARS-CoV-2 lower respiratory tract infection were enrolled. These patients were randomized to receive 10 d of treatment with remdesivir (200 mg as a loading dose, followed by 100 mg daily) *vs* placebo. The data obtained showed a significant reduction in recovery time compared to placebo (10 d *vs* 15 d). According to the results of this analysis, this effect was greater with the initiation of treatment in the early phase (first 10 d), and in patients in the 5$^{th}$ stage of severity. No clear results were obtained on its effect on mortality[11].

The Solidarity study carried out by the World Health Organization (WHO) confirmed the absence of effect of remdesivir on mortality in comparison with placebo

and in comparison with hydroxychloroquine, lopinavir/ritonavir and interferon[11].

Review articles on this drug have also been published, including information from the current literature and from smaller studies. A systematic review carried out by the American College of Physicians suggested that, according to the reviewed bibliography, there are studies that would demonstrate a similar benefit between the 5-d *vs* the established 10-d treatment regimen, with a consequent reduction in the reported adverse effects in patients with respiratory infection caused by SARS-CoV-2 who do not require mechanical ventilation or extracorporeal oxygenation[12].

### Lopinavir/ritonavir

Lopinavir is a protease inhibitor antiviral drug used against human immunodeficiency virus; its combination with ritonavir increases its plasma half-life.

This drug has shown *in vitro* activity against SARS-CoV-1 and was used during the MERS epidemic, demonstrating efficacy in terms of clinical and radiological improvement and reduction of viral load[13].

Despite its initial compassionate use, clinical trials have shown lack of efficacy against SARS-CoV-2.

The RECOVERY clinical trial is one of the largest studies conducted to date. It included 26 hospitals in the United Kingdom, and has studied the efficacy and safety of various drugs against COVID-19 (hydroxychloroquine, azithromycin, dexamethasone and lopinavir/ritonavir). In this study, 1616 patients were randomized to receive lopinavir/ritonavir *vs* 3424 patients receiving the standard treatment at that time. This study confirmed lack of efficacy of this drug in terms of mortality reduction, clinical improvement or time to discharge, concluding with a recommendation against its use in COVID-19 patients[14].

### Hyperimmune plasma

Convalescent plasma (hyperimmune plasma, with active antibodies against SARS-CoV-2) has been proposed as a treatment for COVID-19 due to its direct antiviral neutralizing effect, its ability to modulate viral activity in the acute moment and its ability to indirectly activate antiviral functions of the immune system such as the complement cascade, NK cells, *etc.* Hyperimmune plasma has been successfully used for the treatment of influenza pneumonia and, more recently, for SARS-CoV-1. The RECOVERY group has assessed mortality at 28 d with hyperimmune plasma in comparison with standard of care, concluding that there are no significant differences; neither when analysing by subgroups. They propose as a limitation for the study that only hospitalized patients are included, so most are not in the viral replication phase, where theoretically hyperimmune plasma would have more effect[15].

Piechotta *et al*[16] made a review of 20 studies comparing hyperimmune plasma and standard of care. In a preliminary analysis, they did not find any benefit in terms of mortality, death time or improvement of clinical symptoms, concluding that there is insufficient evidence on efficacy and safety[16].

### Plitidepsin

The antiviral activity of plitidepsin is mediated by the inhibition of eukaryotic translation initiation factor 1, establishing it as a possible drug target. Thus, as observed both *in vitro* and *in vivo* in the article by White *et al*[17], plitidepsin can reduce viral replication by two orders of magnitude and lung inflammation *in vivo*, showing clinical potential against COVID-19. Clinical studies are needed to see if it is effective in human patients.

## TREATMENTS IN THE INFLAMMATORY PHASE

### Corticosteroids

Corticosteroids have been proposed as a possible treatment for COVID-19 due to their anti-inflammatory and immunosuppressive properties, being able to reduce the systemic damage produced in the inflammatory phase. In the systematic review by Budhathoki *et al*[18], 83 articles were included. It attempted to assess which patients would benefit the most from corticosteroid treatment according to the severity of the disease. It was observed that severely ill patients were more likely to receive corticosteroids in their treatment, with the groups receiving corticosteroids presenting a longer hospitalization and higher mortality; without being able to rule out bias because of the non-randomization of the patients[18].

The RECOVERY group assessed mortality from all causes at 28 d, comparing standard of care with the daily administration of dexamethasone 6 mg for 10 d. It demonstrated that mortality was lower in patients who received dexamethasone. In addition, they saw that this benefit was greater in those patients requiring oxygen therapy, with or without positive pressure therapy, and in those patients recruited after more than 7 d of symptoms. Likewise, it was observed in those patients with oxygen therapy that the administration of dexamethasone decreased their risk of needing invasive mechanical ventilation (IMV) and increased their possibility of IMV withdrawal if they were already receiving it[19].

Finally, it should be noted that a WHO work group has published a meta-analysis. Out of 1703 randomized patients, 678 received corticosteroids and 1025 received conventional treatment, showing an absolute risk of mortality at 28 d of 32% and 40% respectively. Also, mortality was lower in those patients who received low doses of corticosteroids (29%) than in those who received high doses (36%). No increase in adverse effects was perceived in the group receiving corticosteroids.

The Food and Drug Administration, WHO, European Medicines Agency and National Institutes of Health recommend the use of corticosteroids for the treatment of COVID-19 in patients requiring oxygen therapy. The WHO also established that a corticosteroid regimen should be part of the standard treatment of critically ill patients [20].

### *Tocilizumab*

Hypoxia and severe respiratory failure that occurs in patients with COVID-19 infection have been related to a disproportionate increase in acute phase reactants and pro-inflammatory cytokines such as Interleukin-6 (IL-6) or IL-1[21].

Therefore, it is believed that specific immunomodulatory substances against these cytokines could stop the mentioned inflammatory cascade and slow down the clinical deterioration of these patients.

Tocilizumab is a monoclonal antibody used in rheumatological diseases such as Rheumatoid Arthritis. It blocks the IL-6 membrane and soluble receptors, with the consequent reduction of the associated inflammatory response[22].

Its efficacy in patients with COVID-19 infection is still uncertain. To date, multiple clinical trials have been conducted, with disparate results.

In October 2020, Stone *et al*[23] published the results of its randomized clinical trial, conducted in 7 hospitals in the city of Boston (United States). They included a total of 243 patients with moderate COVID-19 infection (who did not require mechanical ventilation), randomized with a 2:1 ratio to receive conventional treatment *vs* placebo, or a single dose of 8 mg/kg of tocilizumab (maximum 800 mg). This study did not demonstrate any beneficial effect on the use of tocilizumab in mortality, IMV requirements or decrease in clinical deterioration. It should be noted that, at the time of this study, the results of the RECOVERY study on the efficacy of dexamethasone had not been published, so corticosteroids were not included as standard treatment [23].

In February 2021, Malhotra's group published the results of its phase 3 clinical trial. This was carried out in 61 centers between the United States and Europe, in patients with severe COVID-19 infection, randomized with a 2:1 ratio to receive tocilizumab 8 mg/kg *vs* placebo. In this study, no results were obtained that demonstrated an additional benefit of tocilizumab on mortality, or improvement in clinical status according to the ordinal severity scale (Table 1) at 28 d. It suggests a possible reduction in hospitalization time and ICU stay time in the treatment group, but more extensive research is needed[24].

Salama *et al*[25] conducted a phase 3 trial in 6 countries, with 389 patients of different age groups and ethnicity. This trial has demonstrated a decrease in the progression of the clinical deterioration and the need for IMV, mainly in patients with moderate or severe disease without mechanical ventilation. No reduction in mortality was demonstrated compared to the placebo group.

The RECOVERY group has recently published the results of the randomized trial carried out in the United Kingdom, with the participation of 131 hospitals belonging to the National Health System. The trial included 4116 patients who were randomized to receive tocilizumab *vs* standard treatment. The results of this study have shown a significant decrease in mortality at 28 d in the group randomized to receive tocilizumab and in patients with hypoxia and elevated acute phase reactants. It also improved the odds of hospital discharge before 28 d and a lower rate of progression toward IMV. In this study, the use of corticosteroids was included as standard medical treatment against COVID-19, also suggesting a possible benefit of the synergy of these two drugs[26].

**Table 1 Coronavirus disease 2019 treatments**

| Drug | Mechanism of action | Recommendation | Posology | Benefits |
|---|---|---|---|---|
| Remdesivir[1] | RNA replication inhibition | Hospitalized patients in the first 10 d of infection requiring supplementary oxygen, without mechanical ventilation or extracorporeal oxygenation | Loading dose of 200 mg, followed by 100 mg daily for 5 d | Reduction in recovery time compared to placebo (10 d *vs* 15 d) |
| Corticosteroids[1] | Anti-inflammatory and immunosuppressive effects | Hospitalized patients requiring oxygen therapy. Also beneficial in patients with higher requirements of respiratory support | Dexamethasone 6 mg daily for 10 d | Reduction of mortality at 28 d. Decrease the risk of IMV and days of IMV |
| Tocilizumab[1] | Antagonist of IL-6 receptor. Immunomodulatory effect | Hospitalized patients with hypoxia and elevated acute phase reactants | 8 mg/kg in a single dose (maximum of 600 mg). A second dose might be administrated if lack of effect | Reduction of mortality at 28 d. Reduce progression to IMV |
| Anakinra[2] | Antagonist of IL-1 receptor. Immunomodulatory effect | Not clear recommendations. Hospitalized patients with hypoxia and elevated acute phase reactants | - | Some data show some effect on clinical improvement in patients with NIMV requirements. |
| Sarilumab[2] | Antagonist of IL-6 receptor. Immunomodulatory effect | Not clear recommendations. Hospitalized patients with hypoxia and elevated acute phase reactants | - | It might reduce mortality in critical patients (unclear data) |
| Bariticinib[2] | Janus kinase (JAK) 1/2 inhibitor. In-vitro activity against SARS-CoV-2, given its inhibitory effect on cytokine release and its inhibition of virus entry into pneumocytes | Not clear recommendations. Hospitalized patients with moderate-severe COVID-19 infection | - | In combination with corticosteroid, it improves $SpO_2/FiO_2$ |
| Colchicine[2] | Lipid soluble alkaloid, with anti-inflammatory effect | Not clear recommendations. Non-hospitalized patients with COVID-19 | - | Some data show reduction of mortality and hospitalization in patients with mild infection. |
| Otilimab[2] | Monoclonal antibody, anti-granulocyte macrophage colony-stimulating factor | Not clear recommendations. Hospitalized patients with severe disease | - | Might have beneficial effects in elderly patients with severe disease |
| Plitidepsin[2] | Inhibition of eef1a, reduce viral replication | More studies needed, not clear recommendations | - | - |
| Hydroxychloroquine[3] | RNA replication inhibitor | Not recommended | | |
| Azithromycin[3] | Immunomodulatory effect | Not recommended | | |
| Lopinavir-Ritonavir[3] | Protease inhibitor. | Not recommended | - | - |
| Hyperimmune plasma[3] | Convalescent plasma with active antibodies against SARS-CoV-2 | Not recommended | - | - |

[1]Recommended ones.
[2]Need more evidence.
[3]Not recommended treatments. COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2; IMV: Invasive mechanical ventilation.

### *Anakinra*

Anakinra is an antagonist of the IL-1 receptor, with the ability to inhibit the pro-inflammatory activity of IL-1 alpha and beta. This drug is approved for the treatment of rheumatologic diseases such as Still's disease or familial Mediterranean fever. It is believed that it could be a therapeutic target against the inflammatory cascade produced by COVID-19, and especially against macrophage activation syndrome[27].

So far, this drug has shown effectiveness in patients with sepsis criteria and signs of hyperinflammation[28].

In the retrospective study carried out by Cavalli *et al*[29], they analyzed 29 patients admitted to the San Rafaelle hospital in Milan with NIMV requirements. This showed a certain improvement of the clinical status of the patients, without finding a reduction in mortality.

The CORIMUNO-ANA-1 clinical trial included 153 patients across France with moderate-severe COVID-19 infection, without mechanical ventilation (category 5 on the WHO severity scale). It did not demonstrate any beneficial effect of anakinra, indicating the need for further studies in other groups of patients with greater severity [30].

Therefore, according to the literature, so far there is no clear evidence that supports the use of anakinra in any specific group of patients. Currently, there are ongoing clinical trials with this drug in different subgroups of patients.

### Sarilumab

Several studies prove that elevated levels of interleukin-6 are related to greater severity of COVID-19 infection and higher mortality[31].

Sarilumab is a recombinant monoclonal antibody against the IL-6 receptor (soluble and membrane), approved for rheumatoid arthritis[32].

Many publications and trials have shown a benefit with the use of IL-6 antagonist drugs on severe COVID-19 infection. The study carried out by the REMAP-CAP group on 895 patients with COVID-19 demonstrated a reduction in mortality and a higher clinical improvement in critically ill patients randomized to receive an IL-6 antagonist. However, it should be noted that in this trial only 48 patients received sarilumab, while 366 patients received tocilizumab[33].

The results of the clinical trial carried out by Lescure *et al*[34] for the Sarilumab COVID-19 Global Study Group were recently published. In this Phase 3 trial, 431 patients with severe SARS-CoV-2 pneumonia (categories 5, 6 or 7 on the WHO severity scale) were randomized. This trial compared the use of sarilumab (200 or 400 mg) *vs* placebo. Sarilumab did not show to be effective in reducing mortality, improving the clinical severity scale, or reducing the length of hospital stay.

### Baricitinib

Baricitinib is another drug used in rheumatology as a Janus kinase 1/2 inhibitor. Multiple *in vitro* studies have been carried out with this molecule. The results of these studies suggest *in vitro* activity against SARS-CoV-2, given its inhibitory effect on cytokine release and its inhibition of virus entry into pneumocytes[35].

Studies in animal models show a significant reduction in cytokine production by alveolar macrophages, which translates into a reduction in the local inflammatory cascade and neutrophil recruitment[36].

The Oxford study, carried out by Rodriguez-Garcia *et al*[37], suggests a beneficial effect of the combined use of baricitinib with corticosteroids in patients with moderate-severe COVID-19 infection, by observing an improvement in lung function measured by $SpO_2/FiO_2$. It might produce a certain lung protective effect, as lower D-dimer values are observed in this group of patients.

The study carried out by Kalil *et al*[38] suggested a benefit from the combination of baricitib together with remdesivir in patients with COVID-19 infection. In this clinical trial, 1033 patients were randomized to receive remdesivir in combination with baricitinib or placebo. The results demonstrated a greater benefit with the association of the two drugs in terms of improvement in clinical status and in the days to recovery, with a greater benefit in patients requiring high-flow therapy or NIMV at the beginning of treatment.

Right now, there are multiple ongoing studies about the efficacy of this drug, alone or combined with others.

### Colchicine

Colchicine is a lipid soluble alkaloid that accumulates in granulocytes and monocytes. It reduces chemotaxis of inflammatory cells, blocks the expression of E-selectin, responsible for leukocyte binding to endothelial cells, and it is also in charge of the inflammasome activation and superoxide production. It has shown anti-inflammatory activity in pathologies such as pericarditis or gout.

McEwan *et al*[39] conducted a systematic review of the infectious complications of the use of colchicine and the use of colchicine for the treatment of infectious diseases, concluding in the case of COVID-19 that mortality at 21 and 28 d was lower in the colchicine group than in the standard treatment group. However, it is unknown whether this potential benefit is due to the antiviral or anti-inflammatory action of colchicine.

Likewise, the preliminary results of the COLCORONA study (Tardif *et al*[40]) were recently published confirming that in non-hospitalized patients with COVID-19, colchicine reduces mortality and hospitalization.

### *Otilimab*

This monoclonal antibody that inhibits granulocyte macrophage colony-stimulating factor (anti-GM-CSF) is currently under investigation in patients with severe SARS-CoV-2 infection.

The OSCAR clinical trial, which is about to start Phase 3, has shown promising results in Phase 2, ensuring the safety goals and suggesting a benefit in groups with older patients[41].

## OTHER TREATMENTS

### *Hydroxychloroquine*

Hydroxychloroquine has shown *in vitro* antiretroviral activity against several viruses, including SARS-CoV-2, it has an acceptable adverse effect profile and is inexpensive. It has not shown clinical efficacy in animals, but there are several studies that have suggested clinical benefits from the association of azithromycin with hydroxychloroquine.

The Oxford RECOVERY group compared all-cause mortality at 28 d in two groups, one of which received hydroxychloroquine ($n$ = 1561) and the other, standard treatment ($n$ = 3155). The risk of progression to non-invasive mechanical ventilation was found to be higher in the group taking hydroxychloroquine. Likewise, mortality was higher in the group taking hydroxychloroquine, determining that hydroxychloroquine is not an effective treatment for COVID-19. In addition, there is a risk of cardiovascular toxicity, which is exacerbated by co-administration with azithromycin [42].

Tleyjeh *et al*[43] studied the cardiovascular risk of the use of chloroquine and hydroxychloroquine in patients with COVID-19, establishing a significant risk of drug-induced QT prolongation and increased incidence of Torsades de pointes, ventricular tachycardia and cardiac arrest. Therefore, they do not recommend this treatment by routine for COVID-19.

The meta-analysis by Kashour *et al*[44] establishes with moderate certainty that hydroxychloroquine, with or without azithromycin, does not reduce short-term mortality in hospitalized patients with COVID-19 or the risk of hospitalization in patients treated on an outpatient basis.

Fiolet *et al*[45] also analysed the mortality of hydroxychloroquine alone, hydroxychloroquine and azithromycin, and standard treatment, showing that hydroxychloroquine alone does not modify mortality over standard treatment. However, when it is combined with azithromycin, mortality increases.

### *Azithromycin*

Once the benefit of the use of corticosteroids in COVID-19 had been evaluated, it was assessed whether other treatments that suppress or modulate the immune system could be effective against the disease. Azithromycin, besides being an antibiotic of the macrolide family, has shown an immunomodulatory effect by reducing the production of pro-inflammatory cytokines and inhibiting the activation of neutrophils.

The RECOVERY group studied mortality at 28 d, the time to discharge and the need for invasive mechanical ventilation in hospitalized COVID-19 patients. No significant differences between the azithromycin group and the standard treatment group were observed, nor were significant differences in subgroup analysis. Thus, they consider that azithromycin is not an effective treatment in hospitalized patients with COVID-19 and should be reserved for those who have an indication of azithromycin for antibiotic purposes[46].

Verdejo *et al*[47] conducted a systematic review on the use of macrolides in COVID-19, evaluating articles in which they are used alone or in combination with other drugs such as hydroxychloroquine. They evaluated all-cause mortality, the need for invasive mechanical ventilation and extracorporeal membrane oxygenation, hospitalization time, respiratory failure, serious adverse events, and SARS-CoV-2 PCR time to negativize. Although the quality of the evidence for most of the results was low, they concluded that macrolides do not show any beneficial effect compared to standard treatment.

### *Anticoagulation and thromboprophylaxis*

So far, there is wide evidence that confirms a higher risk of thromboembolic events in patients with severe COVID-19. For this reason, despite not being a direct COVID-19 treatment, the use of anticoagulation in these patients has been a controvert topic.

These thrombotic events are cause by the infection itself, but also by the proinflammatory response, the hypoxia and the critical illness. Some of these mechanisms are still unknow.

Most of the recent guidelines recommend keeping a high level of suspicion of thromboembolic events in hospitalized patients, monitoring laboratory parameters such as D-dimer and blood count. It is important to point out also the risk of haemorrhage in some patients, with its consequent implications. Tools like Wells score and IMPROVE-bleeding score could be useful to predict the risk of thrombosis and bleeding.

According to the article published by Skeik *et al*[48], patients with low or no suspicion for VTE calculated by Wells score (0 for deep vein thrombosis or < 2 for pulmonary embolism), they recommend regular antithrombotic prophylaxis. In patients with higher risk, imaging should be considered. If the result is negative, or imaging is not available, we should consider the bleeding risk. If this one is high, also regular thromboprophylaxis is recommended; if it is low, we should consider anticoagulation. In patients with high suspicion of VTE (Wells > 2 for VDT or 6 for PE) and without imaging available, the anticoagulation is also recommended according to the bleeding risk. Direct oral anticoagulations are usually preferred[48].

Guidelines like the CHEST Guidelines or the American College of Cardiology also recommend thromboprophylaxis in hospitalized patients depending on the thrombotic and bleeding risk of each patient. More studies are still needed.

## CONCLUSION

Currently, multiple pharmacological studies continue to be carried out. For the moment, the evidence recommends treating patients with remdesivir in the viral phase and with dexamethasone, tocilizumab or baricitinib in the inflammatory phase. Nevertheless, we are sure that in the following months we will be able to have more therapeutic weapons to tackle COVID-19.

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*REVIEW*

# Oncolytic virus therapy in cancer: A current review

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## Abstract

In view of the advancement in the understanding about the most diverse types of cancer and consequently a relentless search for a cure and increased survival rates of cancer patients, finding a therapy that is able to combat the mechanism of aggression of this disease is extremely important. Thus, oncolytic viruses (OVs) have demonstrated great benefits in the treatment of cancer because it mediates antitumor effects in several ways. Viruses can be used to infect cancer cells, especially over normal cells, to present tumor-associated antigens, to activate "danger signals" that generate a less immune-tolerant tumor microenvironment, and to serve transduction vehicles for expression of inflammatory and immuno-modulatory cytokines. The success of therapies using OVs was initially demonstrated by the use of the genetically modified herpes virus, talimogene laherparepvec, for the treatment of melanoma. At this time, several OVs are being studied as a potential treatment for cancer in clinical trials. However, it is necessary to be aware of the safety and possible adverse effects of this therapy; after all, an effective treatment for cancer should promote regression, attack the tumor, and in the meantime induce minimal systemic repercussions. In this manuscript, we will present a current review of the mechanism of action of OVs, main clinical uses, updates, and future perspectives on this treatment.

**Key Words:** Oncolytic viruses; Antitumor response; Tumor lysis; Tumor cells; Mechanism; Therapy

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**Core Tip:** Oncolytic viruses are organisms able to infect and lyse the tumor cells beyond stimulating the immune system to combat the disease. The clinical use of oncolytic viruses has shown to have positive results in the treatment of some types of cancers, contributing to reducing the tumor. Furthermore, the combined use of these viruses and other antitumor therapies have contributed to better prognosis in the patient's clinical condition.

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## INTRODUCTION

The first theories about the possible use of viruses to combat tumor cells date from the early 20th century with the description in 1904 of a woman with acute leukemia who presented remission of the clinical picture and a patient with cervical cancer in 1912 that demonstrated extensive tumor necrosis, both after a viral infection[1]. Thereafter, between 1950 and 1980, influenced by the possibility of developing a therapy for cancer, many studies were performed with different types of wild viruses aiming at an oncolytic action; however, the goal was not achieved due to the non-existence of necessary tools to control the viral pathogenesis and direct the virus to specific targets [2]. Viruses can be used to infect cancer cells, specifically over normal cells, to present tumor-associated antigens, to activate "danger signals" that generate a less immune-tolerant tumor microenvironment, and to serve transduction vehicles for expression of inflammatory and immunomodulatory cytokines[3]. Currently, in order to overcome these obstacles, the updates in the field of genetics seek to increase the specificity and efficacy of some viruses in infecting the abnormal cells through mechanisms such as gene deletion and the combined use of viruses and immune checkpoint inhibitors (ICIs)[4].

The oncolytic viruses (OVs) are organisms able to identify, infect, and lyse different cells in the tumor environment, aiming to stabilize and decrease the tumor progression. They can present a natural tropism to the cancer cells or be oriented genetically to identify specific targets[5]. Several OVs are being studied as a potential treatment for cancer in clinical trials[6]. Moreover, the OVs are capable of contributing to the stimulation of the immune system against the tumor cells, influencing the development of an antitumor response[7].

It is known that there are several evasion mechanisms in the tumor environment that contribute to the downregulation of the immune system, positively influencing the stability and progression of the disease even in immunocompetent patients[8]. Antigen presenting cells can be prevented from presenting tumor antigens to the T cells correctly, which contributes to the non-activation or discouragement of these cells [9]. Moreover, certain types of tumors can promote an abnormal stimulation of immune checkpoint receptors in T cells, like the cytotoxic T lymphocyte-associated antigen 4 and the programmed cell death protein 1/programmed death ligand 1(PD-L1), both related to the negative regulation of the inflammatory response and immune system homeostasis contributing to apoptosis and inhibition of proliferation of T cells [10]. In addition, the excess of tumor-associated macrophages, main lymphocytes regarding the inflammatory response against the tumor, are also an important mechanism of immune evasion since they have some similar functions and features to type M2 macrophages, which are responsible for tissue repair and immune response regulation. Thus, the abnormal rise of tumor-associated macrophages has been related to the downregulation of inflammation and increase of tumor growth rates[11].

Therefore, the clinical use of OVs emerges as an alternative to modifying the tumor environment from a state of immune desert caused by the evasion mechanisms that contribute to tumor progression, to an inflamed state, where the immune system is able to kill the abnormal cells[12]. In addition, the viruses present different mechanisms that would lead the infected cells to a cell lysis process, contributing to tumor cell death and increasing the efficacy of the immunotherapy[4]. This review will

encompass the viral mechanisms responsible for the oncolytic action of OVs, the clinical use of these viruses in certain tumors, and the future perspectives about their use.

## MECHANISM

### *General mechanism*

OVs are able to infect abnormal cells through specific targets, such as nuclear transcription factors and among them human telomerase reverse transcriptase, prostate specific antigen, cyclooxygenase-2, osteocalcin, and surface markers as prostate-specific membrane antigen, folate receptor, CD20, endothelial growth factor receptor, and Her2/neu, which are substances produced by the tumor cells[5]. Furthermore, the deletion of pathogenic viral genes in the laboratory in order to increase the selectivity to the tumor cells and decrease the aggressiveness of the OVs to normal tissues is also possible[13].

The administration route of OVs is intrinsically related to the type of tumor to be treated, given that the virus pathway directly influences the effectiveness of the therapy due to the virus availability on-site and the natural barrier of the organism of combat to antigens. The distribution can occur *via* intraperitoneal, intrathecal, subcutaneous, intratumoral, which provides greater control of viral quantity in the tumor environment and less adverse effects, and intravenous, which is related to the treatment of distant metastases[14].

Regarding the mechanisms of immune evasion by the tumor, the cancer cells can present certain alterations in the expression and activation of some mechanisms, such as protein kinase R and interferon 1 signaling pathway, which interferes in the response to viral infections, programmed apoptosis, and maturation of inflammatory cells. The modifications in the antiviral response, allied to viral factors capable of preventing apoptosis, allow OVs to survive longer in cancer cells and consequently to conclude the life cycle and maturation to the lytic phase[15].

The presence of viruses in the human organism stimulates the recognition of different immune signs related to the virus structure, such as viral proteins, RNA, DNA, and viral capsid, the pathogen-associated molecular patterns (PAMPs)[16]. Dendritic cells, upon recognition of the PAMPs through toll-like receptors (TLRs), which are pattern recognition receptors, stimulate production of inflammatory molecules with antiviral characteristics, like the type 1 interferons, tumor necrosis factor alpha (TNF-alpha) and cytokines such as interleukin 2 (IL-2), important mechanisms of recruitment of immune cells, and maintenance of the inflammatory environment[17].

TNF-alpha is related to response to the viral infection, positively regulating the expression of class 1 major histocompatibility complex in the cell membrane and positively influencing the action of caspase enzyme and cell apoptosis on some tumors [18]. This interferon is capable of stimulating cancer cell death through mechanisms that contribute to necrosis and apoptosis, generating thrombotic events through its antiangiogenic effects, which can lead to the destruction of some blood vessels responsible for the blood supply of the tumor[19]. TNF-alpha is also related to the stimulation of T helper cells type 1 (Th1) response, increase of the cytotoxicity of natural killer cells, and maturation of antigens presenting cells[18].

Studies have shown that IL-2 is related to the stimulation of cytotoxic lymphocytes and activation of T cell response, contributing to maturation and expansion of CD8+ T cells (TCD8) and natural killer cells, along with positive regulation of CD4+ T cells (TCD4). IL-2 is also capable of regulating T regulatory cell action and homeostasis, creating an inflammatory environment favorable for combating the tumor[20]. Furthermore, the Th1 inflammatory profile was also related to the decrease of T regulatory cells, increased rates of TCD4 and TCD8 effector cells, stimulation and differentiation of T lymphocytes as well as the maturation of dendritic cells, which contributes to the reversal of the immunosuppressive state of the tumor and promotes an inflammatory response[21].

In addition to the damage caused by the inflammatory response, the viral action inside the cell is also an important factor in the lysis and death of the aberrant cells. The presence of OVs could stimulate some dysfunction of organelles, such as the endoplasmic reticulum, mitochondria, or lysosome, compromising the normal cellular function. Moreover, the virus can stimulate oxidative stress through the production of reactive nitrogen species and endoplasmic reticulum stress, which is related to an increase of intracellular calcium levels[17], contributing to the stabilization and

decrease of the tumor.

The combined use of cell checkpoint blockers and OVs is an important mechanism to increase viral survival rates in the human organism, given that it contributes to the stimulation of an inflammatory response against the tumor. Through negative regulation of PD-L1, the tumor can circumvent the immune system, avoiding the maturation of T cells. In this way, PD-L1 inhibition was capable of stimulating a response with a Th1 profile, contributing to the appearance of TCD8 cells against the tumors and stimulating natural killer cell action[22]. Furthermore, studies have demonstrated that the administration of the OVs and monoclonal antibodies that inhibit the action of cytotoxic T lymphocyte-associated antigen 4 contributed to enhancing the effectiveness of immunotherapy[21].

The aforementioned mechanisms contribute to different types of elimination of the tumor cells, such as autophagic cell death, apoptosis, pyroptosis, and necrosis, leading to the production of immune signs related to the cell damage: damage-associated molecular patterns (DAMPs), like high mobility group box 1 protein and ATP. The DAMPs are important elements in the stimulation of the dendritic cell maturation process and contribute to the presentation of tumor-associated antigens to the immune cells through the cross-presentation between DAMPs and tumor-associated antigens, which leads to the perpetuation of the inflammatory response process[23]. Therefore, cellular lysis allows the liberation of the viruses in the extracellular environment and subsequent infection of other tumor cells, creating a chain reaction of combat to the tumor[16]. Besides that, the cell death contributes to the release of tumor antigens liable to be identified by immune cells in the inflammatory environment, stimulating a response against tumor cells, even in the uninfected ones, by the OVs[15].

The main mechanisms of action of OVs are represented in (Figure 1).

## OVs

**Adenovirus:** The adenoviruses are non-enveloped organisms with double-stranded linear DNA and an icosahedral capsid with three main proteins, hexon, penton base, and fiber, which when identified by the immune system contribute to the emergence of an antiviral response. There are more than 80 human types of adenoviruses that belong to the *Adenoviridae* family[24]. These viruses have a high tropism for different tissues of the organism, including ocular, respiratory, enteric, renal, and lymphoid and are able to use several receptors, such as human coxsackie-adenovirus receptor, CD86, CD46, and CD80 to enter the host cells[25]. Moreover, due to its capacity of serving as a viral vector[24], allied to their chemical and thermal stability outside the cell, various mechanisms of cellular entry, and the great knowledge about their biology, the adenoviruses have been used for the development of different immune therapies[26].

The viral replication process starts inside the cellular nucleus, inducing the expression and liberation of some proteins in the cytoplasm such as E1a and E1b, which are related to the stimulation of the autophagy process. This mechanism induces the production of some autophagosomes that can later merge with lysosomes resulting in the death of organelles or even the full cell[27]. Furthermore, research has shown that in tumor cells the expression of E1a can be related to the stimulation of the production of autophagic complexes, and E1b possibly supports the potentiation of action of these complexes, both contributing to the stabilization and decrease of the tumor[28].

When identifying and responding to different proteins of the viral capsid of adenoviruses, the human organism starts producing several inflammatory cytokines, such as IL-12 and TNF-alpha[29], which are related to the stimulation of cytotoxic cells like natural killer cells and TCD8, besides contribution in the maturation of immune cells and against the tumor. The type 5 Ad is commonly used for oncolytic therapy, since it can be detected by TLRs in the cellular membrane (TLR-2) or inside the cell (TLR-9) teasing the stimulation of different mechanisms in order to create a Th1 profile inflammatory response[29]. Moreover, the *Adenoviruses* can activate other pathways of the immune system, such as the complement system stimulating the opsonization processes, increasing the migration rates of inflammatory cells and production of inflammatory cytokines[23], which contributes to destroying infected cells.

Finally, the cellular stress caused by the viral infection and the inflammatory process lead to tumor cell death through necrosis, autophagy, or apoptosis and further liberation of DAMPs or PAMPs in the inflammatory environment, stimulating the maturation and migration of inflammatory cells as well as the production of cytokines. Furthermore, in addition to the direct tumor cell killing, the adenoviruses are capable of initiating the formation of an antitumor immune memory that contributes to the combat in metastatic sites[25]. Table 1 shows some genetic modifications to improve the adenoviruses oncolytic action.

**Table 1 Genetic modifications in the adenovirus**

| Ref. | Virus | Updates | Aim |
|---|---|---|---|
| Rojas *et al* [219] | COVIR -7/-15 | Insertion of E2F-binding sites in the gene *E1A* | Specific targeting to the tumor cells, which express E2F and increase viral replication rate and antitumor action |
| Sarkar *et al* [220] | CTV-m 7 | Insertion of the transgene MDA-7/IL-24 | Expression of the protein MDA-7/IL-24 increases the cytotoxic action in the tumor sites and lyse the metastatic cells. The studies have shown greater effectiveness in the therapy of prostate cancer |
| Sarkar *et al* [220] | tCCN1 -CTV - m 7 | Replacement of E1A by tCCN1 | Specific targeting and cytotoxicity against the tumor cells, which express the promoter tCCN1 in prostate cancer |
| Choi *et al* [221] | Ads armed with inhibitors of tumoral angiogenesis | Incorporation of the gene *FP3* | Increase of the antiangiogenic capacity, which decreases the vascular endothelial growth factor production and suppresses the rate of tumor growth |
| Lucas *et al* [222] | Ad5 armed with the peptide CKS17 | Replacement of HVR5 by the peptide CKS17 | Specific target to the TGFBRII in the liver cancer cells, increasing the viral cytotoxic action and decreasing the liver sequestration |
| Garofalo *et al*[223] | AdV-D24-ICOSL-CD40L | Insertion of *D24*, *ICOSL* and *CD40* genes in the chimeric virus, AdV-D24, serotype 5/3 | Selectivity to infect the cancer cells through DSG-2 receptor and stimulation of the immune system by ICOSL and ICOS, both contributing to the immunogenic cell death in melanoma |
| Vera *et al* [224] | VCN-01 | Selectivity to the pRB pathway and ability to express hyaluronidase | Specific viral replication, decreasing the side effects and degradation of the extracellular matrix by the enzyme hyaluronidase in solid tumors |
| Yang *et al* [225] | Ad5/3-CXCR4-TIMP2 | Replacing Ad5 knob with Ad3 knob and incorporating the gene *TIMP2* | Selective replication in the cancer cells, which reduces the action over the normal cells and the expression of inhibitors of metalloproteinases, contributing to the degradation and remodeling of the extracellular matrix, preventing tumor growth and metastasis |

Ads: Adenoviruses; CD40L: CD40 ligand; DSG-2: Desmoglein 2; FP3: Farnesylated protein 3; HVR5: Hypervariable region 5; ICOSL: Inducible co-stimulator ligand; IL-24: Interleukin 24; MDA-7: Melanoma differentiation-associated gene-7; pRB: Retinoblastoma protein; tCCN1: Truncated cellular communication network factor 1; TGFBRII: Transforming growth factor-beta receptor II; TIMP2: Tissue inhibitor of metalloproteinases 2.

**Protoparvovirus**: The Protoparvoviruses are single-stranded DNA, non-enveloped viruses that belong to the *Parvoviridae* family. They are capable of infecting mammalian cells, including human beings, through fixation factors such as the transferrin receptor or glycosidic substances like the N-acetylneuraminic acid that is expressed on the cellular membrane and contributes to an environment favorable to viral fixation in the cell[30].

The major capsid protein VP1 is a protein that coordinates the penetration of protoparvoviruses in the host cell by an endocytosis process and enables the destruction of the endocytic vesicle inside the cell and further liberation of viral proteins in the cytoplasm. Moreover, VP1 has nuclear localization signals responsible for assisting the viral protein displacement to the cell nucleus[31]. From this point, the virus can remain inert until the beginning of the cellular division process when during the S/G2 phases through protein NS1 action, it can block the cell genome replication and allow the integration of viral material with the host genetic material to ensure the viral survival[31].

H-1PV can produce an oxidative stress state through the increase in levels of reactive oxygen and nitrogen species through NS1 protein action inside the cell. NS1 is also related to the regulation of RNA viral replication, leading to the destruction of genetic material and activation of apoptosis pathways with later cell death. Furthermore, the virus can stimulate the liberation of proteases from the lysosome to the cytoplasm causing cellular necrosis of tumor cells[17].

In addition, the protoparvoviruses are capable of triggering an inflammatory response with antitumor characteristics generating the production of cytokines with a Th1 profile like IL-2 and TNF-alpha, which[32] sets an inflammatory environment able to deal with the tumor cells. H-1PV also contributes to the stimulation of T lymphocytes like TCD8, cytotoxic cells, and the auxiliary cells TCD4 and formation of an immune memory against the tumor[33].

During the lytic phase, the viral action enables the increase of membrane permeability of lysosomes that allows the passage of the cathepsins enzymes to the cytoplasm and decreases the action of inhibitory agents of these proteases. Both factors play an important role in the gathering of cathepsins in the cellular cytoplasm, stimulation of their action, and contribution to the apoptosis pathways and to tumor cell death[34]. Moreover, the expression of NS1 contributes to cellular apoptosis

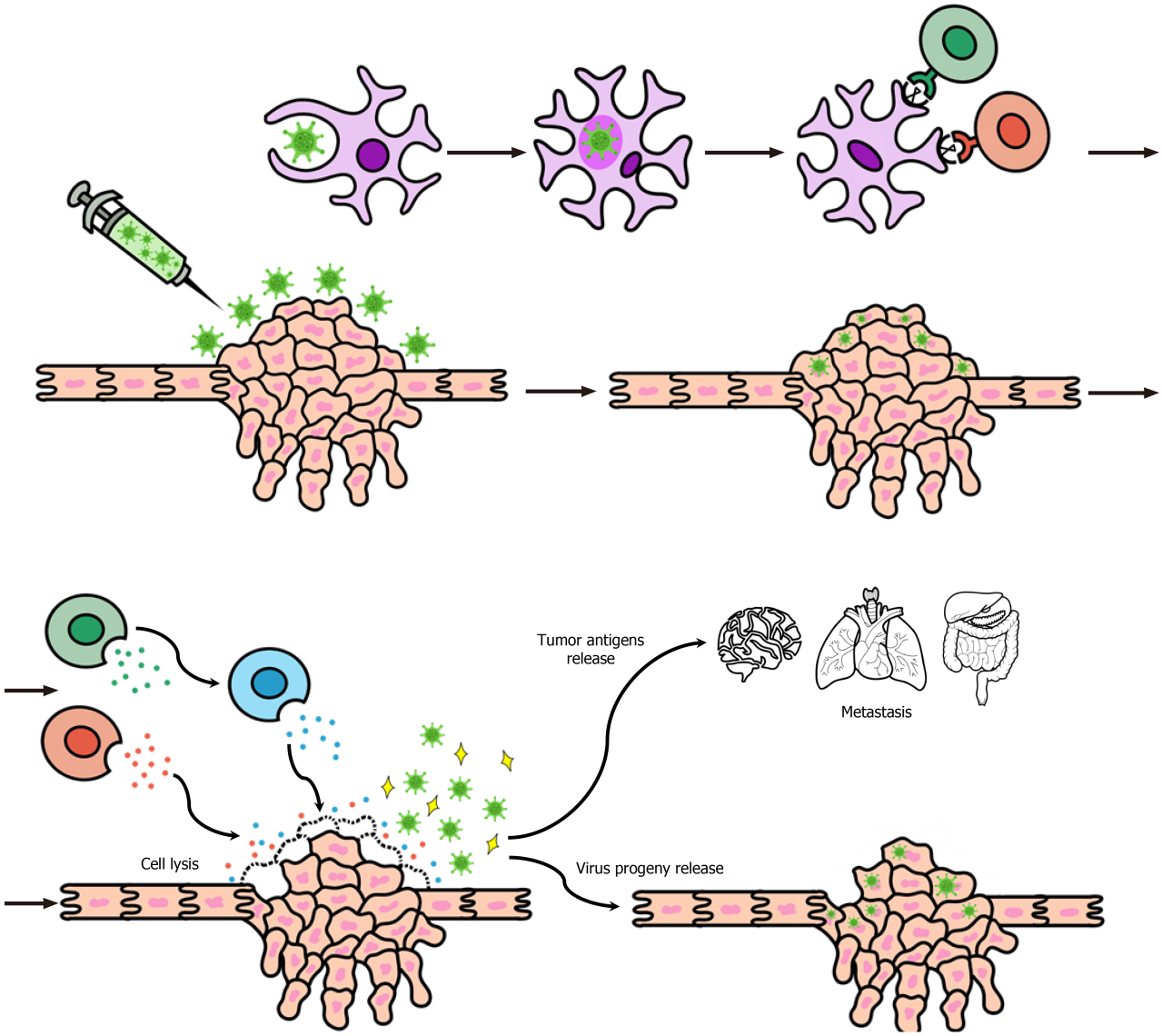


**Figure 1 Mechanism of action of oncolytic viruses.** Initially, oncolytic viruses can be administered by different pathways, such as intratumoral, subcutaneous, intraperitoneal, and intrathecal. Natural tropism and genetic targeting are responsible for favoring the arrival of oncolytic viruses to the tumor cells. Thereafter, the oncolytic viruses start to recognize the abnormal cells through substances expressed in the tumor environment and can use different receptors to connect and infect the host cell. From this point, the virus starts to use the cellular machinery for its replication process, producing viral proteins, reducing the cell function, stimulating oxidative stress states and contributing to the activation of some pathways related to the autophagic processes. At the same time, the antigen-presenting cells encompass some viral organisms, generating the formation of an endosomal vesicle that will merge with a lysosomal vesicle and will cause the digestion of the virus, providing smaller viral particles to be processed inside the cell. Later, the expression of the major histocompatibility complex class 2 together with the viral proteins on the cell surface occurs, creating a favorable environment for the antigenic presentation and subsequent activation and stimulation of the CD4+ T cells and CD8+ T cells, the first related to the production of cytokines responsible for contributing to the migration and maturation processes of inflammatory cells, and the second related to the direct action against the infected cells. Finally, the viral action and the immune response contribute to the destruction of the tumor cells releasing the viral progeny in the host organism allowing it to infect other abnormal cells and restart the process of combatting the tumor. Furthermore, cell death also releases tumor antigens that the immune system can identify, contributing to the formation of new inflammatory responses capable of acting both in the tumor environment and even in metastatic sites.

through damage to the genetic material, activation and stimulation of caspase action, and the generation of oxidative stress processes, bypassing the apoptotic evasion mechanism of the tumor cells[35].

**Vaccinia virus**: The vaccinia viruses (VACVs) are enveloped viruses with double-stranded linear DNA and belong to the *Poxviridae* family. They were used for smallpox vaccination in 1796, and currently after the eradication of this disease, their scientific use is aimed at the creation of vaccines and therapies for other pathologies[36]. One of the members of this family is the Pexa-Vec (pexastimogene devacirepvec, JX-594), which is genetically modified to possess the granulocyte-macrophage colony-stimulating factor (GM-CSF) along with thymidine kinase (*TK*) gene deletion in order

to increase the tropism to the tumor cells and limit the replication to the cells that express aberrant levels of TK[37].

The administration of VACVs in the tumor environment was related to the stimulation and expression of GM-CSF and IL-24, factors that together could contribute to stabilize and provide tumor cell death. GM-CSF is related to the maturation and differentiation of immune system cells like dendritic cells and neutrophils, which create an inflammatory environment that enables the combat of the tumor, and IL-24 inhibits tumor angiogenesis, positively influencing the apoptosis pathways and the formation of an antitumor response while inhibiting the formation of tumor metastases[38].

The viral action of some VACVs strains stimulate different cell death pathways such as necrosis and apoptosis, leading to the liberation of substances related to damage and danger, like ATP and high mobility group box 1 protein, that provides an immunogenic environment. Thereafter, the DAMPs support the cross-presentation between them and the tumor antigens, stimulating the TCD8 cell action and contributing to the stimulation of the antitumor response[39]. Furthermore, the Pexa-Vec has a tropism for endothelial cells that are responsible for tumor growth through the expression of vascular endothelial growth factor or fibroblast growth factor. It leads to the destruction of vasculature that irrigates the tumor and consequently a tissue necrosis process and decreasing of the tumor extension[40]. Some genetic modifications in the VACVs and updates in oncolytic therapy are listed in Table 2.

**Reovirus**: Respiratory enteric orphan virus (Reovirus) is a non-enveloped and double-stranded RNA virus that belongs to the *Reoviridae* family, which has a wide range of hosts (fungi, plants, fish, mammals, among others)[41,42]. This name is due to the isolation of the pathogen in the respiratory and gastrointestinal tract and the inability to cause any known human diseases[43,44]. Interestingly, this last characteristic is strongly correlated to the successful use of reoviruses in oncolytic therapy as well. The primary connection of reoviruses to an oncolytic role was found in 1977 when a study demonstrated that they have a tropism for "transformed cells" and that normal cells are resistant to the virus[45]. This information led, consequently, to further studies in order to evaluate the possibility of reoviruses as an alternative for cancer treatment.

There are three different reovirus serotypes: type one Lang, type two Jones, and type three Abney and Dearing[44]. Among them, the T3D is the most widely studied as a possible therapeutic for cancer treatment and is also known as Reolysin[46]. Furthermore, reoviruses are dependent on a mutation in the *ras* gene in order to replicate properly in the tumor cells[47], a fact that limits its use, given that only approximately 30% of the human tumors have these mutations. However, the Ras pathway can be activated by some elements, which means that more types of cancer can be subjected to viral oncolytic therapy by reoviruses (up to 80%)[48].

Regarding the mechanism in which reoviruses replicate in tumor cells, the Ras pathway plays an important part, given that it inhibits protein kinase R and therefore enables viral protein synthesis[49]. Moreover, studies also show that the epidermal growth factor receptor, more specifically the tyrosine protein kinase signaling pathways, increases reovirus infection and viral peptide synthesis[50]. In addition, reovirus-resistant NIH 3T3 cells capable of being infected and enhance protein production when transfected with the gene encoding epidermal growth factor receptor or with the v-erbB oncogene are also documented[51]. Thereby, these works on reoviruses clarified their possible use in oncolytic therapy, given that they are also non-pathogenic in humans, which makes it an attractive option.

The main mechanism of tumor lysis by reoviruses is virus-induced apoptosis, along with the immunomodulatory characteristics of the virus. The viral capsid proteins are able to activate an apoptotic pathway in the tumor cells through release into the cytosol of cytochrome *c* and smac/DIABLO from the mitochondria[52]. In regard to the immune response, once the reoviruses start protein synthesis, there is a secretion of proinflammatory cytokines and chemokines through PAMPs and DAMPs, which eases the generation of an adaptive antitumor immune response[15,53]. Then, cytotoxic TCD8 cells recognize the reovirus antigens and lyse the cells, along with a maturation of dendritic cells[54], consequent activation of natural killer cells, and further cytotoxicity[55].

**Herpes simplex virus type I**: The herpes simplex virus-1 (HSV-1) is a double-stranded DNA virus with a large genome of 150kb encoding for 70 or more genes that belongs to the alpha-herpesviruses subfamily[56,57]. Its large genome is very important, given that it can be easily modified in order to improve oncolytic properties and safety for the patient[56]. Unlike the reoviruses, HSV-1 is pathogenic to humans and can cause

**Table 2 Genetic modifications in the vaccinia virus**

| Ref. | Virus | Updates | Aim |
|---|---|---|---|
| Parato *et al* [226] | JX-594 | Express *GM-CSF* and *lacZ* transgenes | Increase lytic activity and antitumor immunity |
| John *et al* [227] | vvDD-GFP | Insertion of an Ab specific for the costimulatory molecule 4-1BB | Increase antitumor responses with myeloid cells, greater infiltration of CD8+ effector T and NK cells |
| Zhang *et al* [228] | GLV-1 h68 | Insertion of three expression cassettes into the A56R, F14.5L, and J2R | Increased tumor targeting specificity and reduced toxicity |
| Yoo *et al*[229] | CVV | Deletion of viral thymidine kinase genes | Regression of liver tumorigenicity and metastasis to the colon |
| Ricordel *et al* [230] | deVV5 | TK-deleted chimeric VV armed with the suicide gene *FCU1* | Union of different VV strains, with increased oncolytic properties, with more efficient replication in human tumor cells |
| Ge *et al*[231] | vvDD-IL-12 | Oncolytic VV delivering tethered IL-12 | Increase tumor infiltration of activated CD4+ and CD8+ T cells, decrease the transforming growth factor β and increase interferon γ |
| Deng *et al* [232] | VG9 | The oncolytic potency of VG9 was evaluated in various cell lines | Evaluate replication and cytotoxicity in vitro, antitumor effects and process of biodistribution of VG9 in a B16 tumor model |

Ab: Agonist antibody; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IL-12: Interleukin 12; NK: Natural killer; TK: Thymidine kinase; VV: Vaccinia virus.

infections of the mucosa or skin and central nervous infections, which reveals the need of deletions and insertions of additional transgenes in order to produce a viable oncolytic virus therapy[58].

In that context, a large number of oncolytic HSVs-1 have been developed and tested, with good outcomes, and among them the Talimogene Laherparepvec (T-VEC) is approved by the Food and Drug Administration[59,60]. T-VEC is one of the most studied HSV-1 oncolytic virus; it is created through deletion of γ34.5 and ICP47 and insertion of GM-CSF to inactivate neurovirulence factors and enhance the virus replication and immunogenicity[61,62]. It was also found possible to link HSV-1 to the *ras* signaling pathway in order to provide viral replication[63].

The mechanism of action of these viruses, especially T-VEC, is dual. The first aim is to perform direct tumor cell killing in which the viruses are able to enter the tumor environment, normally by local injection, and then start replication and consequent lysis of the infected tumor cell, release of tumor antigens, and local immune response [64]. In addition, the GM-CSF expression enables an accurate migration and maturation of dendritic cells to the environment and further antigen presentation to CD4+ and CD8+, which are capable of reaching distant metastases[65,66]. Studies also demonstrate that interferon response increases PD-L1 expression, and consequent T cell infiltration in the tumor environment is also possible[66,67]. Table 3 lists some genetic modifications in HSV-1 and impacts in the oncolytic action.

## CLINICAL USES

### *Pancreatic cancer*

Worldwide, the occurrence of pancreatic cancer is low, and the disease is not recommended for screening by the World Health Organization[68]. The survival rate of pancreatic ductal adenocarcinoma, responsible for 95% of pancreatic cancers[69], is 6% in 5 years[70], and the only potential cure for pancreatic ductal adenocarcinoma (duodenopancreatectomy) does not offer a big change in mortality[69].

Reolysin® (Oncolytics Biotech Inc., Calgary, AB, Canada) is the name of a reovirus that is in a Phase II clinical trial in pancreatic cancer[71]. The studies are not yet conclusive. However, intraperitoneal administration of reovirus has been shown to be effective and safe in the control of peritoneal metastases in hamsters with pancreatic ductal adenocarcinoma carcinomatosis[72].

Measles viruses depend on overexpression of CD46, a viral entry receptor also found in many cancer cells[73]. In a previous study, a modified measles virus showed oncolytic activity in pancreatic tumor xenografts in mice with tumor regression and increased survival[74]. In another study, the virus was modified to target prostate stem cell antigen, which is a protein expressed in pancreatic cancer and was armed

**Table 3 Genetic modifications in the herpes simplex virus-1**

| Ref. | Virus | Updates | Aim |
|---|---|---|---|
| Liu *et al*[61] | T-VEC | Insertion of GM-CSF and deletion of γ34.5, US12 | Increase lytic activity and antitumor immunity |
| Ushijima *et al* [233] | HF10 | Insertion of UL53, UL54 and deletionof UL43, UL49.5, UL55, UL56, LAT | Reduce neurovirulence and increase immunogenicity |
| Ebright *et al* [234] | NV1020 | Incorporation of the HSV-1 TK gene and deletion of α0, α4, γ34.5, UL56, UL24 | Reduce neurovirulence and provide susceptibility to antiviral chemotherapy |
| MacKie *et al* [235] | HSV 1716 | Incorporation of γ34.5 | Reduce neurovirulence |
| Mineta *et al* [236] | G207 | Insertion of lacZ and deletion of γ34.5 | Avoid ribonucleotide reductase encoding and reduce neurovirulence |

GM-CSF: Granulocyte-macrophage colony-stimulating factor; HSV-1: Herpes simplex virus 1; LAT: Latency-associated transcript; T-VEC: Talimogene laherparepvec; TK: Thymidine kinase.

with the drug purine nucleoside phosphorylase. The authors concluded that viral therapy demonstrated antitumor activity in immunocompromised mice[75].

A study using H-1PV, a parvovirus, associated with gemcitabine in mice showed a reduction in tumor growth, in addition to increased survival and absence of metastases in imaging studies[76]. In another previous study using parvovirus, the infection increased natural killer-mediated cell death in pancreatic ductal adenocarcinoma[77]. However, many studies still need to be done to obtain a conclusive answer since current studies only suggest the viral oncolytic action of parvoviruses[76]. However, the myxoma virus demonstrated *in vitro* lysis of pancreatic ductal adenocarcinoma cells[78] and prolonged the survival of mice, especially when the therapy was combined with gemcitabine[79].

Adenoviruses are the main viral vectors used to treat cancer, as they are able to bind to a target cell receptor with great affinity[80]. This great affinity is due to the possibility of building the ideal selectivity using two techniques: excluding viral genes necessary for replication in normal cells and introducing fundamental proteins accompanied by specific tumor promoters[81]. In preclinical tests, ONYX-15, an adenovirus, had a deletion mutation of the E1B gene and showed increased survival and antitumor efficacy in murine animals[82], in addition to showing viability and tolerability when combined with gemcitabine. However, its development was interrupted due to its limited clinical activity[83]. The LOAd703 virus, a parvovirus with the deleted E1A gene, has shown that it can change the tumor microenvironment from immunosuppressive to immunocompetent[84]. Tests have also shown its ability to elicit immune responses by releasing tumor-associated antigens while positively regulating favorable chemokines as well as dendritic cells[85].

HSVs are recognized for infecting and killing tumor cells quickly[86]. In addition, HSV has exhibited strong tumor reactivity mediated by T cells, indirectly causing an immune response to cancer[87]. In 1999, preclinical data showed that G207, an HSV-1 virus with gene deletions and inactivations, lysed pancreatic ductal adenocarcinoma cells *in vitro*[88] and induced complete tumor eradication by 25% when injected into mice xenograft tumors[89]. L1BR1, an HSV-2 with deletion of the US3 gene, replicated in pancreatic ductal adenocarcinoma cells and induced apoptosis cytolysis, especially when combined with 5-fluorouracil and cisplatin[90]. In a phase I study, HF10, a natural HSV-1 mutant, was injected into pancreatic tumors in 6 patients. Biopsies revealed a greater number of infiltrating CD4+ and CD8+ lymphocytes. In addition, an objective response was observed in 1 patient, while disease stabilized in 3 patients, and in the remaining 2 cases there was disease progression[91]. Finally, two phase I trials were performed to test the safety of the intratumoral injection of T-VEC (OV HSV-1 with multiple deletions) and Orien X010 (OV hGM-CSF HSV-1 recombinants) in advanced pancreatic cancer patients[92-94]. However, unfortunately, the results have not yet been reported to the scientific community.

### Melanoma

Melanoma is a potentially fatal malignant skin disease that continues to have greater incidences in the world, while the scenario of other tumors is the opposite[95]. The average risk of melanoma is 1 in 50 in several western countries[96] and is more frequent in light-skinned populations[97].

Regarding OV therapy, the vaccinia virus is a prototypical poxvirus with high clinical relevance, which can be easily attenuated by deleting virulence genes and inserting therapeutic genes[98]. Two phase I studies using JX-594, an OV vaccinia modified to activate local macrophages and dendritic cells[99], involved a total of 17 patients with unresectable cutaneous melanoma. The studies concluded that JX-594 replicated successfully in the tumor microenvironment, led to local oncolysis, and that increasing doses of JX-594 were safe and effective[100,101]. In two other similar phase I clinical trials, they used the vaccinia virus, which encodes B7.1 T cell co-stimulating molecules[102], in 25 patients with unresectable melanoma. As a result of these tests, the rate of complete objective response was 20% with limited toxicity and low-grade reactions[102,103].

The herpes simplex virus is an attractive option for OV in melanoma since the large genome has several non-essential genes that can be deleted in order to reduce pathogenicity and insert genes of interest[104]. Currently, T-VEC is the first oncolytic virus approved by the United States Food and Drug Administration for melanoma cancer therapy[105]. Phase I, II, and III clinical trials were concluded with positive results from the use of T-VEC in the treatment of melanoma[106-108]. Biopsies of injected lesions were performed in phase I and showed significant tumor necrosis caused by T-VEC[107]. In phase II, the overall objective response rate was 26% with a 1 year survival rate for all patients of 58% and mild side effects in 85% of patients[107]. Finally, in phase III, the objective response rate for the T-VEC arm remained at 26% with 11% complete responses, but unfortunately the final survival data are not available[108]. Even so, this was the first randomized clinical trial to reveal beneficial therapeutic use of OV for patients with advanced or unresectable melanoma[104].

HF10, a spontaneously mutated strain of HSV-1 with a deletion mutation in some viral genes[109], was used in an *in vitro* study that revealed that murine and human melanoma tumor cells had relevant cytolytic effects after HF10 infection[110]. In that same study, immunocompetent mice with advanced melanomas received HF10 intratumorally. Tumor growth was reduced in injected and non-injected tumors, which suggests direct oncolysis and induction of a systemic antitumor immune reaction [110]. HF10 was associated with dacarbazine to assess the oncolytic efficacy of the virus in mice prepared with subcutaneous melanoma models. The combined treatment of dacarbazine with HF10 showed a very fast and strong cytotoxic effect compared to monotherapy since a robust systemic antitumor immune response was induced and prolonged survival[111].

Other viruses with fewer highlights have been tested and have shown good results. Coxsackievirus A21 demonstrated in preclinical studies oncolytic activity in melanoma cells, maintaining tolerability and low viral pathogenicity[112]. CVA21, a commercial version of coxsackievirus A21, was studied clinically in phase I and II in patients with advanced and unresectable melanoma who received the virus intratumorally for 15 wk. As a result of these trials, the treatment was generally well tolerated with low-grade reactions, being able to observe complete therapeutic responses and an acceptable safety profile[113,114]. Finally, a phase II trial evaluated the oncolytic action of Reolysin® in 21 patients with metastatic melanoma who received intravenous injections[71]. All patients tolerated the injections well, and in 2 patients viral replication was evident when evaluating post-treatment biopsy samples from 13 patients. However, the study did not obtain observed objective responses nor did it achieve its primary efficacy objective, although the trial data support the use of reovirus in combination with other therapies to treat malignant melanoma[71].

### Breast cancer

Breast cancer (BC) is a multifactorial and heterogeneous disease in which the interaction between family history, lifestyle, and hormonal components has a fundamental role in its development[115,116]. Worldwide, the numbers of the disease are increasing, partly due to the increase in life expectancy of the population but also associated with the increase in early diagnosis techniques. Currently, 1 in 8 women have a chance of being diagnosed with BC in the world, making it the most common cancer among women[117].

There are prospects for treatment of more advanced forms of the disease since to date oncolytic virotherapy has demonstrated a wide variety of options for action at the cellular and molecular level[118]. Among the options currently most sought for this purpose, there are double-stranded DNA viruses that replicate and transcribe in the cell nucleus, without the integration of its genetic material with that of the host cell [118]. In addition, it is essential that OVs are extremely selective to replicate in cancer cells[15], a fact corroborated by tests that show the good tolerability and selectivity of genetically modified viruses for this purpose, such as the vaccinia virus[119]. Another

important OV, adenovirus, one of the most studied for BC, is still controversial. Preclinical studies show efficacy in tumor reduction by inhibiting the growth of its cells in addition to controlling metastases in mice[118]; however, other phase I trials demonstrate low efficacy for BC either in monotherapy or in combination with other drugs[119]. In addition to these, T-VEC approved in the United States and Europe for use in some types of melanomas[120] has been clinically tested in BC and shows good tolerability by the patient as well as relative success in inducing tumor necrosis and immune response[119,121].

RNA viruses such as Pelareorep (Reolysin) have also been studied for BC[119]. Although inconclusive, the trials show that there is safety in its use, in addition to an efficiency in viral replication and in its induction of cell death[122]; however, they suggest that the administration of Pelareorep in combination with the drug paclitaxel is more effective when compared to its isolated use[123]. An important point of this virus is its optimized form of intravenous administration, which favors its development even more and extends its use when compared to most of the OVs that are still administered in clinical trials by intratumoral route[119]. Also very promising against BC is the marabá virus, a strain of rabdovirus. Its MG1 variant was developed to have a greater oncolytic action and also little replicative action in normal cells, achieving success in these objectives[118]. As for tumor control, trials have shown an important association of positive results in the use of MG1 for the prevention of metastasis in the preoperative period[124] as well as in the safety of its use and the possibility of having a good systemic efficiency[125].

### *Liver cancer*

A highly malignant tumor type, liver cancer is still a major challenge to current medicine[126]. Its most common form is hepatocellular carcinoma (HCC)[126,127], which represents one of the six most prevalent and four most lethal types of cancer in the world[128-131]. Linked to this, HCC is attributed to an increase over the years [128], related to a high worldwide prevalence, concentrated mainly in underdeveloped countries[130]. The unfavorable numbers corroborate to a high rate of disease recurrence after conventional therapies currently used, with just over 10% of patients surviving after 5 years[129].

The literature shows OVs as promising in the possibility of overcoming HCC, especially in more advanced stages, in a safe manner and with the least possible chance of recurrence[129,131]. One of the most widely used is adenovirus, which shares a relevant tropism for liver cells[128]. Among this type of virus, there are several lines of studies with particular modifications aiming at a better viral adaptation to the obstacles found in tumor cells. One of them is the Ad5 viral vector integrated with the GP73 and SphK1-shRNA promoters[130], in which through preclinical tests it was able to induce cell apoptosis and inhibit tumor expansion considerably, improving the survival of mice[131]. The adenovirus ZD55 vector was modified to overcome the high resistance of HCC cells to tumor necrosis factor-related apoptosis ligand and successfully managed to reduce the tumor size by associating ZD55-tumor necrosis factor-related apoptosis ligand with ZD55-Smac, a variant that has a second mitochondrial caspase activator in its constitution[128].

The vaccinia virus has also been studied for HCC. The JX-594 variant has been proven safe and effective through preclinical studies in rabbits by eradicating lung metastases and liver tumors in these animals[126,128]. In addition to this, the vaccinia virus may also be associated with cytokines, such as recombinant VV-IL-37, which with interleukin 37 associated with its genome also inhibited liver tumor growth[130]. Among the therapeutic options, it is also worth highlighting the findings in trials using HSV. A study using mice developed Ld0-GFP, a more selective and more oncolytic vector for liver cells, which has safely demonstrated an important potential in the induction of cell apoptosis and in the release of DAMPs related to immunogenic cell death[129].

### *Glioblastoma*

Glioblastoma is the most common malignant primary brain tumor in adults, with a median age of approximately 55 to 60 years and has a 10% survival rate after 5 years [6], even with important advances in recent years in cancer therapy. Thus, oncolytic therapy has been highlighted in the treatment of glioblastoma, once it kills tumor cells *via* direct oncolysis and *via* stimulation of antitumor immune response[132].

Regarding the use of OVs, studies have shown its use with combined therapy and monotherapy. A research conducted at clinicaltrials.gov, Martikainen *et al*[133] found more than fifteen clinical studies at different stages. A phase II study, using the modified DNX2440 adenovirus, combining oncolytic virus with tumor-targeting

immune checkpoint modulators, demonstrated that the virus was able to specifically increase T cell activation, facilitating tumor recognition. In other studies, HSV (phase I), vaccinia virus (phase I/II), poliovirus (phase I/Ib), parvovirus H-1PV (phase I/II), and unmodified human reovirus were also used[134-137]. The study using attenuated (Sabin) poliovirus with internal ribosomal entry site from human rhinovirus 2 was applied to 61 patients over a period of 5 years with the result of increasing their patients' survival rate by 24 and 36 mo compared with the rate among historical controls. On the other hand, the study with unmodified rat parvovirus indicated that H-1PV treatment was safe and well tolerated. It showed favorable pharmacokinetics, induced antibody formation in a dose-dependent manner, and triggered specific T cell responses. There was an increase in survival compared to recent studies. Furthermore, researchers who used unmodified human reovirus reported that 10 of the 12 patients had tumor progression and 1 had stabilized, while the median survival was 21 wk. Finally, the preclinical study involving HSV-1 and rats used the modern approach of viral redirection with IL-12, resulting in increased overall survival and complete tumor elimination in 30% of the animals.

### Prostate

Prostate cancer is the most common cancer among men and the second type of cancer that kills men the most in Western countries[138]. In view of the therapies currently available, the OVs are an attractive way of treating prostate cancer, either as monotherapy or in combination with other immunotherapies (for example, anti-programmed cell death protein 1 and anti-PD-L1 inhibitors)[139]. This is due to the immunological events induced by the administration of OVs in cancer-bearing animals that bring down multiple tumor immune evasion mechanisms and induce strong, multiclonal, and protective anti-prostate cancer immunity. The effect of OVs on prostate cancer occurs because of abnormalities in antiviral defense pathways, including those attributed to impaired tyrosine-protein kinase Janus kinase, a signal transducer and activator of transcription signaling.

To date, there are several clinical trials in phase I and II using adenovirus, reovirus, HSV-1, vaccinia virus, fowl pox virus, and Sendai virus[140]. Among the studies with adenovirus, one was able to insert mk5 (the mutational kringle5 of human plasminogen) into a DD3-promoted (differential display code 3) oncolytic adenovirus, showing that mK5 has been proven to be able to inhibit the tumor angiogenesis and inhibit cell proliferation[141]. Currently, a number of Ad5-CD/TK OVs have been developed and tested as a therapeutic for prostate cancer. These viruses provide two suicide genes, cytosine deaminase and HSV-1 TK, to tumor cells. Studies using a reovirus in patients with metastatic castration-resistant prostate cancer, on the other hand, showed an increase in the secretion of inflammatory cytokines[138].

### Colorectal cancer

Colorectal cancer is the third most common cancer in the United States and the second leading cause of cancer-associated mortality[142]. There is currently no effective treatment for this type of cancer, so OVs can be an interesting option in this way. Heavily pretreated colorectal cancer patients were treated with the oncolytic vaccinia virus alone or combined, by increasing the expression of GM-CSF (a hematopoietic growth factor) and reached stable disease in 67% of patients[143,144]. Another study using oncolytic HSV2 performed an *in vitro* and *in vivo* analysis. In the first, oncolytic HSV2 effectively inhibited the growth of CT-26 cells. In the second, hepatic metastasis was reduced in mice models with xenograft tumor[145].

## FUTURE CHALLENGES AND PERSPECTIVES

A wide variety of OVs are going through studies in phase I/II clinical trials or in preclinical cancer models[2,146]. According to clinicaltrials.gov, there are currently 114 clinical trials listed at the time of this writing showing considerable progress in this field. Despite all the advances, some limitations still have to be surpassed to enhance OV-based immunotherapy[37,119,147]. Thus, to overcome these challenges, research scientists are creating new strategies, which will be presented below.

### Choosing the optimal OV species

As aforementioned, a range of virus species has been developed as OVs recently. It is essential to comprehend the exclusive biological aspects to establish the most relevant antitumor oncolytic virotherapy, considering that distinct kinds of viruses have

different sizes, genetic materials, shapes, and pathogenicity[148]. First, the size of the virus must be considered; larger viruses are more suitable for the therapeutic gene insertion, but they are less inclined to infiltrate the physical barriers, whereas smaller viruses can penetrate and spread throughout the tumor more easily, though they are not as susceptible for genetic administration[148]. In addition, the viral genome is important; RNA viruses replicate faster than DNA viruses and are able to kill tumor cells because they do it in the cytoplasm and do not have to reach the nuclei of the target cells[149]. Nevertheless, they have shown fewer tumor-selective properties due to the same reason[150]. Likewise, the existence of a viral capsid is also a crucial factor in OV selection because enveloped viruses are less oncolytic and are more likely to be eliminated by the host immune system[149].

Therefore, during the past decade, some improvements have emerged in the area, such as capsid development, genome engineering, and chemical modifications[151]. The capsid can be altered to improve the binding between the virus and the entry receptors from the target cell. For example, researchers have noticed that genetically inserting protein domains or peptides into the viral capsid can benefit transduction efficacy in some cells and improve the attachment of the OVs to target tumor cells membranes, boosting viral tropism, and internalization[151-153]. Furthermore, viral cytotoxicity needs to be considered since the high capacity to generate cell injuries can decrease viral replication rates and consequently interfere in the effectiveness of therapy[154]. Meanwhile, all of those strategies still have limitations and need to be improved.

### *Effective delivery methods*

Finding an ideal route for OV administration still constitutes one of the major challenging issues in virotherapy[60]. The two leading delivery platforms include local intratumoral, which the OVs are injected directly into the tumor site, and systemic method (intravenous or intraperitoneal)[4,55]. Local intratumoral is the most common delivery route in preclinical or clinical trials due to its safety and to decrease the chance that preceding circulating antibodies might overcome the virus before it reaches its target[2,155,156]. However, this platform cannot be utilized for inaccessible or multifocal tumors, such as pancreatic or brain tumors, so it is not always a viable option[157]. On the other hand, the systemic injection is, theoretically, an ideal delivery method, because of the broad distribution of viruses, allowing the OVs to reach not only primary but also metastatic tumors, and it is relatively non-invasive and highly repeatable[155,157]. Nonetheless, its bioavailability and efficiency at the moment is unsatisfactory, and the viral particles in this route do not specifically target cancer because they can be rapidly sequestered and degraded by the host immune system before they reach the tumor[158].

In this way, several strategies have been studied to overcome these hurdles. For example, capsid modifications have been explored as a way to deliver OVs to tumor sites, like the changing of the viral envelope by polyethylene glycol polymers that prevent its recognition by macrophages[151,157,159]. Thus, considerable new approaches such as the use of nanoparticles, complex viral particle ligands, liposomes, polymeric particles, and immunomodulatory agents have been used and designed [160-163]. Another hopeful strategy is the utilization of ultrasound image guiding and magnetic drug-targeting systems[164-166]. These are all different kinds of approaches for improving the delivery methods.

### *Immune response*

The immune response is an obstacle capable of preventing the effectiveness of OVs, given that it can limit infection and viral replication, whether by the specific immunity from viral infections or by pre-existing immune memory[167,168]. There are many cases in which antiviral immunity already exists from previous infections or vaccinations since many of the OVs used in anticancer therapy are originally pathogenic to humans[159,169]. Besides that, the excessive administration of OVs can induce antiviral immunity that eliminates it more quickly than supposed[159]. The presence of coagulation factors FIX, FX, and complement protein C4BP and the large number of immune cells infiltrated into the cancerous stem cells impair selective viral replication as well[149,170].

To overcome such problems, new treatment strategies were developed and showed promising results as genetic manipulation of OVs, cytokines, nanoparticles, complex viral particles binders, immunomodulatory agents, use of decoy viruses for sequestering pre-existing antibodies, and multiple administration of different serotypes[120, 168]. However, it is relevant to emphasize that viral immunity can be beneficial in some cases by recruiting immune cells for tumor microenvironment (TME) and

reversing the immunosuppressive TME. Therefore, there must be an adjustment in the balance between OV-induced antitumor immunity and antiviral immunity[147,169,171].

### Physical barriers

Another major challenge that OVs need to overcome is physical barriers, as viruses must pass through the endothelial layer to reach target cells. Studies have identified several physical barriers that limit effectiveness, such as chemotherapeutic agents, monoclonal antibodies, antitumor immune cells, and genetic therapies[149,172,173]. Furthermore, abnormal lymphatic networks and epithelial cell tumors are protected by extracellular matrix, which results in interstitial pressure and may impair the ability of OVs to spread themselves throughout the tumor mass, negating its effectiveness[174,175].

Therefore, strategies to achieve efficient penetration and dissemination of OVs are highly necessary for significant improvements in this therapeutic modality[176]. To increase the viral spread, oncolytic adenovirus genetically modified to express molecules such as relaxin and hyaluronidase were generated in order to stop angiogenesis of the extracellular matrix and have shown promising preclinical results [174]. An intravenous administration of the OVs can bring numerous benefits for the vascularization of the tumor, being able to be superior to intratumoral injections[176]. Studies show efficiency in the spread of OVs in solid tumors through changes in the viral envelope or by increasing the diffuse transport of the virus through changes in the interstitial space[177]. These data provide strong evidence of the significant antitumor effects of the therapy.

### Clinical use of OVs allies to other therapies

Since OVs showed limited efficacy in monotherapy, the combination of immunotherapy drugs and virotherapy has become a potential direction and appealing choice [158,178]. In this way, some preclinical studies in animal models and early clinical trials have confirmed the therapeutic responses increased with combination approaches, showing considerable response rates and tolerable safety profiles[120,179]. The following sections discuss these diverse combination strategies.

### Combination with chemotherapy

The combination of virotherapy with chemotherapy agents is a promising approach. For example, adenovirus combined with chemotherapeutic agents such as cisplatin, 5-fluorouracil, doxorubicin, temozolomide, irinotecan, and paclitaxel has successful results and enhanced antitumor effects compared to the response rate of the virus alone[179-181]. Concomitantly, a combination strategy also showed less risks and higher safety, extending the patient's survival[182]. Likewise, vaccinia virus combined with paclitaxel also revealed a harmonious effect[183]. In some models, the combination of sorafenib and vaccinia virus demonstrated good antitumor results, while patient trials showed remarkable safety and clinical response, and it has been approved for use in kidney, liver, and thyroid cancers[184].

### Combination with radiotherapy

Radiotherapy combined with OVs has shown potential effects in cancer treatment[185-187]. Initially, the propitious result was observed in studies with oncolytic HSV[188-190]. In addition, the forceful combination effects can also be observed in radiotherapy and vaccinia virus. For example, a study reported that VACV-scAb-vascular endothelial growth factor was able to boost the radiation therapy's sensitivity of tumor locations, increasing the antitumor response[191].

### Combination therapy with adoptive cell therapy

Another promising strategy is the combination of OVs and adoptive cell therapy since OVs can kill cancer cells specifically and have the potential of turning the TME into an immunostimulatory environment that is susceptible to T cell entry and activation [192]. A recombinant oncolytic adenovirus, OAd-TNF-a-IL-2 combined with meso-chimeric antigen receptor T cells in an animal model of pancreatic ductal adenocarcinoma caused considerably better tumor regression and expanded the antitumor effectiveness of chimeric antigen receptor T cells[193]. Furthermore, a preclinical trial of this combination approach utilizing GD2-chimeric antigen receptor T cells and a recombinant oncolytic adenovirus in a mouse model revealed substantial elevated overall survival of mice as with both monotherapy ways[194]

### *Combination therapy with OVs and immune checkpoint inhibitors*

One of the most common strategies to increase the effectiveness of OVs is to combine them with ICIs as the combination of the two therapies relieves the tumor immunosuppressive environment. The infection caused by OVs triggers an anticancer immune response, increasing the effectiveness of ICIs, which in the process interrupt the ligand-receptor interaction of cancer cells exposing T cells to attack[169,194,195]. In short, the objective of this combination is to make the local microenvironment more conducive to the proper functioning of ICIs through infections caused by OVs[195-197]. This synergistic relationship has led to the development of several studies with promising results.

For example, a phase II study (clinicalTrials.gov: NCT02978625) studied how biological therapy T-VEC and the immunotherapy with monoclonal antibodies nivolumab worked in 68 patients with lymphoma who have not responded to treatment or non-melanoma skin cancers that have spread to other parts of the body or have not responded to treatment. In addition, the combination of ICIs with various OVs, such as vaccinia virus, coxsackievirus, adenovirus, marabá virus, reovirus, and vesicular stomatitis virus, is being evaluated in different phase I or phase II clinical trials[167,198]. Thus, new treatment options through this combination continue to be awaited with expectations of promising paths.

### *Combination therapy with OVs and bispecific T cell engagers*

In recent decades, there has been great clinical progress in immunotherapy with bispecific antibodies and effective therapeutic applications[199]. By definition, bispecific T cell engagers (BiTEs) are proteins that, through DNA recombination, form bispecific antibodies with two variable fragments of single chain antibodies, one directed to a cell surface molecule in T cells (for example, CD3) and the other targeting antigens on the surface of malignant cells[172,200]. BiTE-mediated interaction triggers the formation of immune synapses, which ultimately result in tumor specific cell death and release of effector Th1 cytokines[201]. However, BiTEs have low penetration in solid tumors, in addition to the risk of toxicity in hematological cancers[172,200]. In this sense, the combination of BiTEs and OVs is considered in order to increase therapeutic efficacy since OVs are able to selectively replicate and infect malignant cells, thus alleviating the immunosuppressive state of the TME[172,201].

Currently, several BiTEs delivered by OVs have been tested on several types of hematological and solid tumors reported by preclinical research, and promising tests were obtained with a BiTE that recognizes fibroblasts associated with cancer (*via* fibroblast activation protein)[202]. In addition, preclinical studies also provided evidence of the effectiveness of OVs in combating the side effects of therapy with BiTEs through the redirection of T cells, in addition to improving antitumor activity [203]. Such efforts should lead to the development of new anticancer agents as it is believed that this combination is powerful to address unmet clinical needs[199].

### *Biosafety on oncolytic virotherapy*

Although OV therapy has shown potential to be a safe treatment for cancer patients, some biosafety issues *in vivo* still remain a concern as a treatment strategy. Primarily, some adverse events were associated with this therapy[14]. A few symptoms, such as mild flu-like syndromes[204,205], local reactions commonly manifested as pain, rash, peripheral edema, and erythema, are the most common events linked to the treatment [124,206]. Some of them disappeared without intervention after a few days or with the administration of nonsteroidal anti-inflammatory drugs during the treatment course[4, 207]. In addition, other common adverse events, like leukopenia, liver dysfunction, anemia, lymphopenia and more, were noticed in the trials of HSV, reovirus, and adenovirus[208,209]. Besides this, few OV therapies have caused severe adverse reactions that brought harm to patients' health[162,210-212], and they have been manageable and rarely caused a severe impact on the patients or threatened their lives [162,213]

Moreover, the transmission and shedding of OVs during the treatment is also a potential safety issue. During the therapy, viruses such as T-VEC, Ad5- Δ24-RGD, HSV, adenovirus, pox, and reovirus, can be transmitted to people in close contact with the patient, such as the family and health care staff who are more likely to be exposed to the patient's fluids, such as saliva or urine, or be shed to other parts of the patient's body[214-216]. Another challenge in the biosafety of the use of OVs is the application of the treatment in specific populations, given that the studies in this area are currently limited[14]. Therefore, in order to reduce risks, the viruses observed are highly attenuated, in addition to being of the utmost importance that the health professionals

who administer OVs carefully follow the safety standards for the procedures[215,216]. On the other hand, the trials and preclinical studies of several viruses, like the T-VEC, HSV-1, and H-1PV indicate that pregnant women and people with low immunity should avoid using them[214,217].

Lastly, aiming to improve the biosafety of oncolytic viral therapy and decrease its side effects, the use of viruses that do not present pathogenicity to humans are being evaluated. H-1PV, for example, demonstrated no inducement of the production of specific antibodies when inoculated in humans, which means little chance of generating an active infection. Nevertheless, the virus has shown specificity to the tumor cells[218]. Furthermore, the recombinant therapies between different OVs, such as adenovirus and parvovirus, have shown satisfactory results in terms of biosafety since the synergistic action generated from the viral specificities, such as the infectivity of adenoviruses to the tumor cells and the lack of harmfulness of parvovirus to the normal cells, contributes to greater therapeutic efficacy and reduction of collateral damage[14].

## CONCLUSION

OVs emerge as a way of bypassing the immune evasion mechanisms of the tumor, aiming to improve the clinical condition of patients through the stimulation of the host immune system or direct lysis of abnormal cells. The modern techniques of genetic engineering have made it possible to improve the construction of OVs, increasing the safety and the efficiency, targeting the virus to the tumor, and decreasing the adverse effects of their use. Furthermore, it is possible to observe significant effects of the clinical use of OVs, whether in single or combination therapy, to the treatment of tumors. Therefore, upgrading antitumor therapies and consequently improving patient prognosis with contributions from the areas of molecular biology, structural biology, immunology, genomics, and bioinformatics lays a solid foundation for future clinical success of OVs.

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*MINIREVIEWS*

# Ambisense polarity of genome RNA of orthomyxoviruses and coronaviruses

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## Abstract

Influenza viruses and coronaviruses have linear single-stranded RNA genomes with negative and positive sense polarities and genes encoded in viral genomes are expressed in these viruses as positive and negative genes, respectively. Here we consider a novel gene identified in viral genomes in opposite direction, as positive in influenza and negative in coronaviruses, suggesting an ambisense genome strategy for both virus families. Noteworthy, the identified novel genes colocolized in the same RNA regions of viral genomes, where the previously known opposite genes are encoded, a so-called ambisense stacking architecture of genes in virus genome. It seems likely, that ambisense gene stacking in influenza and coronavirus families significantly increases genetic potential and virus diversity to extend virus-host adaptation pathways in nature. These data imply that ambisense viruses may have a multivirion mechanism, like "a dark side of the Moon", allowing production of the heterogeneous population of virions expressed through positive and negative sense genome strategies.

**Key Words:** Virus genome; Ambisense RNA; Influenza; Coronavirus; Virus diversity; Virus genes



**Core Tip:** A novel genes identified in viral genomes in opposite direction, as positive in influenza and negative in coronaviruses, are considered. The identified novel genes colocolized in the same RNA regions of viral genomes, where the previously known opposite genes are encoded, a so-called ambisense stacking architecture of genes in virus genome. It seems likely, that ambisense gene stacking in influenza and coronavirus families significantly increases genetic potential and virus diversity to extend virus-host adaptation pathways in nature. These data imply that ambisense

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## INTRODUCTION

Orthomyxo- and coronaviruses are two families of enveloped viruses containing single stranded linear RNA genomes. Orthomyxovirus family includes seven genera: Alphainfluenzavirus, Betainfluenzavirus, Deltainfluenzavirus, Gammainfluenzavirus, Isavirus, Thogotovirus, and Quaranjavirus. These viruses infect wide range of hosts including mammals, birds, rodents, fish, ticks and mosquitoes. Orthomyxoviridae viruses contain six to eight segments of negative-sense single stranded RNA with a total genome length of 10-15 Kb[1]. Coronaviridae is divided into the four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus. Alpha- and betacoronaviruses infect mammals, while gamma- and deltacoronaviruses primarily infect birds. The size of genomic positive sense RNA of coronaviruses ranges from 26 to 32 kilobases, one of the largest genome among RNA viruses[2]. Here we mainly consider alphainfluenza viruses and betacoronaviruses as a typical members in both families.

## INFLUENZA A VIRUS AMBISENSE GENES

Genome of influenza A viruses is composed of 8 segments of single-stranded RNAs with mol. wt. 0.7-2.8 × $10^3$ kilobases/segment. Each segment encodes one or several unique polypeptides through the canonical negative sense genome strategy (Table 1). It means that genome RNA of negative sense polarity is transcribed by the virus polymerase to produce positive sense mRNAs, which recognized by ribosomes to translate individual viral proteins (Figure 1). In addition to the negative sense genes, influenza A virus genome segments were found to contain long open reading frames (ORFs, genes) in opposite positive sense orientation. These ORFs have all ribosome translation elements: canonical start codon AUG or noncanonical CUG, termination codons (UAG, UAA, or UGA), internal ribosome entry sites (IRES), and Kozak-like sequences at the initial start codon[3-9].

There are three groups of data showing *in vivo* expression potential of these negative stranded genes. (1) The template function of the full length "negative sense" genome RNA of segment 8 (NS) was demonstrated in a cell-free translation system of rabbit reticulocyte lysate. It was shown that influenza A virion RNA of segment 8 can initiate synthesis of major polypeptide negative stranded protein (NSP8) (mol.wt. 23 kD) specifically reacted with antibody to the central domain of the NSP8[10]; (2) The NSP8 encoded in the 8'th influenza A virus segment NS could be expressed in vivo, in insect cells (ovary cell line of *Trichplusia ni*) infected with recombinant baculovirus (insect nuclear polyhedrosis virus) carrying influenza virus sequence NSG8 in the virus DNA genome. This gene appeared to express ~20 kD influenza-specific polypeptide NSP8, which was intracellularly stable and accumulated in the perinuclear zone of infected cells[11]. Later, it was also supported that influenza A virus NSP8 could be efficiently expressed from either a plasmid or a recombinant vaccinia virus in mammalian cells and the synthetized NSP8 was localized in the perinuclear endoplasmic reticulum (ER) and post-ER cellular compartments[12]; and (3) There are data that mice infected with influenza virus produce CTL response specific to epitopes presented in the influenza NSP8 protein[12-14]. These findings also demonstrate that translation of sequences locating on the negative RNA strand of a single-stranded RNA genome of influenza A virus can develop *in vivo* and can initiate antiviral CTL response and immunosurveillance.

**Table 1 RNA segments of influenza A virus genome and encoded polypeptides**

| Viral RNA segments and their length (nt)[1] | Positive sense polypeptides (mol. wt., kDa)[2] | Negative stranded polypeptides, NSPs (mol. wt.; a.a.)[3] |
|---|---|---|
| PB1 (2341) | PB1 (86.6); PB1-N40 (89.4); PB1-F2 (10.5) | NSP1 (174, 239) |
| PB2 (2341) | PB2 (85.7); PB2-S1 (55) | NSP2 (116, 121, 130, 137) |
| PA (2223) | PA (84.2); PA-X (29); PA-N155 (62); PA-N182 (60) | NSP3 (95, 109) |
| HA (1778) | HA (61.5) | NSP4 (n.d.) |
| NP (1565) | NP (56.1); eNP (56.8) | NSP5 (117, 154) |
| NA (1413) | NA (50.1); NA43 (48.6) | NSP6 (91, 154) |
| M (1097) | M1 (27.8); M2 (11); M42 (13) | NSP7 (99, 102, 109) |
| NS (890) | NS1 (26.8); NEP (14.2); NS3 (21); tNS1 (17) | NSP8 (93, 167, 216) |

[1]RNA segments and nucleotide (nt) calculations were made for the A/PR8/34 (H1N1) virus.
[2]Canonical influenza A virus polypeptides synthesized through the negative genome strategy (Figure 1; for review see[1]).
[3]Negative stranded genomic open reading frames (ORFs) and predicted negative stranded proteins (NSPs) have been calculated for A/PR8/34 (H1N1) and A/Aichi/2/68 (H3N2) viruses[3-8]. Negative stranded ORFs were identified by in silico approach using the Open Reading Frame Finder program ( https://www.ncbi.nlm.nih.gov/orffinder/). These ORFs can be realized through the positive genome strategy. The amino acid length (a.a.) of NSPs were based on the data presented mainly in ref.[8]. A.a. values reflect variations among human, avian and other mammalian virus strains. N.d. means the absence of ORFs longer than 90 a.a. NSP: Negative stranded protein.

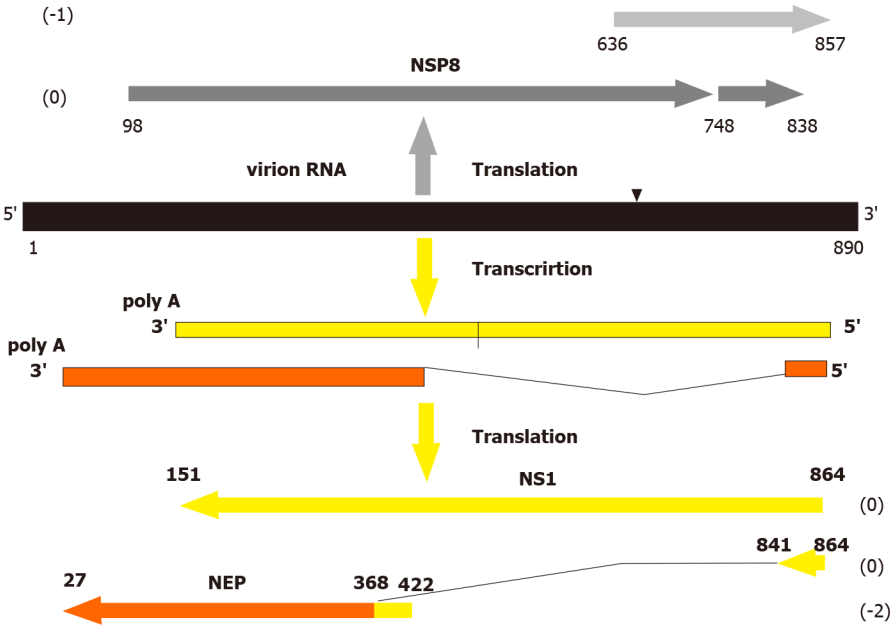


**Figure 1 The scheme of expression of the genome negative sense segment of influenza A virus.** The negative sense (NS) segment of influenza A/Aichi/2/68 (H3N2) virus is displayed. The horizontal arrows show the open reading frames (ORFs) of the negative strand protein 8, non-structural anti-interferon protein (NS1), and nuclear export protein (NEP). Numbers in brackets indicate the ORF translation phase. Numbers under the lines indicate nucleotide positions from the 5' end of the virion genome RNA. The broken line shows the splicing segment of the *NEP* gene mRNA. Triangle in the virion RNA molecule shows a site position of possible translation frameshifting[10]. NS: Negative sense; NSP8: Negative strand protein 8; NS1: non-structural anti-interferon protein.

The mature product of the NSP8 gene has not been yet identified in biological systems such virus-infected cells and animals. The failure to detect NEG8 protein could be due to a number of factors other than the complete absence of translation from genomic RNA. The properties of the NSP8 as an "escaping protein" may be explained either by its low synthesis and a short period of life or/and strong tissue-specific expression in certain cell types containing factors which are necessary for the regulation of expression of these "negative sense" genes. It would not be surprising if negative polarity genes are only expressed physiologically under special circumstances *in vivo* determining host cell tropism of influenza viruses.

## NOVEL NEGATIVE SENSE GENES IN THE RNA GENOME OF CORONAVIRUSES

Recently, similar ambisense polarity has been revealed in coronaviruses genomes[15]. It is well known that these viruses possesses a linear positive sense genome RNA of 25-29 × $10^3$ kb length[2]. The coronavirus genome RNA contains two groups of genes expressing proteins through the positive sense strategy. The first ones (nonstructural genes for nsp1-nsp19 proteins) are localized at the 5'-region of the virion genome RNA and directly translated by host ribosomes. The second ones (mostly the structural proteins genes N, S, HE, M, E and several accessorial proteins, such as 3a/b, 6, 7a/b, 8a/b, 9b, *etc.*) occupy a 3'-region of the virion RNA and express proteins through the translation of subgenomic mRNAs, which was transcribed on the anti-genomic RNA template[16] (Figure 2A). In addition to the positive sense genes, we have identified numerous long open reading frames in negative sense orientation (Table 2; Figure 2B). Like in the case of the ambisense genes of flu viruses, coronavirus negative sense genes have all elements characteristic of the mRNA molecules which are recognized by host ribosomes: classical AUG or alternative CUG[17] start codons, termination codons, IRES, and Kozak-like sequences at the start area[18,19]. However, unlike to influenza A viruses, coronavirus ambisense polarity has opposite configuration: a positive sense genome strategy and a negative sense orientation of the novel negative sense genes, so called a negative sense genes or negative gene proteins (NGPs).

The identification of coronavirus negative-polarity genes implies two possible mechanisms of their expression and synthesis of the corresponding mRNAs and proteins. These mechanisms include either direct translation of a replicative (-)copy of genomic (+)RNA (replication pathway II) or the transcription of genomic (+)RNA by viral polymerase with the formation of subgenomic mRNAs of "negative polarity" for their subsequent translation to synthesize specific viral polypeptides (transcription pathway I). To realize pathway I coronavirus genome contains poly A sequence (positions 11935-1194 nt) functioning as a viral polymerase binding site and transcription initiation signal (Figure 2B).

## BIOLOGICAL SIGNIFICANCE OF THE AMBISENSE GENES

The function and role of the newly discovered ambipolar viral genes have not yet been determined. In the case of influenza viruses, there are indirect data that the identified new ambisense genes can be involved in the regulation of the host immune response against viral proteins and/or in the regulation of the stability of viral proteins in infected cells through the protein deubiquitinating system[5,12]. The possible functional significance of the novel ambisense genes is not yet generally clear. However, the stability and retaining of these type of genes in field viruses genomes for more than 100 years at the high variability of virus population suggest the functional necessity of these genes and their biological evolutionary determination[20]. Notably, the influenza NSP8 has high synonymous/nonsynonymous (dN/dS) mutations rate (> 1.5), which was similar to that one for the most variable surface virus glycoproteins HA and NA representing major target for antiviral host adaptive immune response. The elevated variability of the NSP8 implies that it undergoes positive selection and host adaptation, which influence its evolution[5].

The discovery of new ambisense genes has raised a number of important questions regarding its origin, functions, and evolutionary variability. One of the essential questions is how the novel genes have emerged in the genomic region to encode two opposite sense genes. The appearance of the ambipolar gene suggests the existence of yet unknown correspondence principle (or reverse determination rule) for the expression of oppositely directing genes locating in the same region of RNA molecule. This principle implies that a certain pre-existing gene can predetermine the emergence mechanism and the properties of a new ambipolar gene[5]. Without this rule, chaotic accumulation of mutations will result in the appearance of a new functional gene and its further evolutionary selection, that seems to be unlikely. Moreover, the probability for such chaotic event is low, considering the ambipolar overlapping of several preexisting genes, when changes in one of them would cause changes in the coupled ambipolar genes. In this case, gene variability and selection of mutations should be interconnected in all opposite viral genes (in the case of influenza virus for NS1, NEP, and NSP8). These considerations incline to the assumption of the existence of a rule of reverse determination, when both ambipolar genes can have linked structural motives and functions. Further studies are necessary to clarify this idea.

**Table 2 Negative sense genes in genomes of coronaviruses**

| Virus genera | Viral genomes | Number of NSGs in virus genome[1,3] | M.W. range of the NGPs[2] |
|---|---|---|---|
| Alpha-coronaviruses | HCov-229E: https://www.ncbi.nlm.nih.gov/nuccore/NC_002645.1 | 29/1/29/5 | 12.4-14.4 |
| Beta-coronaviruses | SARS-CoV-1: https://www.ncbi.nlm.nih.gov/nuccore/NC_004718.3 | 34/0/35/2 | 11.5- 15.0 |
| | SARS-CoV-2: https://www.ncbi.nlm.nih.gov/nuccore/MT635445.1 | 21/1/26/4 | 10.9- 17.2 |
| | MERS: https://www.ncbi.nlm.nih.gov/nuccore/NC_019843.3 | 32/8/23/3 | 11.1- 18.6 |
| | Pangolin-CoV: https://www.ncbi.nlm.nih.gov/nuccore/MT040335.1 | 29/3/17/4 | 10.8-19.9 |
| | HCov-HKU1: https://www.ncbi.nlm.nih.gov/nuccore/NC_006577.2 | 15/1/13/2 | 11.5- 15.0 |
| | Bat coronavirus RATG13: https://www.ncbi.nlm.nih.gov/nuccore/MN996532.1 | 17/2/29/1 | 10.9- 19.7 |
| | Bovine coronavirus BCoV-ENT: https://www.ncbi.nlm.nih.gov/nuccore/NC_003045.1 | 25/1/26/0 | 20.8 |
| | Murine hepatitis virus A59: https://www.ncbi.nlm.nih.gov/nuccore/FJ884687.1 | 29/5/42/7 | 11.2-36.8 |
| Gamma-coronaviruses | Avian infectious bronchitis virus: https://www.ncbi.nlm.nih.gov/nuccore/NC_001451.1 | 20/6/8/3 | 12.7- 26.5 |
| Delta-coronaviruses | Porcine coronavirus HKU15: https://www.ncbi.nlm.nih.gov/nuccore/NC_039208.1 | 26/5/29/3 | 11.2- 17.4 |

[1]Negative sense genes (NSGs) were identified by in silico approach using the Open Reading Frame Finder program (https://www.ncbi.nlm.nih.gov/orffinder/). First and second digits show overall and numbers of the large gene open reading frames (ORFs) starting with classical AUG, respectively. Third and fourth numbers show overall and large gene numbers ORFs having noncanonical CUG, respectively. Large genes were assumed to have more than 300 nt long. GenBank ac.n. of the viral genomes are indicated.

[2]A range of mol. wt. (kDa) of negative gene proteins encoded by the large negative sense genes (≥ 300 nt) starting either with AUG or CUG codons are outlined.

[3]The data were partially presented in[15]. These partial elements were used here with the Publisher's permission. NSGs: Negative sense genes; SARS-CoV: Severe acute respiratory syndrome coronavirus.

Ambisense stacking of genes revealed in coronavirus and influenza virus genomes significantly increases virus diversity, genetic potential and extend virus-host adaptation pathway possibilities. Existence of numerous ambisense genes opens up a new avenue for virus reproduction where one virus genome can produce a multiple progeny population of virions possessing identical genome RNA and different protein compositions. In this case, a part of virions decorated with one of the NGPs proteins (in the case of coronaviruses) could be hidden from us, as "the dark side of the Moon". The expression of coronavirus "negative" and flu "positive" genes may have a host (tissue)-dependent regulation facilitating immune escape of overcovered virions and specific pathogenetic pathways in the host(s) where the up-expression of the virus NGP or NSP genes occurs. Further studies will shed light on this ambisense concept of human and animal orthomyxo- and coronaviruses.

For the current time, there are four ambisense virus genera (phlebo-, tospo-, arena-, and bunyaviruses), which are well known to realize both positive- and negative-sense genome RNA strategies to encode viral proteins[12,21]. Ambisense genes of these virus genera locate in separate areas of the genome RNA without their overlapping and stacking. The ambisense genes locating in the genome in the stacking manner were found in influenza viruses, in which, similarly to coronaviruses, direct expression of these genes has not yet been identified, but there are indirect signs of such expression during natural viral infection *in vivo*[12-14]. Location of genes with opposite polarity in the same region of the RNA molecule makes it possible to significantly increase the genetic capacity of the viral genome and opens new ways for virus diversity, increasing virus adaptability to the host and biological evolution in nature[15]. The presence of potential ambisense genes in genomes of influenza and coronaviruses raises the question of the classification of these families. The detection in infected cells or infected organisms of protein products expressed by the ambisense manner will give grounds for classifying the coronavirus and orthomyxovirus families as the ambisense viruses with a bipolar genome strategy.

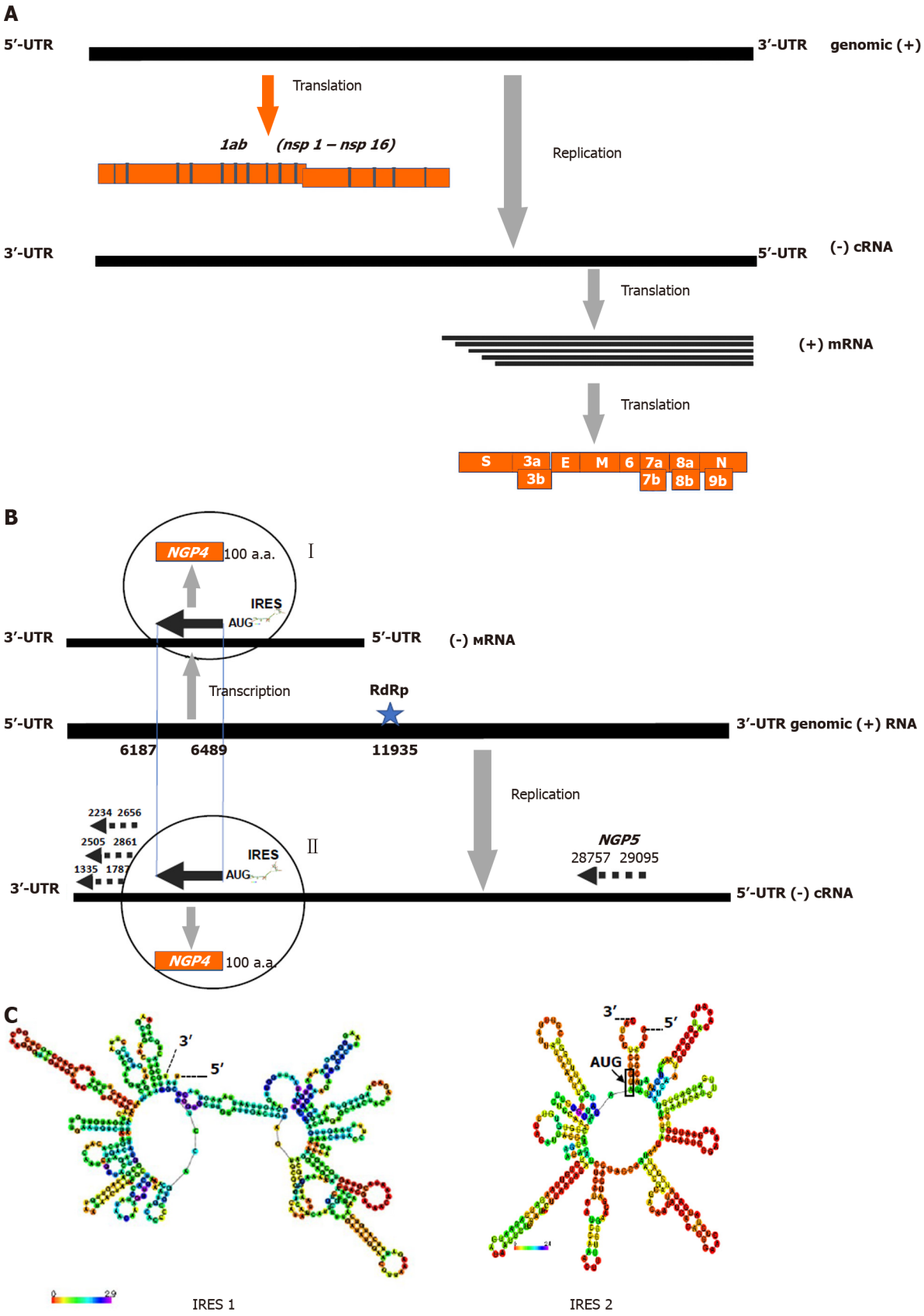


**Figure 2 Positive sense genome strategy and translation cassette unit at the 3' end of the negative sense complimentary RNA of coronavirus severe acute respiratory syndrome coronavirus 2 genome.** A: Replication scheme of the RNA genome of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) coronavirus (ac.n. MT890462.1). UTR means untranslated RNA region; B: A 3' end area of the subgenomic (-)cRNA complimentary to the virus genome 5' end (+)vRNA of SARS-CoV-2 (ac.n. MT635445.1) is displayed. Five ORF containing cassette for NGP1-NGP5 beginning either with classical AUG (NGP4) or noncanonical CUG (NGP1-3, NGP5) codons are shown by arrows. Nucleotides counting from the 5' end of (+)vRNA are shown for each ORFs. Phases of the translation frame (fr) are estimated regarding the frame of NGP4 (fr.0) as follows: NGP1and 2 (fr. +1), NGP3 (fr.0). Poly A tract (11935-

11940 nt) functioning as a viral RNA dependent RNA polymerase binding site is shown by star; C: IRES-like structures enriched with 16 and 10 canonical "hair-pins" RNA elements in the regions 8100-8599 nt (IRES 1) and 6488-6792 nt (IRES 2), respectively, were predicted by the IRESpred program[22]. The IRES-like structures 1 and 2 have significant free energy value as low as -99,4 and -73,8 kkal/mol, respectively. The data were partially presented in[15]. These partial elements were used here with the Publisher's permission.

## CONCLUSION

The manuscript data suggest that ambisense gene stacking in influenza and coronavirus families significantly increases genetic potential and virus diversity to extend virus-host adaptation pathways in nature. These data imply that ambisense viruses may have a multivirion mechanism, like "a dark side of the Moon", allowing production of the heterogeneous population of virions expressed through positive and negative sense genome strategies.

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*MINIREVIEWS*

# COVID-19 in dialysis units: A comprehensive review

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## Abstract

The coronavirus disease 2019 (COVID-19) pandemic has been challenging for healthcare professionals worldwide. One of the populations affected by the pandemic are patients on renal replacement therapy, as kidney disease is an independent risk factor for severe COVID-19 and maintenance dialysis (a life-sustaining therapy) cannot be interrupted in the vast majority of cases. Over the past months, several authors and medical societies have published recommendations and guidelines on the management of this population. This article is a comprehensive review regarding the measures to prevent, contain and deal with a COVID-19 pandemic in the dialysis setting. We recapitulate the epidemiology and pathophysiology of COVID-19 in kidney dysfunction and present the main recommendations concerning the screening of healthcare personnel, dialysis patients and visitors as well as measures to improve the safety of the dialysis facilities' environments. In addition to preventive measures, this article briefly describes actions directed towards management of an outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) within a dialysis facility, the management of complications in dialysis patients with COVID-19 and overall data regarding the management of children with kidney disease.

**Key Words:** COVID-19; SARS-CoV-2; Renal dialysis; Renal replacement therapy; Hemodialysis units, Hospital

**Specialty type:** Urology and nephrology

**Country/Territory of origin:** Brazil

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**Core Tip:** Dialysis patients are more vulnerable to develop severe coronavirus disease 2019 (COVID-19) infection. To minimize risks, some measures should be followed by dialysis units, healthcare personnel, patients and visitors. Until vaccination against COVID-19 is widely available to dialysis patients worldwide, an evidence-based approach is required to avoid the spread of the virus and consequently more death of patients.

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## INTRODUCTION

The outbreak of the coronavirus disease 2019 (COVID-19) pandemic in early 2020 proved to be a massive challenge for healthcare professionals all around the world. Clinically, its symptoms range from pulmonary (*e.g.*, cough and dyspnea) to extrapulmonary manifestations (*e.g.*, fever, myalgia, anosmia and ageusia), revealing the systemic nature of the aforementioned malady[1,2].

Due to the aforementioned variety of clinical manifestations attributed to the infection by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), different areas of the medical field became highly interested in the better comprehension of COVID-19, one of them being nephrology. The glomerular epithelium, the proximal tubular cells of the nephrons and endothelial cells have considerable levels of angiotensin-converting enzyme 2, which explains why COVID-19 patients may develop renal injury[3-6].

Such interest emerged as doctors recognized the necessity for guaranteeing the safety of patients treated with renal replacement therapy (RRT) during the pandemic, focusing on preventing an outbreak in dialysis units. The attentiveness to COVID-19 by nephrologists was reinforced when multiple studies from different countries suggested that patients who acquire the disease have a significant risk for developing acute kidney injury (AKI)[7-9].

In this article, we review the epidemiology of COVID-19 in dialysis centers as well as the main recommendations concerning the screening of healthcare personnel (HCP), dialysis patients and visitors, the safety of the dialysis facilities' environments, the conduct regarding an outbreak of SARS-CoV-2 infection within a dialysis facility, the management of complications in dialysis patients with COVID-19 and the conduct directed towards children with kidney disease.

## EPIDEMIOLOGY OF COVID-19 IN DIALYSIS UNITS

As of March 2021 there have been over 125 million confirmed cases of COVID-19 and over 2.7 million deaths, giving the disease a case fatality rate of 2.22%[10]. The currently available literature suggests that the frequency of COVID-19 among dialysis patients is approximately between 2% and 20%, a difference possibly explained by the region in which each study was conducted[11-14]. Meanwhile, the proportion of infected individuals appears to be lower in other health services, for both HCP and patients, and also in the general community[13-17].

Infection by SARS-CoV-2 in dialysis units does not seem to depend on sex, ethnicity, time of dialysis or presence of diabetes but is likely associated with in-center dialysis and older patient age; the higher risk of infection in healthcare facilities has been attributed to a higher rate of self-reported illness among the staff[11,18]. Chronic kidney disease (CKD) patients, especially those in dialysis, are more vulnerable to SARS-CoV-2 infection, given that a decrease in the estimated glomerular filtration rate has been associated with death by COVID-19 in one large cohort study that obtained data using OpenSAFELY[19]. The mortality of dialysis patients who contracted COVID-19 is approximately between 21% and 33%, being above both the general population's death rate due to SARS-CoV-2 infection[12,14,20,21]. Some studies have also shown that hemodialysis (HD) patients are more likely to contract the disease

than peritoneal dialysis patients, something that is at least partially explained by the fact that HD patients cannot perform dialysis at home, while peritoneal dialysis patients can[13,14].

COVID-19 can also cause AKI. It has been documented that about one fifth of patients with the disease end up developing AKI treated with RRT (AKI-RRT). CKD is associated with higher risk of developing AKI-RRT among COVID-19 patients as well as diabetes mellitus, hypertension, higher body mass index and high levels of D-dimer. The mortality is extremely high among AKI-RRT patients with COVID-19, even more than in the previously mentioned group, reaching levels above 60%[22].

## HEALTHCARE PERSONNEL, PATIENTS AND VISITORS

It is known that AKI and RRT increase the risk of complications and death in COVID-19, so it is necessary to follow specific rules to avoid infection[23,24]. In addition, HD units are classified as high risk of contagion, hence the need to further tighten these measures in these environments[25]. It is possible to divide protective actions into measures for HCP, for patients and visitors.

The first group includes doctors at the HD unit, nurses, technicians and cleaning staff[26], and they must receive the following instructions: (1) The use of personal protective equipment (PPE: surgical or N95 masks, gloves, hair caps and clothing with waterproof insulation) must be mandatory and constant[26-28]; (2) Educational actions on how to properly use PPE, how to properly sanitize hands and how to dispose of contaminated items should be promoted[26]; (3) Updates and training on new knowledge related to the epidemic need to be encouraged[26,27]; (4) Nurses must be trained to collect the nasopharynx swab to perform the COVID-19 polymerase chain reaction (PCR)[26]; (5) Groups of face-to-face activities, including discussion groups, ought to be avoided and should be done digitally[27,29]; (6) Teams from different parts of the health unit must have meals at different times in order to avoid contact [27]; (7) The team should, if possible, avoid using public transport as well as participating in large agglomerations[27,29]; (8) The presence of COVID-19 symptoms in the team as well as in their family members should be monitored closely. Members with suspected infection should notify the unit, perform the PCR for COVID-19 and quarantine themselves in order to avoid contaminating patients[26,27]; and (9) HCP vaccination against SARS-CoV-2 should be implemented on a large scale as soon as possible[30,31].

Patients also need to take several protective measures in order to further mitigate the possibility of contagion, such as: (1) The use of surgical masks, N95 or similar should be mandatory and the use of homemade cloth masks should be discouraged. However, due to economic reasons and the low availability of surgical masks, N95 or similar, some emerging countries recommended universal use of cloth masks for dialysis patients[32]. Although these are a better option than not using masks, surgical masks are about three times more effective in blocking the transmission of the virus [26,27,29,33-35]; (2) Educational measures, such as avoiding the use of public transport, practicing social isolation, wearing appropriate face masks, not traveling, staying away from agglomerations, preventing contact with people outside your residence, must be promoted[26,27,29]; (3) It is necessary to instruct, even in the dialysis units, on proper hand hygiene, on the cough etiquette and on the main symptoms of COVID-19[26,27,29,33]; (4) The medicines previously prescribed must be continued, with due medical follow-up. This includes angiotensin-converting enzyme inhibitors, other medications for the treatment of hypertension, glucocorticoids, immunosuppressants, medications for diabetes and anemia and any other necessary for the patient[27,36]; (5) Vaccination against influenza should be encouraged in dialysis units[29]; (6) Measures of attention to psychosocial care must be taken, as dialysis patients are predisposed to problems such as anxiety, depression and insomnia during the pandemic[27]; (7) Vaccination against SARS-CoV-2 in patients with kidney disease should be implemented on a large scale as soon as possible. So far, this is the most effective measure in the prevention and containment of COVID-19[30, 31]; and (8) If possible, the patient should be transferred to a home dialysis program [37].

Dialysis units should be encouraged to decrease the flow of people during the pandemic; therefore, it is not indicated that other individuals accompany patients on dialysis[26,29]. It can be allowed in situations of extreme need, judged on a case-by-case analysis. In this matter, it is recommended that the companions wear surgical masks, N95 or similar and obey the same basic rules as dialysis patients, *e.g.*, social

distancing[26,28,29,33].

## SAFETY OF DIALYSIS FACILITIES

The pandemic reinforced the importance of a safe environment for dialysis. Although current recommendations advise prioritizing the use of telehealth whenever is deemed possible[38], dialysis patients' demands are not always solved by those services alone. Thus, the ongoing scenario required that dialysis units adapted themselves to minimize SARS-CoV-2 infection rates within their installations.

General measures include the patient assessment for COVID-19 symptoms or exposure to diseased individuals in every dialysis session and planning for SARS-CoV-2 viral detection testing. In general, testing for COVID-19 (and other respiratory diseases) in outpatient HD facilities and home dialysis should be considered if the individual presents any signs or symptoms of the illness, even mild and atypical ones, or if there is suspicion of exposure to someone potentially infected with the virus.

It is also the role of the facility to ensure that the screening of staff, patients and visitors is being adequately done, including body temperature checking at entrance (and at both start and end of the dialysis session for patients) and that all rooms are well ventilated[26,29,39-41].

Also, safe patient placement is an important component of the strategy that dialysis facilities have been following. It is highly advisable that the minimum separation of six feet (approximately 180 cm) between patients, either in a waiting area or in the treatment area, is ensured in the whole facility. The same guidance applies to cohorting patients unless the individuals in question are confirmedly infected with the disease, in which case they can be cohorted together. Whenever possible, patients with suspected or confirmed SARS-CoV-2 infection should go through dialysis in a separate room. Also, single use of dialyzers is highly recommended in patients with confirmed or suspected cases of COVID-19; the once widespread (and still a reality in emerging countries) practice of reusing dialyzers should be avoided in patients with SARS-CoV-2 infection[29,42].

Given that the coronaviruses can persist on surfaces like glass, metal and plastic, cleaning and disinfection (C&D) has been frequently recommended to counteract SARS-CoV-2 transmission. The standard C&D course of action is considered satisfactory for COVID-19 cases, but the chemical product used for surface disinfection has to be capable of inactivating SARS-CoV-2, *e.g.*, ethanol, sodium hypochlorite and hydrogen peroxide[42,43]. It has been recommended that bed linens get changed between shifts and that the used ones are correctly contained or laundered, that constantly touched surfaces within the dialysis units are cleaned and disinfected regularly and that the adequate PPE is equipped when C&D is being performed[42,44].

However, it is arguable that too much focus is being directed towards C&D. Studies have suggested that the risk of infection by fomites is low and often exaggerated due to the inapplicability of the circumstances obtained in an artificial lab environment in daily life situations[45-47]. The reasons why C&D remains a constant aspect of many guidelines despite its apparent low impact on the dissemination of SARS-CoV-2 vary from public expectation and reliance on C&D protocols, as seen in cases in which people fumigate and/or wash the streets and sidewalks, measures which have been deemed by health authorities as ineffective[48].

As previously mentioned, telehealth plays a pivotal role in the current pandemic and should be used wherever and whenever possible. Even though it does not satisfy every need a dialysis patient may have, given that it is a complementary practice and not a substitutive one. Its benefits must not be downplayed, especially on the subject of home dialysis. There have been reports regarding the benefits of telehealth in dialysis in patients, released both before and during the pandemic and especially concerning peritoneal dialysis[49-51]. However, the quality of the obtained evidence is disputed[52,53]. Special attention must be given to specificities of home dialysis care, such as the likelihood of shortages of PPE and peritoneal dialysis fluid and the higher possibility of developing a more severe form of COVID-19. Dialysis facilities should also provide useful guidance to patients who are dialyzed at home and update their HCP on the clinical knowledge of COVID-19[27,54].

## DEALING WITH AN OUTBREAK

Despite all the protective measures being taken, it is still possible for a case of COVID-19 to appear in the dialysis units precisely because of the current pandemic. During an outbreak period, several patients and doctors visit dialysis units in general, which makes them a high-risk environment for nosocomial coronavirus infection[55]. In this scenario, it is possible to deal with two types of cases: (1) Suspected infection in patients or visitors; and (2) In HCP.

Still at the entrance to the dialysis unit, patients and visitors must undergo both symptomatic (*e.g.*, presence of fever, dyspnea, myalgia, coughing and sneezing) and epidemiological screening (*e.g.*, contact with people positive for COVID-19 in the last 14 d)[42,56-58]. If the patient is at low risk of infection, he must be referred to dialysis and must obey the protective measures already addressed in this article, *e.g.*, wearing PPE and keeping a minimum distance of 6 feet from other people[42,58]. If the patient has symptoms of COVID-19 or has had contact with someone who is positive for the virus, he must do the PCR for the disease and has to be treated as moderate/high risk for infection. Also, the monitoring of the evolution of the symptoms is mandatory, even if it is absent. In such cases, as previously mentioned, patients have to wear PPE and must be dialyzed in separate environments from other patients, with the door closed. If this is not possible, treatment should be carried out at the end of the day, in places away from the main passage of personnel, such as at the end of the corridor or in a corner[42,56,58,59]. If the patient already has a positive PCR for COVID-19, care must be increased and dialysis in a separate location is highly recommended[42]. These recommendations can be seen in Figure 1.

If the visitors are symptomatic or were in close contact with people with COVID-19 in the last 14 d, their entry should be prohibited. If they are classified as a low risk of infection, they should continue to follow protective measures against COVID-19 within healthcare centers. In addition, it is recommended that only patients confirmed for COVID-19 are dialyzed together whenever the facility's infrastructure enables, thus patients with suspicion of SARS-CoV-2 infection (not yet confirmed) ought to be treated separately from them[42,56,59]. The healthcare team responsible for the treatment of patients suspected or confirmed disease should use N95 or equivalent or higher-level respirator, eye protection, glove and isolation gown[42]. Recommendations towards visitors are shown in Figure 2.

In a situation in which the outbreak originates from the healthcare team, two fronts of action should be adopted. First, the healthcare worker must be immediately and temporarily relieved from work and has to self-quarantine for 14 d or 10 d as long as remains asymptomatic for 3 consecutive days from the last exposure to a contaminated individual[42,60,61]. Furthermore, special attention should be given to patients who were under the care of this HCP. If the patient had contact with the infected individual at a distance of less than 6 feet for more than 15 min, the situation should be treated as a potential exposure. If the patient wears a surgical mask at the moment of contact, he will be considered as a low risk for infection and other symptoms should be monitored without further concern. However, if the mask used is homemade or even without a mask, the patient will be considered at high risk for infection and all the measures described previously must be taken into action (Figure 3)[42].

Finally, it is important to note that the perception of a nosocomial transmission of COVID-19 is challenging due to the large circulation of people and the possibility that they have acquired the infection outside the units. Still, if this type of transmission is identified, the situation must be considered an outbreak and containment measures must be taken immediately[42,55].

## MANAGEMENT OF COMPLICATIONS

It should be reiterated that dialysis patients cannot interrupt RRT. Therefore, aside from the previously mentioned conducts related to preventing the dissemination of the virus within the facility, HCP must be able to know how to deal with possible renal complications in COVID-19 patients. The nephrologist plays a crucial role in the correct management of aggravations such as AKI, electrolyte imbalance and acid-base disorders.

The physiopathology of AKI in COVID-19 is not thoroughly known, but it is believed that it originates from a multitude of factors. Some of the proposed pathophysiological mechanisms relate to both prerenal and renal AKI, such as direct viral-related injury, corporal fluid disbalance, cytokine release syndrome, overstimu-

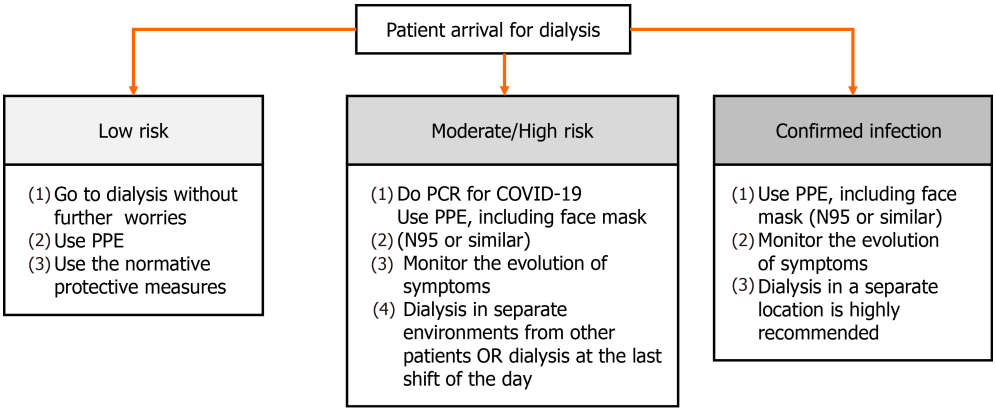


**Figure 1 Conduct related to dialysis patients, stratified according to their risk or current status of being infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).** COVID-19: Coronavirus disease 2019; PCR: Polymerase chain reaction; PPE: Personal protective equipment.

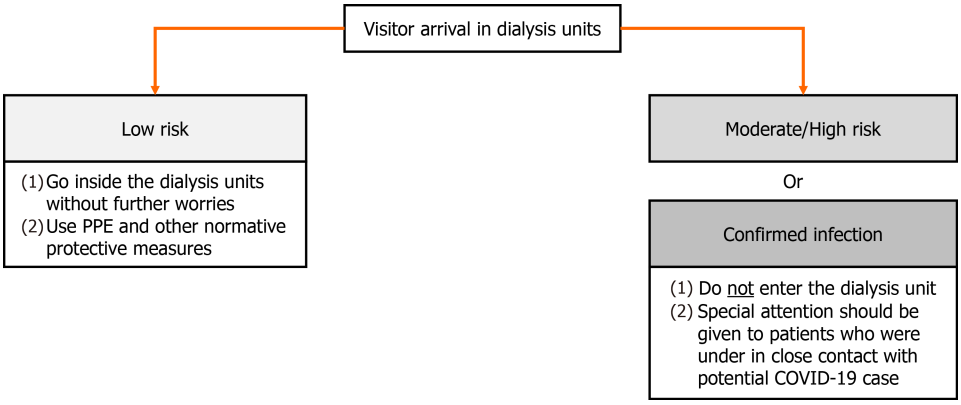


**Figure 2 Conduct in regard to visitors in a dialysis facility.** COVID-19: Coronavirus disease 2019; PPE: Personal protective equipment.

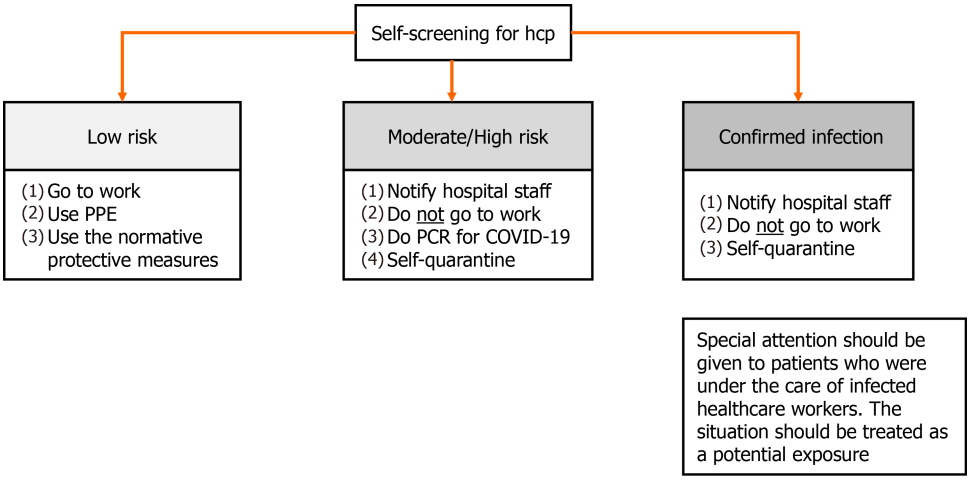


**Figure 3 Self-screening for healthcare personnel, stratified according to their risk or current status of being infected by severe acute respiratory syndrome coronavirus 2.** COVID-19: Coronavirus disease 2019; HCP: Healthcare personnel; PCR: Polymerase chain reaction; PPE: Personal protective equipment.

lation of the renin-angiotensin-aldosterone system, hypercoagulation, complement system dysregulation and multiple organ dysfunction syndrome[62,63].

Regarding RRT, the basic principle that guides all the others is that the entry of HCP in isolated areas must be limited and preference should be given to those who have already developed an effective immune response to SARS-CoV-2. When it comes to

choosing the dialysis modality for AKI patients, continuous RRT offers some considerable benefits regarding less physical contact between HCP and patients. However, due to the variability of resources in each healthcare setting, continuous RRT might not be available for a wide population. Therefore, other modalities might be more logistically adequate to use in certain areas[64]. Vascular access for RRT is usually done in the right jugular vein. While the left jugular vein comes as a natural second option, the femoral access has been suggested for consideration in order to reduce the likelihood of HCP contamination[65]. Also, the intensity of RRT in AKI related to COVID-19 should not be any different compared to the usual one, unless proven different[66]. It has been suggested that early RRT intervention in COVID-19 patients may provide benefits[67], but that assumption is not yet scientifically supported since one previous study detected no significant differences between early and delayed RRT start in general dialysis patients[68].

## COVID-19 IN THE NEPHROPEDIATRIC POPULATION

Although CKD is considerably more frequent in the adult population, children are also susceptible to the development of renal impairment. Data regarding infants and teenagers with CKD is scarce, therefore it is difficult to determine any reliable values for its incidence and prevalence in this population.

As a possible reflex of the rarity of severe COVID-19 cases in children, there are few studies related to the damages of the aforementioned disease in the lives of said individuals, and those who exist are not enough to build a solid evidence-based approach. One of them, an Italian national-scale study, attempted to determine the impact of the pandemic in children with CKD or immunosuppression related to kidney transplant but found no severe cases of COVID-19 among individuals under the age of 18. That same research, on the other hand, estimated that around 80% of children with CKD have a glomerular filtration rate ≤ 60 mL/min/1.73 m$^2$ and that 25% of this fraction are under dialysis treatment[69]. A Spanish retrospective study ($n$ = 16) also concluded that there seems to be no difference in the actual clinical course of the disease between healthy children and children with CKD but reiterated that special attention should be brought upon fluid management and the adjustment of drug doses [70]. Other case reports have been encountered; however, due to the limited methodological design intrinsic to these types of studies, they do not provide any information that can be applied in a larger scenario[71,72].

There were no registries of COVID-related AKI cases among children without chronic renal pathologies. As a result of the relative absence of information or overall existence of clinically relevant COVID-19 cases in pediatric nephrological patients, the guidelines directed to them do not differ much when compared to the ones orientated towards the adult population[73].

## CONCLUSION

In summary, dialysis patients are more vulnerable to develop severe COVID-19 and are at higher risk of a worst prognosis. Because of that, it is necessary to secure that the counteractive measures related to the pandemic are being thoroughly followed by dialysis units and HCP alike as well as ensuring that patients and visitors adhere to this public health commitment. However, even if all is correctly done, an outbreak can still occur in the dialysis unit setting. Until the vaccine against COVID-19 is widely available to dialysis patients worldwide, an evidence-based approach is required to avoid the spread of the virus and consequently the death of patients.

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META-ANALYSIS

# New-onset diabetes in COVID-19 and clinical outcomes: A systematic review and meta-analysis

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## Abstract

*BACKGROUND*

Diabetes mellitus (DM) is associated with adverse clinical outcomes and high mortality in patients with coronavirus disease 2019 (COVID-19). The relationship between diabetes and COVID-19 is known to be bidirectional.

*AIM*

To analyze the rate of new-onset diabetes in COVID-19 patients and compare the clinical outcomes of new-onset diabetes, pre-existing diabetes, hyperglycemic,

and non-diabetes among COVID-19 patients.

*METHODS*

We used the Meta-analysis of Observational Studies in Epidemiology statement for the present meta-analysis. Online databases were searched for all peer-reviewed articles published until November 6, 2020. Articles were screened using Covidence and data extracted. Further analysis was done using comprehensive meta-analysis. Among the 128 studies detected after thorough database searching, seven were included in the quantitative analysis. The proportion was reported with 95% confidence interval (CI) and heterogeneity was assessed using $I^2$.

*RESULTS*

Analysis showed that 19.70% (CI: 10.93-32.91) of COVID-19 patients had associated DM, and 25.23% (CI: 19.07-32.58) had associated hyperglycemia. The overall mortality rate was 15.36% (CI: 12.57-18.68) of all COVID-19 cases, irrespective of their DM status. The mortality rate was 9.26% among non-diabetic patients, 10.59% among patients with COVID-19 associated hyperglycemia, 16.03% among known DM patients, and 24.96% among COVID-19 associated DM patients. The overall occurrence of adverse events was 20.52% (CI: 14.21-28.70) among COVID-19 patients in the included studies, 15.29% among non-diabetic patients, 20.41% among patients with COVID-19 associated hyperglycemia, 20.69% among known DM patients, and 45.85% among new-onset DM. Meta-regression showed an increasing rate of mortality among new hyperglycemic patients, known diabetics, and new-onset DM patients in comparison to those without diabetes.

*CONCLUSION*

A significantly higher rate of new onset DM and hyperglycemia was observed. Higher mortality rates and adverse events were seen in patients with new-onset DM and hyperglycemia than in the non-diabetic population.

**Key Words:** Acute respiratory distress syndrome; COVID-19; Diabetes mellitus; Hyperglycemia; Mortality



**Core Tip:** The relationship between diabetes and coronavirus disease 2019 (COVID-19) is known to be bidirectional. The rate of COVID-19 associated diabetes mellitus (DM) and hyperglycemia was significantly high. Higher mortality rates and adverse events were seen in patients with new-onset DM and hyperglycemia in comparison to the non-diabetic population.

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## INTRODUCTION

The ongoing coronavirus disease 2019 (COVID-19) has infected 93 million patients and claimed the lives of 2.02 million people as of January 19, 2021[1]. Extensive research has been conducted to study the comorbidities associated with increased severity of disease and worse clinical outcomes. Diabetes has consistently been associated with adverse clinical outcomes and high mortality in COVID-19 patients independent of or in association with other comorbidities[2-4]. Such findings have been linked to the alteration of immune and inflammatory responses caused by hyperglycemia among diabetic patients suffering from COVID-19[5]. However, it is now known that the relationship between diabetes and COVID-19 is bidirectional[6]. Not only does having

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diabetes increase the risk of severe COVID-19, but severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is also known to have diabetogenic effects.

Multiple theories have been postulated to explain the increasing rate of new-onset diabetes in COVID-19 patients. One of the proposed mechanisms is that SARS-CoV-2 binds to the angiotensin-converting enzyme-2 (ACE-2) receptors expressed on adipose tissue, lungs, small intestine, kidneys, and pancreas. After endocytosis of the virus, downregulation of ACE-2 occurs, leading to overexpression of angiotensin II, which may impede insulin secretion. Similarly, it has been suggested that the direct entry of SARS-CoV-2 into the islet cells of the pancreas damages the beta cells, which normally secrete insulin[7,8].

In the light of new evidence and theories suggesting that there is increased susceptibility of worsening pancreas function and glucose homeostatic mechanisms in COVID-19 patients, the objective of this study is to analyze the rate of new-onset diabetes in COVID-19 patients and compare their clinical outcomes with those of other COVID-19 patients who had normal or increased blood sugar levels or a pre-existing diagnosis of diabetes.

## MATERIALS AND METHODS

This study was conducted according to the Meta-analysis of Observational Studies in Epidemiology statement[9]. Our protocol was registered in the PROSPERO International Prospective Register of Systematic Reviews (PROSPERO ID: CRD42021219284).

### *Search strategy*

Investigators independently searched databases such as PubMed, PubMed Central, Scopus, Embase, and Google Scholar for all peer-reviewed articles published until November 6, 2020. The terms "New onset diabetes mellitus (DM)", "DM", "hyperglycemia", "SARS-Cov-2" and "COVID-19" connected with "OR" and "AND". Boolean operators were searched under the medical subject headings terms. The reference section of each study shortlisted from this process was checked to identify further studies not found in the previous database searches. Additional studies collected from this method were included if they fulfilled the inclusion and exclusion criteria. Electronic search details are provided in Supplementary Material 1.

### *Selection of studies*

**The studies were selected based on the following criteria:** Inclusion criteria: (1) Study type(s): Observational studies with a comparison of outcomes among individuals with new onset diabetes, pre-existing diabetes, hyperglycemic and non-diabetics with COVID-19 were included in this review; (2) Study participant(s): Individuals of any age, gender, or nationality diagnosed with COVID-19 and new-onset DM; and (3) Objective outcome(s): Mortality, mechanical ventilation/intubation, and intensive care unit (ICU) admission were defined as the primary outcomes of our study. Complications such as Acute Respiratory Distress Syndrome (ARDS), acute cardiac injury, acute liver injury, acute kidney injury, cerebrovascular accident, coagulopathy, and secondary infection were secondary outcomes. Exclusion criteria: (1) Inadequate or unclear descriptions; (2) Animal studies; (3) Review articles; (4) Full text unavailable; and (5) Studies published in a language other than English.

### *Data extraction*

The titles and abstracts of studies retrieved in Covidence during the search were screened independently by two reviewers (PG and SR). The full-texts of potentially relevant studies were then reviewed by two reviewers (SA and SR) according to the eligibility criteria. Any conflict in the first phase of review was resolved by SA and in the second phase by PG. The included studies were then collated, and the three reviewers extracted the data using standardized data extraction formats. The extracted data included: First author, year of publication, country of study, study design, number of patients, age, sex, comorbidities, case definitions, inclusion and exclusion criteria, COVID-19 associated DM, COVID-19 associated hyperglycemia, outcomes, and follow-up duration. The outcomes were mortality and adverse events such as severe COVID-19, intubation, complications and ICU admission. All three reviewers matched their data with each other after extraction and revisited papers in case of disagreements. Discrepancies were resolved through consensus among the reviewers.

**Table 1 JBI bias assessment for observational studies**

| Questions (Yes/No/Unclear/Not applicable) | Smith *et al* [19], 2021 | Zhou *et al* [16], 2020 | Wang *et al* [20], 2020 | Fadini *et al* [17], 2020 | Wang *et al* [21], 2020 | Li *et al* [14], 2020 |
|---|---|---|---|---|---|---|
| Were the two groups similar and recruited from the same population? | Yes | Yes | Yes | Yes | Yes | Yes |
| Were the exposures measured similarly to assign people to both exposed and unexposed groups? | Yes | Yes | Yes | Yes | Yes | Yes |
| Was the exposure measured in a valid and reliable way? | Yes | Yes | Yes | Yes | Yes | Yes |
| Were confounding factors identified? | Yes | Yes | Yes | Yes | Yes | Yes |
| Were strategies to deal with confounding factors stated? | Yes | No | No | Yes | No | Yes |
| Were the groups/participants free of the outcome at the start of the study (or at the moment of exposure)? | Yes | Yes | Yes | Yes | Yes | Yes |
| Were the outcomes measured in a valid and reliable way? | Yes | Yes | Yes | Yes | Yes | Yes |
| Was the follow-up time reported and sufficient to be long enough for outcomes to occur? | No | No | No | No | Yes | Yes |
| Was follow-up complete, and if not, were the reasons for loss to follow-up described and explored? | Yes | Yes | Yes | Yes | Yes | Yes |
| Were strategies to address incomplete follow-up utilized? | Not applicable | Not applicable | Not applicable | Not applicable | Not applicable | Yes |
| Was appropriate statistical analysis used? | Yes | Yes | Yes | Yes | Yes | Yes |
| **Overall appraisal** | Include | Include | Include | Include | Include | Include |

**Data analysis:** The data were analyzed using comprehensive meta-analysis, employing a random effect model. Proportions were presented appropriately using 95% confidence intervals (CI). Forest plots were derived for a visual representation of the analysis. Sensitivity analysis was performed, excluding individual studies to gauge the impact of those studies on the overall results. Meta-regression was undertaken for mortality, considering diabetes status as a moderator among patients with hyperglycemia, patients with new-onset DM, patients with known diabetes, and the non-diabetic population.

**Risk of bias in individual studies:** We assessed the risk of bias using the JBI tool to evaluate the quality of case reports, case series, and retrospective studies (Tables 1,2, 3) [10]. Publication bias across the included studies was evaluated using funnel plot.

## RESULTS

We imported 128 studies after a thorough database search and removed 27 duplicates. The title and abstract of 101 studies were screened, and we excluded 76 irrelevant studies. We assessed the full text of 25 studies and excluded 15 studies with definite reasons (Figure 1). Finally, ten studies were included in our qualitative analysis (Table 4) and seven in our quantitative analysis.

### *Qualitative summary*

A summary of the included studies including type of study, location, study population and the relevant outcomes is presented in Table 4.

### *Quantitative result*

A total of 7 papers were included in the quantitative synthesis.

### *COVID-19 associated DM*

Pooling data from six studies that reported new-onset diabetes among COVID-19 cases using a random effect model showed that 19.70% (CI: 10.93-32.91, $I^2$ = 96.71) of COVID-19 cases were associated with DM (Figure 2). Sensitivity analysis after

**Table 2 JBI critical appraisal for case series**

| Question | Ref. | | |
|---|---|---|---|
| | Suwanwongse and Shabarek [22], 2021 | Kuchay *et al* [23], 2020 | Yang *et al*[24], 2020 |
| Were there clear criteria for inclusion in the case series? | Yes | Yes | Yes |
| Was the condition measured in a standard, reliable way for all participants included in the case series? | Yes | Yes | Yes |
| Were valid methods used for the identification of the condition for all participants included in the case series? | Yes | Yes | Yes |
| Did the case series have consecutive inclusion of participants? | No | No | Yes |
| Did the case series have complete inclusion of participants? | No | No | Yes |
| Was there clear reporting of the demographics of the participants in the study? | Yes | Yes | Yes |
| Was there clear reporting of clinical information of the participants? | Yes | Yes | Yes |
| Were the outcomes or follow-up results of cases clearly reported? | Yes | Yes | Yes |
| Was there clear reporting of the presenting site(s)/clinic(s) demographic information? | No | No | Yes |
| Was statistical analysis appropriate? | Unclear | Unclear | Yes |
| Overall: (Include/Exclude/Seek Further Info) | Include | Include | Include |

**Table 3 JBI critical appraisal checklist for case reports**

| Ref. | JBI critical appraisal checklist for case reports | Remarks |
|---|---|---|
| Marchand *et al*[25], 2020 | Were the patient's demographic characteristics clearly described? | Yes |
| | Was the patient's history clearly described and presented as a timeline? | Yes |
| | Was the current clinical condition of the patient on presentation clearly described? | Yes |
| | Were diagnostic tests or assessment methods and the results clearly described? | Yes |
| | Was the intervention(s) or treatment procedure(s) clearly described? | No |
| | Was the post-intervention clinical condition clearly described? | No |
| | Were adverse events (harms) or unanticipated events identified and described? | Yes |
| | Does the case report provide takeaway lessons? | Yes |
| | Overall: (Include/Exclude/Seek Further Info) | Include |

excluding individual studies is shown in Supplementary Material 2 and Figure 1.

### *COVID-19 associated hyperglycemia*

Pooling data from five studies that reported hyperglycemia among COVID-19 cases using a random effect model showed that 25.23% (CI: 19.07-32.58, $I^2$ = 86.6) of COVID-19 cases were associated with hyperglycemia (Figure 3). Sensitivity analysis after removing individual studies is shown in Supplementary Material 2, and Figures 2 and 3.

### *Mortality outcome*

Pooling data among COVID-19 cases using a random effect model showed a 9.26% mortality rate among non-diabetic (CI: 6.28-13.46, $I^2$ = 50.69), 10.59% among those with COVID-19 associated hyperglycemia (CI: 4.92-21.33, $I^2$ = 77.49), 16.03% among known DM patients (CI: 10.95-22.88, $I^2$ = 54.35), and 24.96% among new-onset DM (CI: 18.10-33.37, $I^2$ = 55.88). The overall mortality rate was 15.36% (CI: 12.57-18.68, $I^2$ = 81.75) among all COVID-19 cases, irrespective of their DM status (Figure 4).

**Table 4 Qualitative analysis of included studies**

| Ref. | Type of study | Country | Population | Outcome |
|---|---|---|---|---|
| Smith *et al*[19], 2021 | Retrospective study, spanning over 7 wk | New Jersey, United States | *n* = 184, M/F = 98/86. Avg age = 64.4 yr (21-100). Below or equal to 60 yr = 75, Above 60 yr = 109. Mean BMI = 29.8 (17.5-61.4). COVID-19 diagnosis based on: 177 patients: Confirmed positive lab test for SARS-CoV-2. Remaining (7 patients): Clinical diagnosis. Case definitions used by the study: New-onset DM: Persistently elevated FBG > 125 mg/dL and requiring insulin therapy; Pre-DM: HbA1C of 5.7% to 6.4%; Non-diabetic patients: HbA1C < 5.7% and FBG ≤ 125 mg/dL | DM = 114/184 (New-onset DM= 29/184). Pre-DM = 44/184. Non-DM = 26/184. HbA1C levels: (1) ≥ 6.5% = 82/171; and (2) 5.7% to 6.4% = 64/171. Among intubated patients (44/184): (1) DM = 35/44 (Newly diagnosed DM = 7/44; New onset DM = 5/44); (2) Pre-DM with high FBG levels = 7/44; and (3) Non-DM = 1/44 (normal HbA1C and FBG levels at admission, but was clinically obese with a BMI > 30). Among intubated patients (44/184): (1) Mean BMI = 32.2 (*vs* 29.3 in non-intubated); (2) Mean HbA1C (%) = 8.0 (*vs* 7.2 in non-intubated); and (3) Mean FBG (mg/dL) = 238.0 (*vs* 163.7 in non-intubated). Death before intubation: 24/184: (1) DM = 17/24; (2) Pre-DM = 4/24; and (3) Non-DM = 3/24 |
| Zhou *et al*[16], 2020 | Retrospective study | Hefei, China | *n* = 80. Euglycemia group: (1) 44 (21 males and 23 females); and (2) Age range was 27-52 yr. Secondary hyperglycemia group: (1) 22 (17 males and 5 females); (2) Conditions of no past histories of diabetes, HbA1c < 6.5%, random blood glucose > 11.1 mmol/L during hospitalization, and normal blood glucose after discharge from the hospital; (3) Age range was 40-70 yr; and (4) 5 patients among them had elevated blood sugar after glucocorticoid therapy. Diabetes group: (1) 14 patients (10 males and 4 females); (2) All were T2DM patients; (3) Treated with oral antidiabetics or insulin before hospitalization and without glucocorticoid therapy during hospitalization; and (4) Ages ranged from 43 to 67 yr | Euglycemia group: 44/80. Secondary hyperglycemia group: 22/80. Diabetes group: 14/80. Non-severe COVID: (1) Euglycemia (*n* = 44): 34 (77.27); (2) Secondary hyperglycemia (*n* = 22): 15 (68.18); and (3) Diabetes (*n* = 14): 6 (42.86). Severe COVID: (1) Euglycemia (*n* = 44): 10 (22.73); (2) Secondary hyperglycemia (*n* = 22): 7 (31.82); and (3) Diabetes (*n* = 14): 8 (57.14). Evidence of pneumonia on CT = 78/80: (1) Euglycemia group = 42/44; (2) Secondary hyperglycemia group = 22/22; and (3) Diabetes group = 14/14 |
| Wang *et al*[20], 2020 | Retrospective study | Beijing, China | *n* = 132. Exclusion criteria: (1) If not tested positive for COVID-19; (2) Receiving glucocorticoids; (3) Hemolytic anemia; (4) Myelosuppression after leukemia chemotherapy; and (5) Median time from onset to admission was 14 (IQR 10.0–17.8) d. Three groups: A, B, and C-(1) Group A had no diabetes and their HbA1c level was 6.0; (2) Group B had no diabetes and their HbA1c level was > 6.0; (3) Group C were diabetic | 41/132 patients in group A. 44/132 patients in group B. 47/132 patients in group C: (1) 31/47 = History of type 2 diabetes; and (2) 16/47 = Newly diagnosed with diabetes. Death = 22/132: (1) Deaths in group A = 4/41; (2) Deaths in group B = 5/44; and (3) Deaths in group C = 13/47 |
| Suwanwongse and Shabarek [22], 2021 | Case series | United States | *n* = 3 (18/M, 51/M , 64/F) | New-onset diabetes was diagnosed after infection with COVID-19. 2 out of 3 cases were diagnosed as Diabetic Ketoacidosis. All were discharged home after successful management of blood glucose levels. None of the cases developed any pulmonary, renal, hepatic or cardiac complications due to COVID-19. Invasive Mechanical Ventilation, ICU Admission, or Death did not occur in any of the three cases |
| Marchand *et al* [25], 2020 | Short communication | France | *n* = 1 | New-onset type-I DM after COVID-19. No information on severity or outcome of COVID-19 |
| Kuchay *et al* [23], 2020 | Case series | Haryana, India | *n* = 3 (30/M, 60/M, 34/M). Follow up duration: 14 wk. Three patients with newly diagnosed Diabetes Mellitus and Diabetic Ketoacidosis with positive SARS-CoV-2 laboratory report. Case Definition: Diabetic Ketoacidosis: DKA was defined as plasma glucose > 250 mg/dL, a positive test for urine or serum ketones, and arterial pH < 7.35 and/or a bicarbonate level less than 18 mmol/L | All three patients responded well to intravenous fluids, antibiotics, and insulin and were discharged after the third week. All three patients were given oral antihyperglycemic drugs after their requirement for exogenous insulin diminished after 4-6 wk. No mortality |
| Fadini *et al*[17], 2020 | Retrospective study | Italy | COVID-19 positive hospitalized patients included: *n* (Total) = 413. Median observation time of 17 d | No diabetes = 306/413. Diabetes = 107/413 (Pre-existing diabetes = 86/413; Newly-diagnosed diabetes = 21/413). Primary Outcome (composite of ICU admission or death): 62/306 (20.3%); 7/86 (31.4%); 13/21 (61.9%). Death: 33/306 (10.8%); 12/86 (14.0%); 3/21 (14.3%). Discharged alive: 238/306 (77.8%); 51/86 (59.3%); 9/21 (42.9%). Mean time to discharge in alive pts: 10.1 ± 5.7 (*n* = 306); 11.6 ± 6.6 ( *n* = 74); 17.4 ± 8.5 (*n* = 18). Mean days of hospitalization in survivors: 11.3 ± 7.1 (*n* = 306); 13.8 ± 8.0 (*n* = 74): 19.7 ± 9.3 (*n* = 18) |
| Wang *et al*[21], 2020 | Multicenter retrospective study | China | Without previous diagnosis of diabetes. *n* = 605 among 1258. Non-survivor = 114. Survivor = 491. Median age: 59.0 yr (IQR 47.0, 68.0). M/F = | Major outcome studied: 28-d mortality. Admission FBG (Total Non-survivor Survivor): (1) < 6.1 mmol/L = 329/605, 35/114, 294/491; (2) 6.1–6.9 |

| | | | | |
|---|---|---|---|---|
| | | | 322/283. Out of total patients included in analysis: (1) FBG < 6.1 mmol/L (*n*) = 329; (2) FBG 6.1-6.9 mmol/L (*n*) = 100; and (3) FBG ≥ 7.0 mmol/L (*n*) = 176 | mmol/L = 100/605, 21/114, 79/491; (3) ≥ 7.0 mmol/L = 176/605, 58/114, 118/491; and (4) Complications 237/605, 114/114, 123/491. With complications: (1) < 6.1 mmol/L = 86/605, 35/114, 51/491; (2) 6.1–6.9 mmol/SL = 48/608, 21/114, 27/491; and (3) ≥ 7.0 mmol/L = 103/605, 58/114, 45/489. Without complications: (1) < 6.1 mmol/L = 243/605, 0/114, 243/491; (2) 6.1–6.9 mmol/L = 52/605, 0/114, 52/491; and (3) ≥ 7.0 mmol/L = 73/603, 0/114, 73/490 |
| Yang *et al*[24], 2020 | Retrospective case series | China | *n* = 69 among 120 evaluated. Exclusion Criteria: (1) Previously diagnosed Diabetes Mellitus; (2) Patients treated with Glucocorticoids; (3) Patients with heart disease (myocardial infarction and heart failure); (4) Patients with kidney disease (maintenance dialysis or renal 20 transplantation); and (5) Patients with liver disease (liver cirrhosis). Median age = 61 (IQR 52-67). M/F = 34/35 | FBG ≥ 7.0 mmol/L for two times during hospitalization and without a history of diabetes in COVID-19 patients: 69/120. COVID-19 Severity: (1) Moderate = 23/69; (2) Severe = 20/69; and (3) Critical = 26/69. Mortality = 16/69 |
| Li *et al*[14], 2020 | Retrospective study | China | Inclusion: Laboratory confirmed SARS-CoV-2 Infection. Exclusion: Incomplete data available, cases without clinical results, patients with pneumonia due to other pathogens. *n* = 453. Non survivor (*n*) = 39. Recovered (*n*) = 414. Median age = 61 yr (IQR 49-68). Divided into four groups: (1) Normal glucose: FBG < 5.6 mmol/L, HBA1c: < 5.7% (*n* = 132); (2) Hyperglycemia: FBG 5.6-6.9 mmol/L HbA1c: 5.7%-6.4% (*n* = 129); (3) Newly diagnosed Diabetes: No history of previous Diabetes. FBG: ≥ 7 mmol/L and/or HbA1c ≥ 6.5% ( *n* = 94); and (4) Known Diabetes: Previously diagnosed Diabetes Mellitus (*n* = 98) | Main clinical outcomes: (1) Invasive mechanical ventilation: 3/132; 6/129; 11/94; 9/98; (2) ICU admission: 2/132, 8/129, 11/94, 4/98; and (3) Death: 2/132, 6/129, 20/94, 11/98. Other outcomes: (1) ARDS: 1/132, 4/129, 10/94, 3/98; (2) Acute Cardiac Injury: 27/132, 26/129, 23/94, 32/98; (3) Coagulopathy: 12/132, 12/129, 15/94, 17/98; (4) Hypoalbuminemia: 14/132, 15/129, 37/94, 36/98; and (5) Length of hospital stay (days): 22.5 (1.19), 21.9 (1.16), 26.5 (1.37), 23.6 (1.37) |

ARDS: Acute Respiratory Distress Syndrome; BMI: Body mass index; COVID-19: Coronavirus disease 2019; CT: Computed tomography; DKA: Diabetic ketoacidosis; DM: Diabetes mellitus; F: Female; FBG: Fasting blood glucose; HbA1C: Hemoglobin A1C; ICU: Intensive care unit; IQR: Inter quartile range; M: Male; N: Total participants; Non-DM: Non-diabetes mellitus; Pre-DM: Pre-diabetes mellitus; T2DM: Type 2 diabetes mellitus; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

### *Adverse events such as severe COVID-19, intubation, complications, and ICU admission*

Pooling data for the occurrence of adverse events among COVID-19 cases using a random effect model showed 15.29% occurrence among non-diabetic patients (CI: 9.06-24.65, $I^2$= 84.47), 20.41% among those with COVID-19 associated hyperglycemia (CI: 6.20-49.86, $I^2$= 93.41), 20.69% among known DM patients (CI: 8.12-43.50, $I^2$ = 90.14), and 45.85% among those with new-onset DM (CI: 22.23-71.50, $I^2$ = 94.21). The overall occurrence of adverse events was 20.52% (CI: 14.21-28.70, $I^2$ = 93.53) among all COVID cases irrespective of their DM status (Figure 5).

### *Meta-regression for mortality outcome*

Meta-regression showed an increasing rate of mortality among newly hyperglycemic patients, known diabetic patients, and new-onset DM compared to non-diabetic patients (Figure 6 and Table 5).

### *Publication bias*

Publication bias across the included studies was evaluated using Egger's test to evaluate funnel plot asymmetry. Publication bias reporting new-onset DM showed some publication bias depicted by the asymmetry of the funnel plot (Supplementary Material 2 and Figure 4). Similarly, publication bias for mortality outcome is shown in Supplementary Material 2 and Figure 5.

## DISCUSSION

Our meta-analysis is the first to pool the prevalence of new-onset DM and compare mortality and adverse events among patients with new-onset DM *vs* patients with hyperglycemia, pre-existing DM, or no DM. Prior meta-analyses have shown DM to be associated with mortality, severe COVID-19, ARDS, and disease progression[11-13]. However, there was a paucity of data to compare the outcomes among infected patients with pre-existing diabetes compared to new-onset DM. We found the pooled

**Table 5 Main results for meta-regression model, random effects, Z-distribution, logit event rate**

| Covariate | Coefficient | SE | 95% lower | 95% upper | Z value | *P* value |
|---|---|---|---|---|---|---|
| Intercept: No DM | -2.3183 | 0.2504 | -2.8091 | -1.8276 | -9.26 | 0 |
| Hyperglycemia | 0.2519 | 0.3788 | -0.4905 | 0.9944 | 0.67 | 0.506 |
| Known DM | 0.6642 | 0.3552 | -0.0319 | 1.3603 | 1.87 | 0.0615 |
| New DM | 1.1865 | 0.3552 | 0.4903 | 1.8827 | 3.34 | 0.0008 |

Test of the model: Simultaneous test that all coefficients (excluding intercept) are zero: Q = 12.51, df = 3, $P$ = 0.0058. Goodness of fit: Test that unexplained variance is zero: Tau² = 0.1610, Tau = 0.4012, $I^2$ = 62.66%, Q = 34.81, df = 13, $P$ = 0.0009. Total between-study variance (intercept only): Tau² = 0.3751, Tau = 0.6124, $I^2$ = 81.75%, Q = 87.66, df = 16, $P$ = 0.0000. Proportion of total between-study variance explained by Model 1: $R^2$ analog = 0.57. DM: Diabetes mellitus.

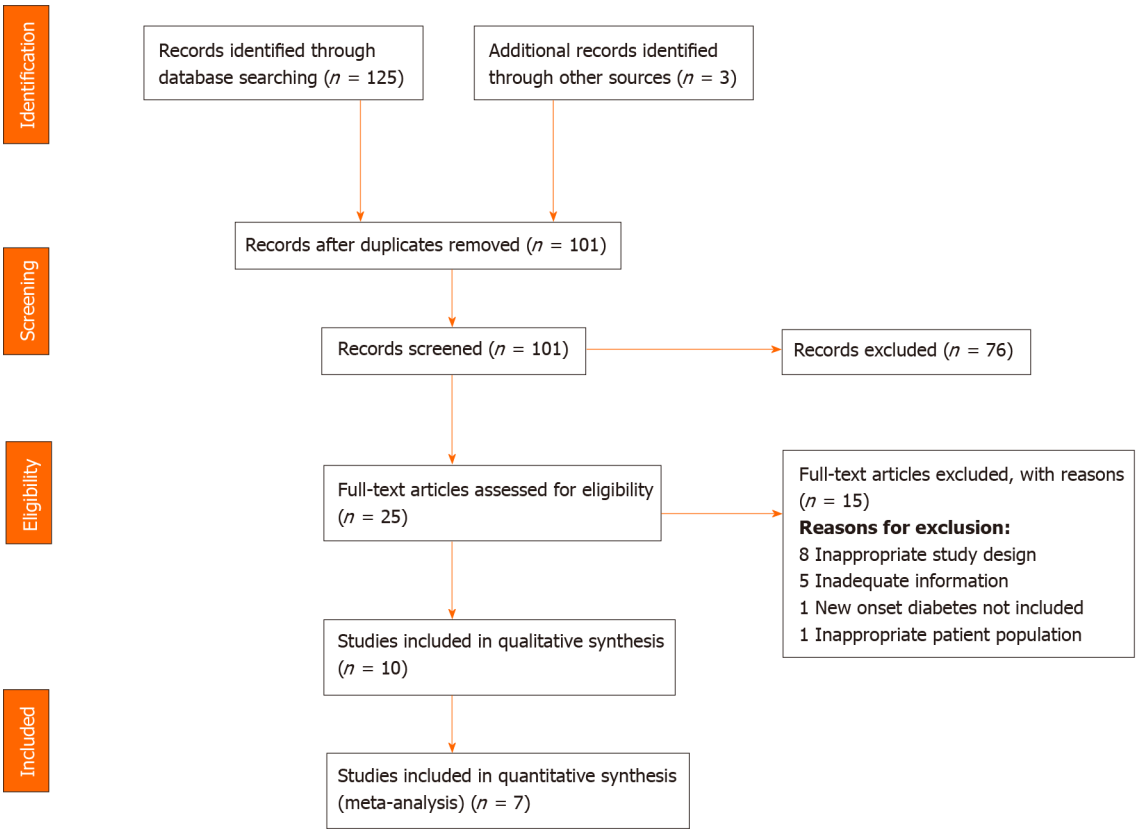


**Figure 1 PRISMA flow diagram.**

prevalence of COVID-19 associated DM (new-onset) to be 19.7%, while the prevalence of COVID-19 associated hyperglycemia was 25.23%. Angiotensin II has been shown to increase hepatic glucose production and decrease insulin sensitivity. A multitude of explanations have been proposed for impaired blood glucose levels among patients infected with COVID-19, including downregulation of ACE-2 receptors leading to increased angiotensin II and defective insulin secretion as well as direct damage to beta cells of islets of the pancreas[7,8]. Infection with the virus itself leads to oxidative stress, resulting in hypoxia and inflammation, which aggravates glucose homeostasis [14]. Additionally, damage to key organs involved in glucose metabolism such as the kidney and the liver resulting in abnormal blood glucose levels, has been observed in cases of COVID-19 infection. The use of corticosteroids is common among COVID-19 patients, especially those with severe COVID-19[15]. However, in our meta-analysis, only one study[16] included patients receiving steroids, which eliminates steroid use as a possible cause of hyperglycemia. The mortality rate was highest among patients with new-onset DM (24.96%), followed by known DM patients (16.03%), patients with COVID-19 associated hyperglycemia (10.59%), and non-diabetic patients (9.26%). The

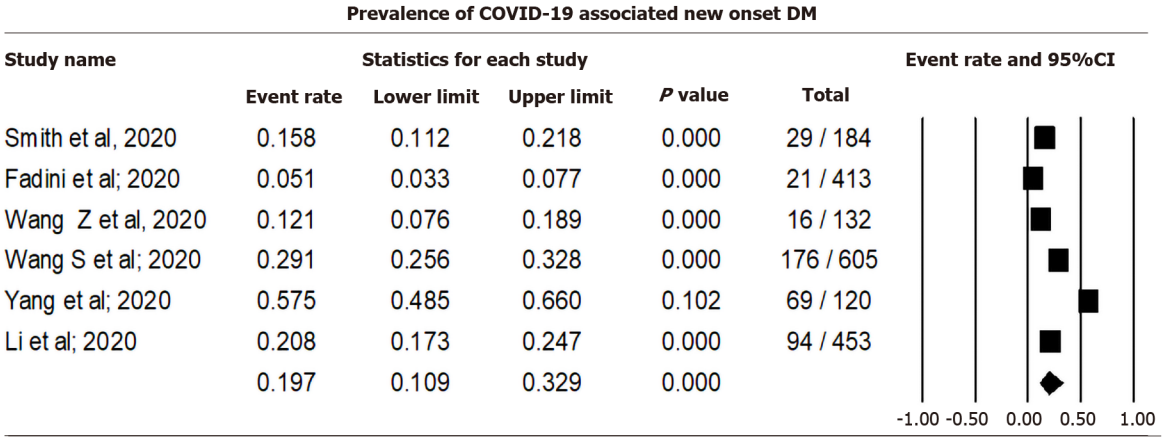


**Figure 2 Prevalence of coronavirus disease 2019 associated new onset diabetes mellitus.** COVID-19: Coronavirus disease 2019; DM: Diabetes mellitus.

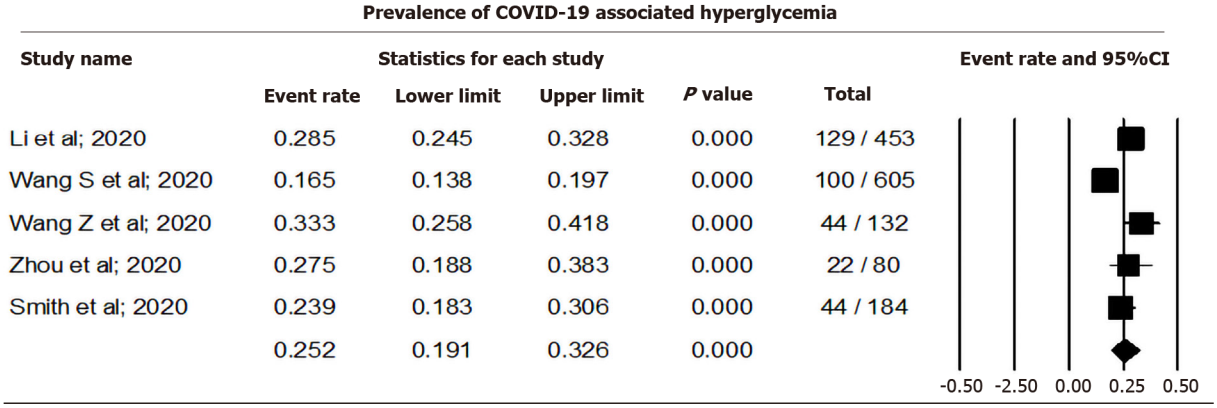


**Figure 3 Prevalence of coronavirus disease 2019 associated hyperglycemia.** COVID-19: Coronavirus disease 2019.

higher prevalence in patients with new-onset DM could be explained by the masked presence of organ damage due to ongoing diabetes, which cannot be accounted for during statistical analysis in contrast to cases of pre-existing diabetes in which organ damage is accounted for statistically[17]. Similarly, metabolic inflammation caused by high blood sugar levels affects the body's immune system and healing process prolonging recovery[14]. Hyperglycemia has been found to affect lung volume and diffusion capacity, causing respiratory deterioration and a decrease in $PaO_2/FiO_2$ ratio [17]. Chronic hyperglycemia causes down regulation of ACE-2, which has a protective effect against inflammation and in turn leads to inflammatory damage by the virus and potential cytokine storm. These are the reasons for increased mortality among patients with diabetes and hyperglycemia compared to non-diabetic patients. The pooled mortality of 16.03% among diabetic patients was lower than that shown in Shang's meta-analysis (21.4%) and higher than that in Miller *et al*[11] (9.9%). Adverse events such as severe COVID-19, intubation, complications, and ICU admissions were highest among new-onset DM (45.85%), followed by known DM patients (20.69%), patients with COVID-19 associated hyperglycemia (20.41%), and non-diabetic patients (15.29%). Our findings concurred with previous studies that have shown a strong association between DM and severe COVID-19, leading to increased complications, including multi-organ dysfunction and ICU admissions[18]. The need for intubation can be explained by the respiratory deterioration noted among patients with hyperglycemia.

Our study has several limitations. Due to the inadequate number of existing studies, we could not include controlled studies, instead using only observational studies, case reports, and case series. The included studies had small sample sizes and low power. Each study had its own limitations, such as the absence of data on body mass index, Hemoglobin A1C in all patients, the possibility of stress hyperglycemia, single-center study, retrospective study design, *etc.*

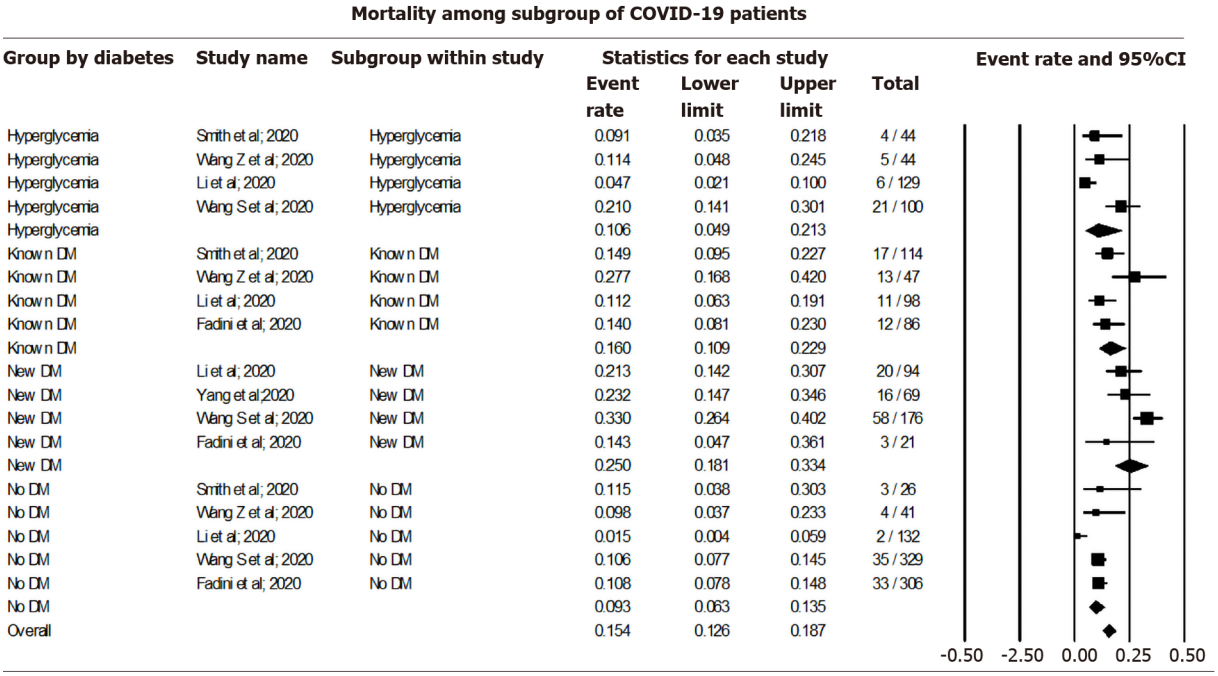


**Figure 4 Mortality among coronavirus disease 2019 cases with subgroup analysis based on their diabetes status.** COVID-19: Coronavirus disease 2019; DM: Diabetes mellitus.

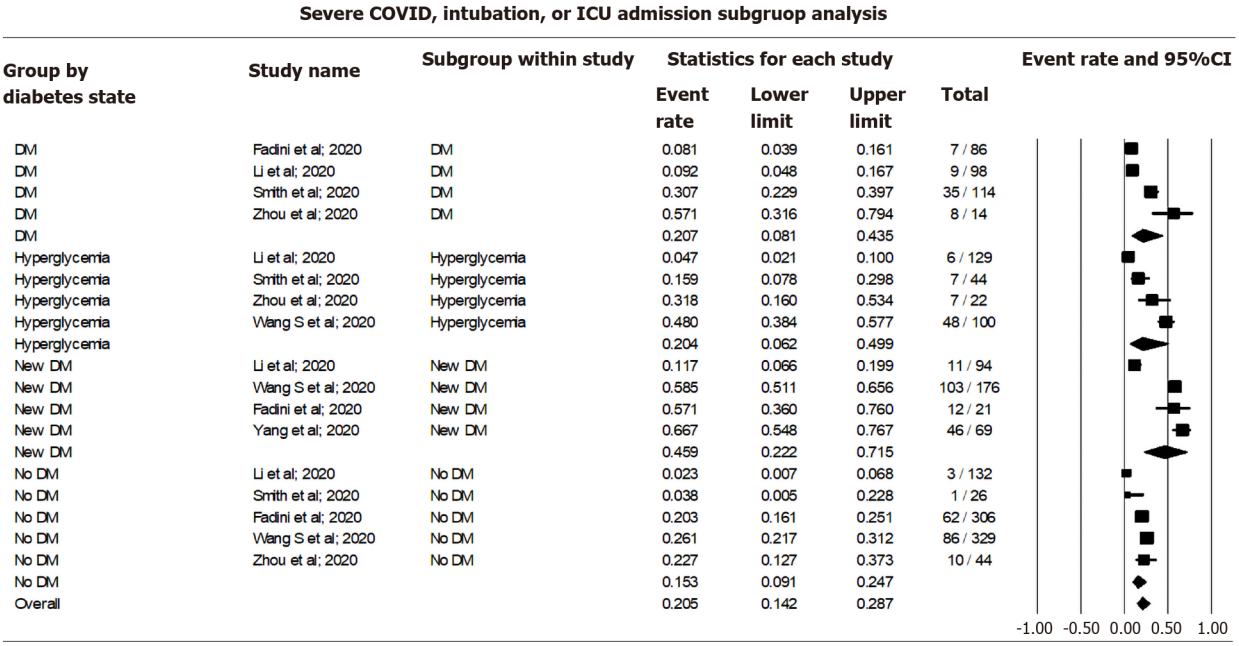


**Figure 5 Occurrence of adverse events among coronavirus disease 2019 cases with subgroup analysis based on their diabetes status.** COVID-19: Coronavirus disease 2019; DM: Diabetes mellitus; ICU: Intensive care unit.

## CONCLUSION

The pooled prevalence of COVID-19 associated DM was 19.70%, and for COVID-19 associated hyperglycemia was 25.23%. Among COVID-19 patients, higher mortality rates and adverse events were seen in patients with new-onset DM compared to those with pre-existing diabetes, those with COVID-19 associated hyperglycemia, and those without diabetes.

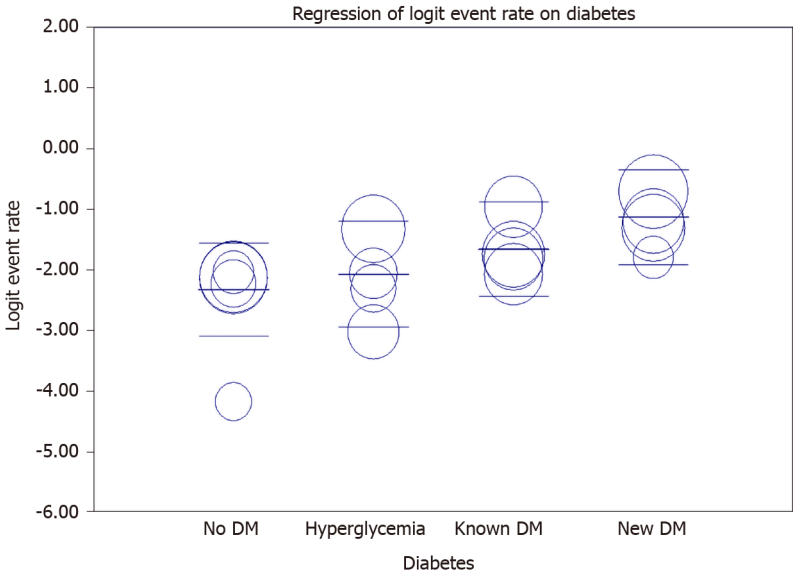


**Figure 6 Meta regression of diabetes status and mortality.**

## ARTICLE HIGHLIGHTS

### *Research background*

Diabetes has been shown to be associated with worsening severity of disease and poor prognosis in coronavirus disease 2019 (COVID-19). Interestingly, various cases of new onset diabetes mellitus (DM) were seen in patients with COVID-19. The virus is believed to bind to angiotensin-converting enzyme-2 receptors leading to increased angiotensin II and subsequent decreased insulin secretion.

### *Research motivation*

In relation to various theories and proposed mechanisms of how COVID-19 may lead to abnormal glucose homeostasis, our study was conducted to evaluate new onset DM in COVID-19.

### *Research objectives*

The study aimed to pool the prevalence of new onset DM and hyperglycemia in COVID-19 patients and compare various outcomes such as mortality, intubation and complications among infected patients who had hyperglycemia or preexisting DM or new onset DM or normal blood sugar levels.

### *Research methods*

Meta-analysis of Observational Studies in Epidemiology was used for the meta-analysis. Studies were screened using Covidence after searching various databases including PubMed, PubMed Central, Embase and Scopus. Comprehensive meta-analysis software was used for data analysis.

### *Research results*

The results showed that 19.70% and 25.23% of patients had COVID-19 associated DM and hyperglycemia, respectively. The mortality rate was highest among COVID-19 associated DM patients (24.96%) followed by patients with preexisting DM (16.03%), and was least in non-diabetic patients (9.29%). The occurrence of adverse events was highest among COVID-19 associated new-onset DM patients followed by patients with preexisting DM, COVID-19 associated hyperglycemia and non-diabetic patients.

### *Research conclusions*

COVID-19 was associated with hyperglycemia and new-onset DM. Infected patients with new onset DM had worse prognosis in terms of mortality and adverse events.

### *Research perspectives*

The findings of this study should alarm clinicians that new onset diabetes and

hyperglycemia is a bad prognostic factor for COVID-19.

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