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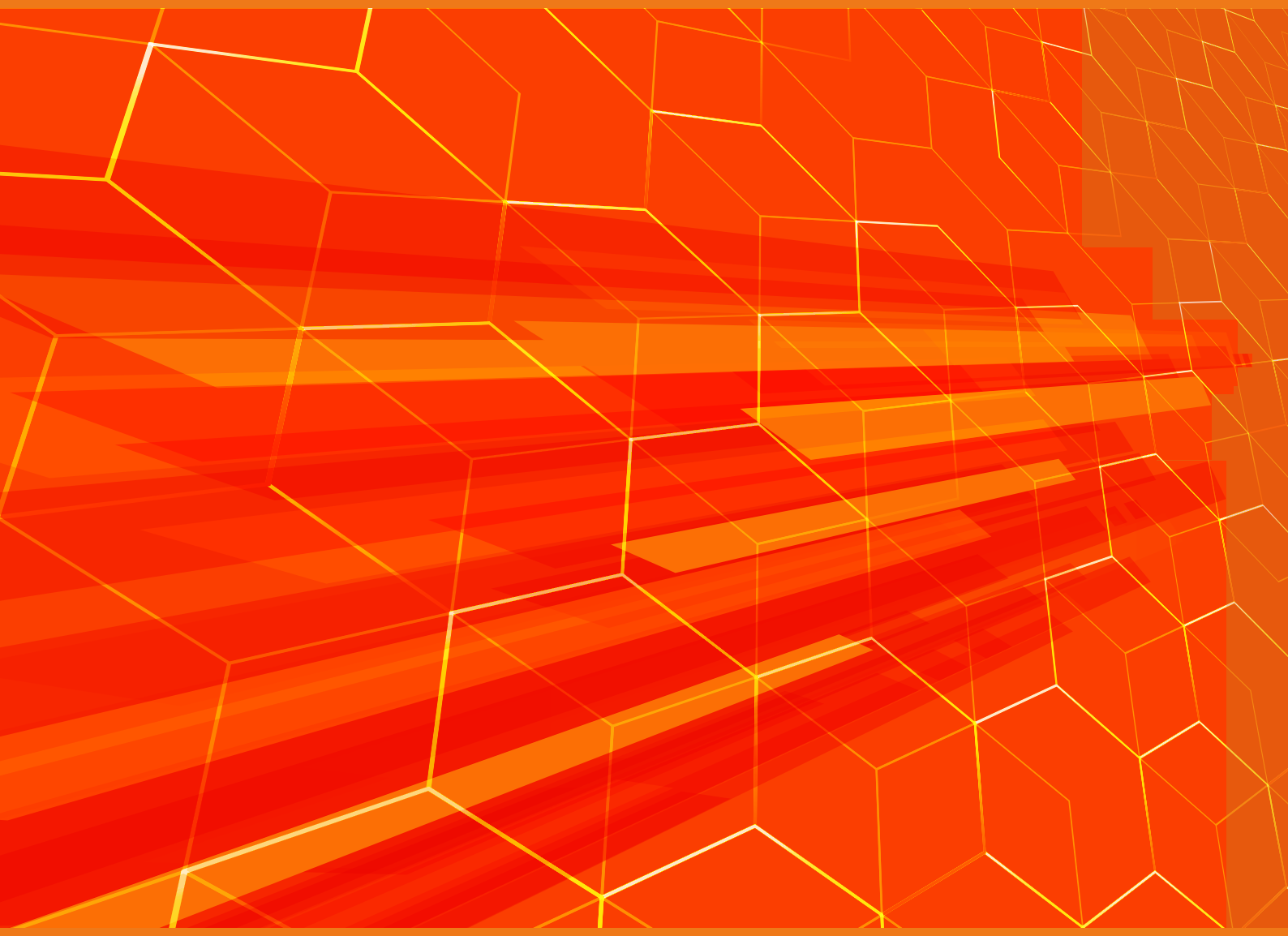

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REVIEW

Corticosteroid treatment reduces headache in eosinophilic meningitis: a systematic review

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ABSTRACT

Background: Eosinophilic meningitis (EOM) is an emerging parasitic disease that can be found worldwide, of which acute severe headache is a presenting symptom. Although such headaches may persist for up to 2 months, studies have found corticosteroid to be effective in reducing this symptom. As the most recent systematic review was published in 2015, the aim of this study was to provide a more up-to-date examination of the role of corticosteroids in EOM.

Methods: We included randomized controlled trials of corticosteroid treatment for EOM regardless of comparators. Research articles published in five databases were searched and evaluated. The primary outcome was headache, which was compared among various treatment regimens.

Results: We found a total of 257 articles after duplication removal. Of those, two met the study criteria. According to these studies, oral prednisolone alone or in a combination of albendazole resulted in fewer patients with headache after a 2-week course of treatment compared with placebo (maximum of 9.1% vs. 45.5%). The duration of headache was also shorter in the prednisolone arm vs. placebo (maximum of 5 vs. 13 days). There were no serious side effects reported.

Conclusion: A 2-week course of treatment with oral corticosteroid with or without albendazole reduced headaches in patients with EOM.

Keywords: *Angiostrongylus cantonensis*, Headache, Prednisolone

Background

Eosinophilic meningitis (EOM) is defined by a cerebrospinal fluid (CSF) eosinophil count of less than 10% of total CSF white blood cells (1). The main cause of EOM is *Angiostrongylus cantonensis* infection. Humans become infected mainly through the consumption of or contact with contaminated food or drink (2). Patients with EOM usually present with acute severe headache (1). As fever and neck stiffness are uncommon in EOM, it may be misdiagnosed if physicians are not sufficiently aware of the condition (1). History of consuming raw freshwater snails or contaminated food is a crucial clue for EOM diagnosis (1,2).

EOM is an emerging and often neglected parasitic disease that can be found worldwide (3). A review published in 2008 found 2,827 cases of EOM reported from 30 countries (4). In addition, there are an estimated 0.3-2 infected people per 100,000 population per year with occasional outbreaks (5). From 1997 to 2008, there were 13 outbreaks in mainland China alone (6). If EOM is left untreated or undiagnosed, individuals are at increased risk of several severe neurological complications such as encephalitis or radiculomyelitis (7-9). The risk of encephalitis in untreated or undetected cases of EOM is 26% per day (7).

If treated with analgesic alone, the severe headaches caused by EOM can last up to 49 days (10). However, this duration can be reduced with corticosteroid administration (11), as it decreases inflammation in the meninges and brain. The most recent systematic review on corticosteroids in EOM was a Cochrane review published in 2015 (12). It included one randomized controlled trial, which compared corticosteroids

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with placebo. It is recommended that systematic reviews be updated every 2 years to include any newly published articles (13). We thus updated and examined studies of corticosteroid treatment in EOM regardless of comparators.

Materials and methods

This study is a systematic review focused on the effects of corticosteroids in human EOM. We included randomized controlled trials of corticosteroid treatment for EOM regardless of comparators. The types of interventions were corticosteroids vs. placebo or other active treatments. The participants in the studies examined were EOM patients aged 15 years or older (12). Those who had already taken corticosteroids prior to study participation were excluded.

We searched five databases in this review: PubMed, Central database, Scopus, CINAHL Plus, and Web of Science. The search terms used were "meningit*, eosinophil*", "*Angiostrongylus cantonensis*", and "randomized controlled trials". The full list of search terms are shown in Appendix 1 (See supplementary material). The final search was performed on November 22, 2019.

After duplication removal, initial screening was carried out for nonrelevant articles. Studies were considered relevant if they had been conducted to evaluate the role of corticosteroids in any form of headache caused by EOM. The full-text reports were subsequently reviewed by two independent authors (SK, KS). Of these, any randomized controlled trials were included in the final analysis. The primary outcomes were number of patients with persistent headache at 2 weeks after treatment and duration of headache by corticosteroid treatment regimen. Other outcomes included number of patients who underwent repeated lumbar puncture and side effects of treatment. We presented frequency with percentage for categorical variables. Continuous variables were described using median and range. Risk ratios (RR) with 95% confidence intervals (CI) were used to compare to compare a risk between two groups from randomized controlled trials (RCTs).

Biases of eligible studies were evaluated across six domains (sequence generation, allocation concealment, blinding of participants/personnel and outcome assessors, incomplete outcome data, selective outcome reporting, and other potential sources of bias) by two authors independently (SK, KS). Biases were categorized as low risk, high risk, or unclear according to the guidelines specified in the Cochrane Handbook for Systematic Reviews of Interventions (12). Disagreements were reviewed and reported by a third reviewer (CN).

Results

We found a total of 288 articles in the following five databases: Scopus (254 articles), PubMed (21 articles), Web of Science (9 articles), Central database (3 articles), and CINAHL Plus (1 article). Two hundred fifty-seven remained after duplication removal (Fig. 1), of which 252 were excluded as nonrelevant. In total, five articles were selected for full-text review (11,14-17). Of these, only two met the study criteria. With regard to the excluded studies, one did not evaluate outcomes or headache, and the other two were not randomized controlled trials.

The first of the remaining studies was published in 2000 and enrolled 110 EOM patients (11). This study found that treatment with prednisolone resulted in fewer patients experiencing headaches at 2 weeks after treatment and shorter duration of headache when compared with placebo (9.1% vs 45.5%; RR 0.20 with 95% CI: 0.08 to 0.48 and 5 days vs. 13 days, respectively). The number of cases of repeated lumbar puncture was also significantly higher in the placebo group than in the prednisolone group (22 times vs. 7 times; RR 0.32 with 95% CI: 0.15 to 0.68), as shown in Table I.

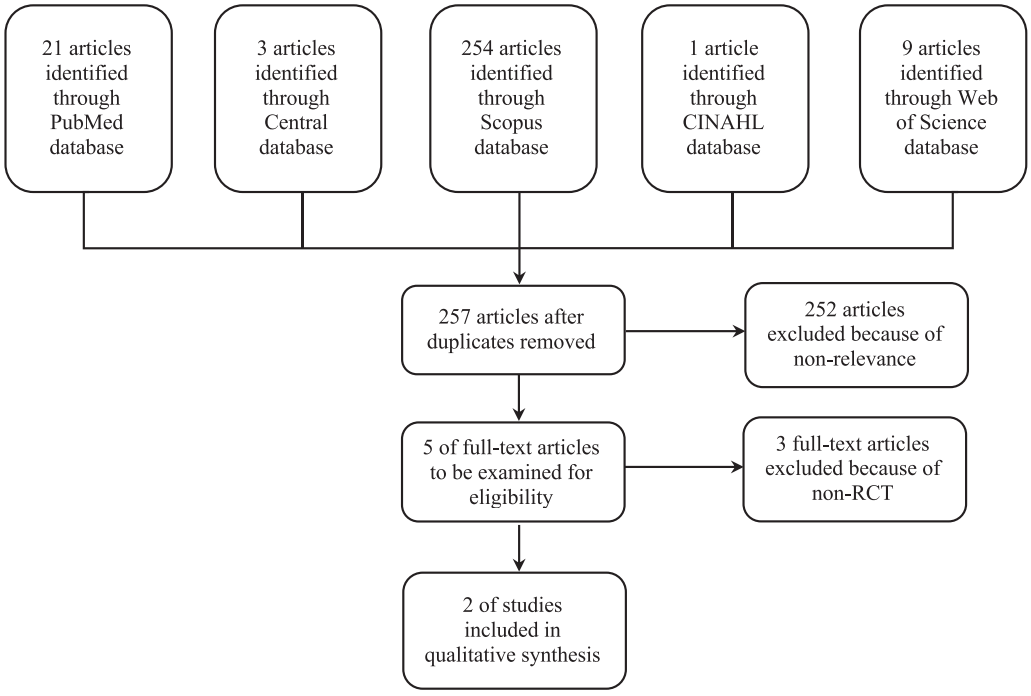

Fig. 1 - Study flow of the systematic review on corticosteroid treatment in eosinophilic meningitis. RCT = randomized controlled trial.

TABLE I - Two randomized controlled trials of corticosteroid treatment in eosinophilic meningitis

Factors	Study 1: 2000		Study 2: 2009	
	Active	Control	Active	Control
Treatment	Pred	Placebo	Pred + albendazole	Pred
No. of patients	55	55	53	51
No. of patients with headache at 2 weeks (n, %)	5 (9.1%)	25 (45.5%)	0	1 (2.0%)
Median (range) duration of headache, days	5 (1-60)	13 (1-56)	3 (1-14)	3 (1-15)
No. of patients with repeated LP (n, %)	7 (12.8%)	22 (40.0%)	NA	NA

LP = lumbar puncture, NA = not available, Pred: prednisolone.

The second study was published in 2009 and conducted in 104 EOM patients (17). It compared prednisolone plus albendazole with prednisolone alone and found no significant difference in terms of the primary outcome. No data on repeated lumbar puncture were reported (Tab. I).

Both studies were conducted in Thailand, used only oral prednisolone, and reported no serious side effects from either prednisolone or prednisolone plus albendazole. Allocation, concealment, and stratification were not reported in either study. The second study was not blinded (Fig. 2).

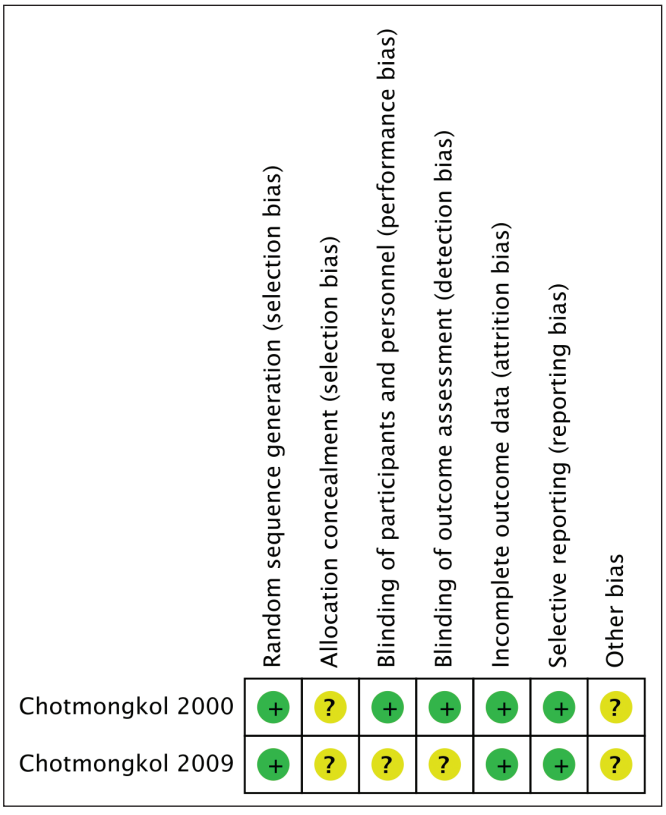

Fig. 2 - Biases of the included studies in the systematic review on corticosteroid treatment in eosinophilic meningitis.

TABLE II - Previous single-arm studies of corticosteroid treatment in eosinophilic meningitis

Factors	Study 1: 2004	Study 2: 2004	Study 3: 2006
Treatment	Prednisolone 1 week	Prednisolone + albendazole	Prednisolone + mebendazole
No. of patients	52	26	41
No. of patients with headache at 2 weeks (n, %)	6 (11.6%)	3 (11.6%)	4 (9.8%)
Median (range) duration of headache, days	NA	4 (1-17)	3 (1-20)
No. of patients with repeated LP (n, %)	1 (2.0%)	0	3 (7.4%)

LP = lumbar puncture, NA = not available.

Discussion

Although there were two eligible studies, we were unable to perform a conventional meta-analysis or network meta-analysis due to the inconsistent treatment arms and incomplete cycle, respectively. As a result, this report was defined as a systematic review.

In both studies, oral prednisolone with or without albendazole yielded favorable outcomes in terms of both numbers of EOM patients with headache and duration of headache after 2 weeks of treatments. These findings were compatible with those of other single-arm studies examining the effects of prednisolone alone vs. prednisolone plus anthelmintics (15,18,19), as shown in Table II. The corticosteroid-based regimen with albendazole and that with mebendazole yielded comparable headache durations in the two studies in this review (3-4 days vs. 3-5 days), but the 1-week prednisolone treatment had higher numbers of patients with headache at 2 weeks (11.6%). There were no serious side effects of corticosteroid found in any of the five studies examined.

There were some limitations in these studies. First, both studies were conducted in northeast Thailand, an area endemic for EOM. Second, some information was not reported in the randomized controlled trials such as concealment or blinding. These items were reported as possible biases (Fig. 2). Finally, only oral prednisolone was administered for a duration of 2 weeks.

Conclusion

Two weeks of treatment with oral corticosteroid with or without albendazole reduced headaches in EOM patients.

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Disclosures

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Conflict of interest: The authors declare that they have no conflicts of interest.

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REVIEW

Tamarind (*Tamarindus indica* L.) Seed a Candidate Protein Source with Potential for Combating SARS-CoV-2 Infection in Obesity

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ABSTRACT

Introduction: Obesity and coronavirus disease (COVID)-19 are overlapping pandemics, and one might worsen the other.
Methods: This narrative review discusses one of the primary mechanisms to initiate acute respiratory distress syndrome, uncontrolled systemic inflammation in COVID-19, and presents a potential candidate for adjuvant treatment. Blocking the S protein binding to angiotensin-converting enzyme 2 (ACE-2) and the 3C-like protease ($3CL^{pro}$) is an effective strategy against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.
Results: Host proteases such as FURIN, trypsin, and transmembrane serine protease 2 (TMPRSS) act in S protein activation. Tamarind trypsin inhibitor (TTI) shows several beneficial effects on the reduction of inflammatory markers (tumor necrosis factor α [TNF-α], leptin) and biochemical parameters (fasting glycemia, triglycerides, and very low-density lipoprotein [VLDL]), in addition to improving pancreatic function and mucosal integrity in an obesity model. TTI may inhibit the action of proteases that collaborate with SARS-CoV-2 infection and the neutrophil activity characteristic of lung injury promoted by the virus.
Conclusion: Thus, TTI may contribute to combating two severe overlapping problems with high cost and social complex implications, obesity and COVID-19.

Keywords: $3CL^{pro}$, ACE-2, COVID-19, FURIN, HNE, Inflammation, TMPRSS

Introduction

The relation between obesity and severe COVID-19

Coronavirus disease (COVID)-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) coronavirus, is a serious threat to health systems around the world. It overlaps with obesity, another global pandemic (1-3). SARS-CoV-2 infection started in December 2019 and spread rapidly because its transmission occurs through the airways.

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The consequences of SARS-CoV-2 infection range from self-limited flu to fulminant pneumonia, respiratory failure, and death (4,5). Although the new coronavirus has mutations, it is still unclear whether they are related to its virulence (6), which might soon be answered through ongoing studies (7).

Advanced age is a risk factor that leads to the worsening of the clinical condition of the disease, placing the elderly as a vulnerable population with a high risk of death. However, severe cases also occur in middle-aged or younger people, and one of the possible contributing factors for this is the already known relationship between nutritional status and the prognosis of viral infections, which can contribute to the improvement or worsening of the disease (8-10).

According to Butler et al (11) and Muscogiuri et al (12) broader access to a healthy and balanced diet rich in vitamins, minerals, bioactive compounds, and antioxidants is essential to assist in reducing susceptibility to SARS-CoV-2 infection, in addition to the complications that can occur in the long term. The regional councils of the four United Nations Agencies (Food and Agriculture Organization of the United Nations—FAO, United Nations Children's Fund—UNICEF, World Health Organization—WHO, and World Food Program—WFA) issued

a joint statement on nutrition in the context of the COVID-19 pandemic in Asia and the Pacific. The recommendations emphasized the need for continuous adoption of advice and strategies ensuring nutritional surveillance, food quality, and food security.

On the other hand, the current moment demands the isolation of the world population as a strategy to contain the infection spread. Isolation can increases consumers' preference for industrialized and ultra-processed foods. This preference occurs due to the practicality, hygienic safety, longer shelf life, of industrialized and ultra-processed foods compared to fresh and unprocessed products (13). This possible trend may increase food insecurity and directly impact weight gain (13-16), increasing the risk of overweight and obesity, which can persist for an extended period (17).

Diet composition and obesity have a fundamental role in the coregulation of adaptive immunity (18), influencing the modulation of the immune response and, consequently, affecting the severity of respiratory diseases and other infections (9). In general, adiposity can impair the ventilation of the base of the lungs, resulting in less oxygen saturation in the blood (19). Thus, in COVID-19, the need for treatment in intensive care units (ICUs) increases, and intubations are technically more challenging in obese patients, in addition to the difficulty in obtaining a diagnosis by imaging techniques.

According to the review study by Johnson et al (20), obesity damages various organs, which increases the susceptibility to several diseases, such as metabolic disorders, cardiovascular diseases, cancer, and viral infections such as influenza and COVID-19 (1,21).

There is an intrinsic relationship between fat distribution and metabolic health status. The location of fat deposition in the body is determinant for health, and the ectopic deposition of triglycerides in the abdominal region, mainly visceral fat, causes a phenotype of high cardiometabolic risk—even for individuals who have a normal body mass index (BMI) but with a high abdominal circumference (AC)—due to unregulated cytokine secretion pathways. In addition, inflammation and increased release of circulating fatty acids is associated with visceral fat. Thus, the fat distribution phenotype contributes to metabolism, leading to metabolically unhealthy individuals (22).

Thus, visceral obesity can be an important risk factor to increase the severity of COVID-19 (23). Studies with patients affected by this disease in Germany and Italy have shown that as the area of visceral adipose tissue increased, there was a greater need for intensive therapy, regardless of other factors, such as age, sex, and associated diseases (24,25). Another study performed in China with COVID-19 patients showed that higher visceral and subcutaneous adipose tissue were independent risk factors for critical illness (26). In addition, visceral adipose tissue positively regulates the expression of plasminogen activator inhibitor 1, thus generating an increased risk of developing thrombosis in patients with visceral obesity affected by COVID-19 (27). Thus, understanding the metabolically unhealthy individual is essential, carefully assessing the response to SARS-CoV vaccination (23), as obesity and impaired metabolic health generate a potentially reduced immune response, which can negatively affect the vaccine's effectiveness (28-31).

Obesity is characterized by a state of low-grade chronic inflammation related to changes in immune cells, including the number and types of cells present in the inflamed tissue. At the beginning of weight gain, immune cells infiltrate adipose tissue, contributing to persistent adipose inflammation and insulin resistance. Macrophages are classified into two subtypes of phenotypes, M1 proinflammatory and M2 anti-inflammatory. In eutrophic individuals, the M2 subtype is distributed throughout adipose tissue, producing interleukin (IL)-10 and expressing arginase-1 for collagen synthesis, which is important for promoting tissue repair. During the progression of weight gain to obesity, the M1 subtype becomes dominant, spreading inflammation through the production of mediators, such as tumor necrosis factor α (TNF-α), IL-1β, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor 1 (PAI-1) and reactive oxygen species (ROS). As a result, M1 macrophages interrupt insulin sensitivity in adipose tissue and the liver (20).

The hormone leptin overlaps when referring to the link between obesity and inflammation, since leptin synthesis is directly proportional to the amount of adipose tissue, and inflammatory cytokines stimulate this synthesis. These inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and interferon gamma (IFN-γ), coagulation factors (fibrinogen and PAI-1), acute-phase protein (C-reactive protein and amyloid serum A [SAA]), white blood cell count, and chemokines are markers of inflammation. In patients with obesity, these markers are commonly elevated, and with the reduction of excess weight, there is a reduction in their plasma concentrations (32). There are also anti-inflammatory adipokines, such as IL-4, IL-5, and IL-10, which can be observed in obesity at low concentrations. Thus, an imbalance between these anti- and inflammatory cytokines can induce the inflammatory response (33).

Considering that obesity negatively affects the immune system, there is, therefore, a clear relationship with the higher susceptibility to the more severe COVID-19. This close relationship occurs due to the increased release of inflammatory cytokines by disrupting the integrity of tissues (adipose and lymphoid tissue, intestines, and lungs), which alters the activation of leukocytes, impairing the action of the immune system. As a result, there is a direct impact on the healing process, which prolongs recovery and increases the risk of evolution from respiratory infection to severe diseases with a high risk of death (34).

It is important to highlight that SARS-CoV is responsible for coding 3C-like protease ($3CL^{pro}$), a cysteine protease (35), formally known as C30 endopeptidase that is chymotrypsin-like (36). It is considered a key component in polyprotein processing (37), which synthesizes nonstructural proteins and structural proteins, such as spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (38). Thus, this protease plays an important role in the replication and transcription of viral ribonucleic acid (RNA) (39).

Recently, Hoffmann et al (40) demonstrated that the new coronavirus enters human cells through glycoprotein S, found on the surface of the virus. This glycoprotein can bind to the angiotensin-converting enzyme 2 (ACE-2) located in human cells. ACE-2 receptors are expressed in the intestine, kidneys, lungs, and blood vessels, becoming targets for SARS-CoV-2 infection. Hoffmann et al (40) also

observed that the cellular transmembrane serine protease 2 (TMPRSS2) is needed for SARS-CoV-2 entrance in host cells. Xu et al (41) investigated the possible routes of SARS-CoV-2 infection in the oral cavity mucosa, exploring the expression of ACE-2 and the proportion and composition of the cells responsible for this function based on RNA-seq profiles and cell transcript-independent data. The results showed that ACE-2 could be expressed in the epithelial cells of the oral cavity, mainly in the tongue, oral and gingival tissues, indicating that the oral cavity mucosa can be a potential route for infection.

In the respiratory system, ACE-2 hydrolyzes angiotensin II to angiotensin 1-7, with an essential role in regulating the system. When ACE-1 activity is increased and ACE-2 is inhibited, intact angiotensin II acts through the angiotensin 1 (AT1) or 2 (AT2) receptor to exert proinflammatory responses and stimulate aldosterone secretion. As a result, these effects increase blood pressure and potentially cause hypokalemia, in addition to intensifying local vascular permeability, increasing the risk of respiratory distress syndrome. On the other hand, angiotensin 1-7 acts on the receptor pathway, leading to anti-inflammatory and antifibrotic responses that would be favorable to the recovery of patients with COVID-19 (42). Studies also describe the importance of the renin-angiotensin system (RAS) in the regulation of metabolism and the development of cardiovascular and inflammatory diseases (43-45). This system also modulates the endocrine and metabolic functions of adipocytes, hypertrophy, and hyperplasticity in obesity (46,47).

Obesity and its related comorbidities facilitate viral replication, increasing the risk of severe complications in SARS-CoV-2 infections. This increased risk occurs in obesity because the enlarged adipose tissue expresses higher levels of ACE-2 and, consequently, serves as a reservoir for the virus (48). The expression of ACE-2 in target tissues (lung, liver, and heart) is increased in diabetes (49), commonly seen in obese individuals. Hyperglycemia and type 2 diabetes also trigger the release of inflammatory cytokines, which in cases of COVID-19 can lead to higher release of cytokines (cytokine storm), generating an immune dysregulation that can lead to multiple organ failure and death (50). Obesity also induces deregulated lipogenesis that promotes the high expression of ACE-2 in the lungs (51). The increased leptin synthesis stimulated by inflammatory cytokines (33) can also worsen the clinical condition of obese patients with COVID-19.

Therefore, drugs that mediate metabolic responses targeting ACE-2 have been considered promising in the modulation of glucose metabolism and blood pressure control. These drugs may also prevent the entry of the new coronavirus through competitive pathways of ACE (9).

Results

Can the Kunitz trypsin inhibitor from tamarind seeds combat SAS-CoV-2 Infection in Obesity?

Considering these dysfunctions in patients with COVID-19, several studies and tests with drugs, traditionally used to control these changes, whether endocrine or metabolic, appear. Some adjuvant therapies for COVID-19 deserve special mention, such as immunomodulatory agents, immunoglobulin therapy, corticosteroids, and anticytokines used to control endocrine and metabolic changes (9,52,53).

A new mechanism related to the inhibition of the transmembrane protease serine 2, encoded by the TMPRSS2 gene, is an additional target for drugs for research. As already discussed, researchers have demonstrated that SARS-CoV-2 uses the SARS-CoV ACE-2 receptor to enter cells. The serine protease TMPRSS2 is also necessary for the initiation of protein S. A TMPRSS2 inhibitor, Camostat mesylate—a synthetic serine protease inhibitor (trypsin), approved in Japan for clinical use, blocked entry and could be another option treatment in COVID-19 (9,40). Other studies also highlight Camostat mesylate for the treatment of pancreatitis (9,52) currently in phase 2 clinical trial in COVID-19.

Furthermore, inhibition of 3CL ^{pro} can block protein S synthesis and coronavirus proliferation (39). Most studies have focused mainly on small-molecule compounds from virtual screening based on a 3CL ^{pro} structure (54). Several inhibitors, which can be classified as peptoids and non-peptidomimetics, have shown good inhibitory activity of this protease (39).

Protease inhibitors have been widely studied. One reason is that they are present in multiple forms in plants, animals, and microorganisms. In addition, protease inhibitors are natural protease regulators that are intrinsically involved in biological processes such as digestion, healing, viral replication, and the blood clotting cascade, among others, and that need precise regulation (55,56).

Thus, the prospect of peptides for use in biotechnology has led to a number of molecules' discovery. Its use in experimental models has exposed mammals to risks that have not yet been evaluated. Although the benefits of proteins and peptides substantially outweigh the potential harmful effects, their use is not without risks. There is a balance between the ability of peptides to induce desirable effects, that is, their bioactivity, and the potential toxic effects associated with their cell-penetrating properties (57).

Among protease inhibitors, trypsin inhibitors are widely extracted from seeds to be purified and characterized for application in various studies (58). These molecules have shown, in recent studies, performance in different mechanisms involving the control of obesity and its related effects, such as the production of hormones related to satiety, affecting the central nervous system and the small intestine; reduction of food consumption and weight gain; improvement of the lipid profile; and reduction of the inflammatory process associated with obesity, regardless of weight loss (59).

A trypsin inhibitor extracted from seeds of the tamarind fruit, *Tamarindus indica* L., belonging to the legume family, occurring in all regions of Brazil (60) has been extensively studied by our research group.

The first study to evaluate the potential of the tamarind trypsin inhibitor (TTI) to reduce weight gain was developed by Ribeiro et al (61). In this study, male eutrophic *Wistar* rats were fed a standard diet and received the isolated TTI by gavage for 11 days at a dose of 25 mg/kg. TTI administration reduced food intake in these animals. To better understand this reduction in consumption, another experiment using the same experimental model evaluated food consumption 1 h, 2 h, and 16 h after gavage with TTI, in addition to serum cholecystokinin (CCK), using doses of 25 and 50 mg/kg. TTI

reduced food consumption 16 h after its administration dose-dependently (61). In this study, TTI did not cause changes in the liver enzymes and serum proteins of *Wistar* rats, in addition to not affecting the histological aspects of the liver, stomach, intestine, and pancreas. In addition, TTI did not cause classical deleterious effects on protein digestion and, consequently, malnutrition at the doses tested, as demonstrated by the measurement of serum proteins.

Obesity is a condition that leads to numerous physiological changes, and the behavior of TTI in this condition needs to be assessed. For this, Carvalho et al (62) used *Wistar* rats with diet-induced obesity, assessing food consumption and other biochemical parameters, using the 25 mg/kg dose of TTI, as proposed by Ribeiro et al (61). In animals with obesity, TTI reduced food consumption without inducing weight loss. TTI reduced plasma TNF-α to undetectable concentrations, showing that the isolated inhibitor also influenced other aspects related to obesity, such as low-grade chronic inflammation (62).

In the same experimental model of obesity, Costa et al (63) observed that animals with obesity treated with TTI decreased food intake in a similar way to eutrophic animals. The animals with obesity treated with TTI showed a slight reduction in the Lee index, which, although not significant, was important because of the consumption of a diet rich in ultra-processed foods called High Glycemic Index and Glycemic Load (HGLI) (64). Although TTI did not alter the plasma CCK, it decreased the expression of the CCK-1R gene and plasma leptin in animals with obesity compared to the group with obesity without treatment (63).

TTI is characterized as a protein isolate with high inhibitory activity for trypsin, obtained in a process that generates enrichment of this protein, but not its purification (61). Therefore, it is possible that other molecules can influence the biochemical and bioactive characteristics of TTI.

Due to the many biological functions of TTI in the context of obesity, new technologies that could potentialize these functions were assessed. Nanotechnology, through nanoencapsulation, can promote the protection of bioactive substances in oral administration, which exposes these molecules to digestive processes, compromising active sites essential to biological activity. In addition, nanoencapsulation provides controlled release at a specific target and further intensification of the biological effect (65).

Thus, TTI was nanoencapsulated to increase the efficiency and stability of antitrypsin activity. The isolated and conjugated effects of chitosan and isolated whey protein on incorporation, antitrypsin activity, and TTI stability at different temperatures and pH conditions were investigated. The combination of chitosan and isolated whey protein (ECW) formed nanoparticles (109 nm), promoted a reduction in the half maximal inhibitory concentration (IC50; 0.05 mg) compared to pure TTI (0.21 mg), and preserved antitrypsin activity up to 80°C (35.0% [3.74]) compared to isolated agents and TTI, which have no inhibitory activity. Besides, the nanoparticles showed stability under different pH conditions. Thus, ECW proved to be an essential strategy to improve the function and stability of TTI (66).

To assess the safety of administration by gavage in addition to maintaining the inhibitory activity, the encapsulated TTI (ECW) was evaluated in a preclinical obesity study. Costa (67) also assessed cytotoxicity using the Caco-2 and CCD-18Co strains (human intestinal cells). The cytotoxicity assay exceeded 70% cell viability for Caco-2 and CCD-18Co when exposed to different concentrations of ECW. For the subacute blood toxicity of the bioactive dose of ECW, through a complete blood count, liver, and kidney function, there was an absence of subacute blood toxicity, demonstrated by the lack of toxic effects on the biochemical parameters evaluated.

Considering that ECW proved to be safe in the face of the parameters evaluated, its biological activity was tested after encapsulation (68). The nanoencapsulated TTI was also offered by gavage in a preclinical obesity model to test whether the isolated inhibitor would maintain its modulating properties on the altered biochemical parameters of the experimental model used. The biological activity was maintained, with emphasis on ECW, which significantly reduced blood glucose, Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), and raised high-density lipoprotein cholesterol (HDL-c), in addition to possibly favoring greater protection to pancreatic tissue.

The purification of these inhibitors is a necessary and critical step to define their structural characteristics and specificity of binding to other molecules. Isolating these proteins from all other proteins that are present in the same biological source is difficult because trypsin inhibitors have great molecular diversity (69). Medeiros et al (70) described the TTI purification process, obtaining a molecule with 100% inhibition for trypsin (pTTI), heat resistant and with a partially identified amino acid sequence (currently complete and with structural modeling—unpublished data), which had high homology with other trypsin inhibitors of the Kunitz family. pTTI also did not affect plasma CCK concentrations but reduced circulating leptin concentrations in animals with obesity at a dose of 730 µg/kg. Leptin is an important hormone in the energy balance, which is elevated in individuals with obesity, leading to the development of a resistance condition (70).

The effect of the purified trypsin inhibitor tamarind was also evaluated in a model of metabolic changes in *Wistar* rats with obesity and dyslipidemia. Obesity was induced using the HGLI diet (64). The animals treated with pTTI at a dose of 730 µg/kg had significantly lower food intake than the untreated group. However, the groups did not show differences in weight gain. pTTI showed great anti-inflammatory potential, reducing the relative expression of TNF-α messenger RNA and positive immunostaining in adipocyte immunohistochemical analysis of obese animals, as well as plasma cytokine concentrations (71). The anti-inflammatory effects of the isolated or purified inhibitor on adipose tissue were observed regardless of weight changes, which may suggest a direct beneficial effect on this tissue that may alter its structure.

This result shows the potential of this molecule since obesity is classically considered a disease that induces a low grade of chronic inflammation, causing changes in tissues, notably the intestinal mucosa (72) and adipose tissue (73). Due to these promising biological effects and safety demonstrated in preclinical studies with partially purified TTI, this inhibitor could be explored in alternative obesity therapies.

The purification process can enhance the functional properties of a molecule, making it necessary to reassess the safety of its use. At a dose approximately thirty times lower, pTTI performed the same biological activities as TTI. This

result emphasizes the need to assess whether its use generated harmful effects on the liver, a target tissue in toxicity studies; pancreas, a classically affected organ by trypsin inhibitors; adipose tissue, where pTTI, in previous studies, has shown anti-inflammatory effects; and intestine, a potential site of action that has not yet been studied.

Trypsin inhibitors have well-reported deleterious effects. Evidence has shown impaired growth due to poor digestion and absorption, metabolic changes in the pancreas, such as increased enzyme secretion, hypertrophy and hyperplasia, and metabolic disturbance in the use of amino acids (74).

Thus, pTTI was evaluated for possible toxic effects, focusing on the histopathological and stereological characteristics of organs involved in its metabolism, processing, and biological activity (liver and pancreas) and the tissues most affected by the obesity model (small intestine and visceral adipose tissue). pTTI at its bioactive dose did not cause signs or symptoms of general toxicity or potential damage to the liver and pancreatic tissue of obese *Wistar* rats. pTTI also promoted a protective effect on the intestines of these animals, reducing the loss of intestinal villi, a well-characterized damage in obesity models. pTTI reduced the presence of inflammatory infiltrates in perirenal (visceral) adipose tissue. Therefore, its use in the tested models is safe and presents anti-inflammatory effects (unpublished data).

Besides the effects mentioned above, TTI is a promising molecule concerning the mechanisms associated with the inhibition of genes involved in the production of ACE-2, such as TMPRSS2 and FURIN (Fig. 1). These genes probably act by

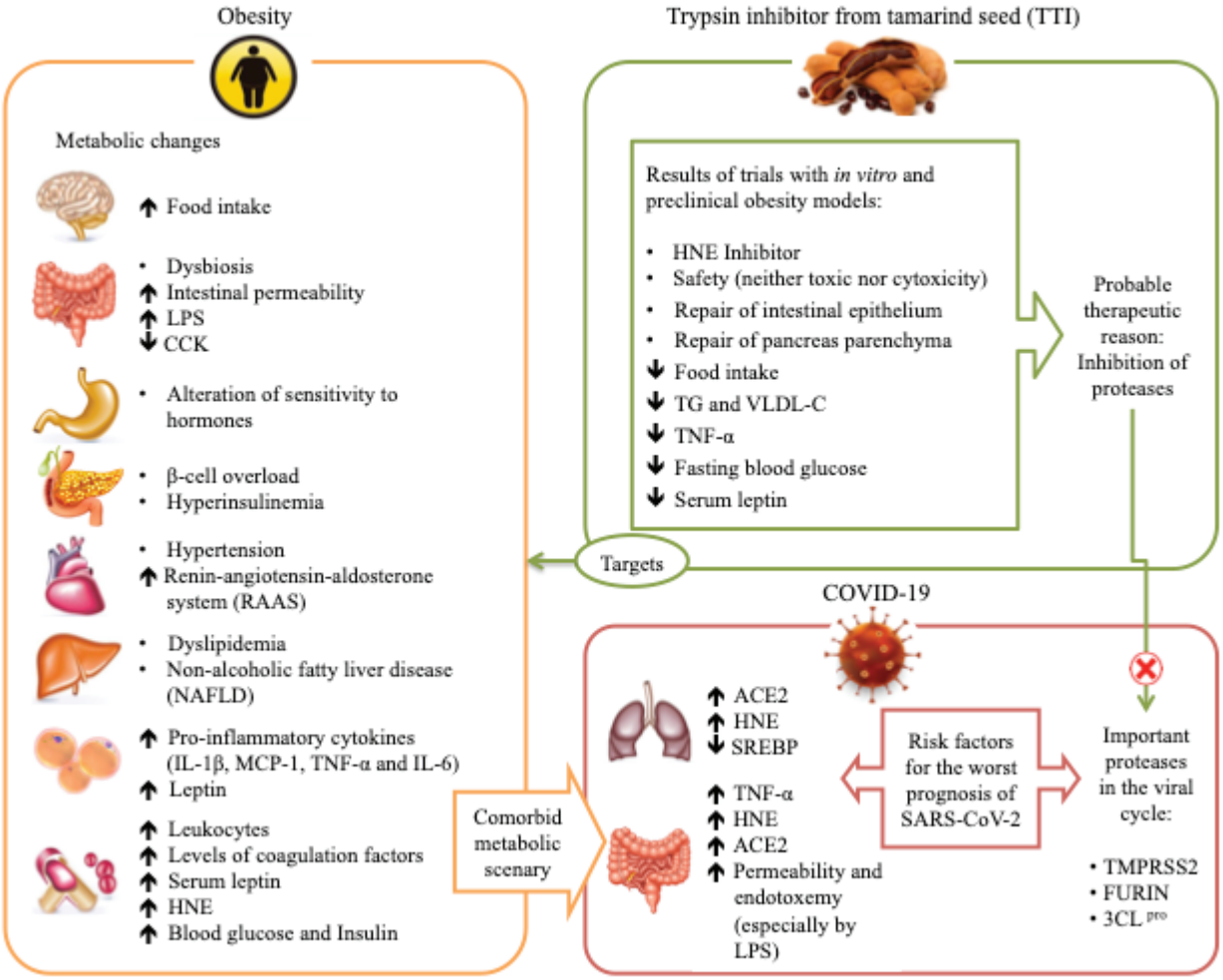

Fig. 1 - Obesity and associated comorbidities as risk factors for complications from SARS-CoV-2 infection, and hypothesis of the TTI mechanism of action. Obesity affects several organs, which have several responses, such as increased inflammation, changes in sensitivity and the action of hormones, dyslipidemia, and others. This metabolic deregulation favors increased expression of ACE-2, which is cleaved in the C-terminal segment by proteases such as TMPRSS2 and FURIN, and there is activation of the spike glycoprotein, so this process facilitates the entry of SARS-CoV-2 into the cells, causing viral infection. Also, 3CL ^{pro} is considered a key component in polyprotein processing and plays an important role in the replication and transcription of viral RNA. The TTI effects in in vitro and preclinical studies show several antiobesity and anti-inflammatory effects and appear to be possible inhibitors of the proteases TMPRSS2, FURIN, and 3CL^{pro}.

ACE-2 = angiotensin-converting enzyme 2; CCK = cholecystokinin; FURIN = member of the mammalian prohormone-protein convertases family; HNE = human neutrophil elastase; IL-1β = interleukin 1-β; LPS = lipopolysaccharide; MCP-1 = monocyte chemoattractant protein 1; SREBP = sterol regulatory element-binding proteins; TG = triglyceride; TMPRSS2 = transmembrane serine protease 2; TNF-α = tumor necrosis factor α; TTI = trypsin inhibitor from tamarind; VLDL-c = very-low-density lipoprotein cholesterol; 3CL ^{pro} = 3C-like protease.

changing the organism's epigenetic system responsible for the increase in ACE-2 expression, a potential target for antiviral intervention by SARS-CoV-2.

Adipokines secreted by adipose tissue can also affect airway function. Leptin is involved in neonatal lung development, surfactant production (75,76), and regulation of ventilatory impulse (76,77). Studies have consistently demonstrated the association of high concentrations of leptin and asthma (78,79). The leptin concentration was reduced with the use of TTI in animal models (63,70).

Several studies with trypsin inhibitors were related to obesity and its complications (59). According to Fook et al. (80), TTI showed selective activity, being highly effective against serine proteinases, especially against bovine trypsin and neutrophil elastase isolated from humans. The IC50 value was determined to be 55.96 µg/mL. The inhibitor also showed no cytotoxic or hemolytic activity in human blood cells. In addition, it exhibited different inhibition of the release of elastase by platelet-activating factor (PAF; 44.6%) and release by *N*-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP; 28.4%), preferentially affecting elastase release by PAF stimuli. This may indicate selective inhibition in the receptors of the PAF (80). The same research group in 2010 conducted another study and demonstrated that the soy inhibitor (SKTI) reduced lipopolysaccharide (LPS)-induced acute lung injury in a preclinical model, significantly suppressing the inflammatory effects caused by elastase in a dose-dependent manner, suggesting the route of inhibition of human neutrophil elastase as a promoter of the improvement (81).

Several computational molecular docking studies have been carried out with some compounds to model binding interactions of various $3CL^{pro}$ inhibitors and other proteases, such as TMPRSS2 (37,82-84). pTTI-derived peptides are also shown to be strong candidates for blocking these proteases since TTI is known to inhibit serine proteases such as trypsin and chymotrypsin, as previously demonstrated.

Conclusions

Therefore, trypsin inhibitors are promising alternatives, in addition to others already discussed in the scientific community, which can be used as adjuvants in COVID-19, especially in obese patients. Thus, the tamarind seed trypsin inhibitor may also be a preventive or adjuvant drug in the context of COVID-19, especially in worsened inflammatory conditions, such as obesity.

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REVIEW

The role of patient preferences in adherence to treatment in chronic disease: a narrative review

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ABSTRACT

Adherence to prescribed medication is important to the management of all diseases, especially those of chronic nature. Drug effectiveness is substantially compromised by therapy nonadherence. We reviewed the available evidences on the impact of patient preferences for therapy on adherence to a prescribed treatment in chronic diseases requiring long-term treatment. A search on PubMed retrieved 699 publications, leading to a selection of 12 publications: 6 on osteoporosis, 2 on moderate-to-severe asthma, 1 on type 1 diabetes, 1 on type 2 diabetes, 1 on kidney transplantation, and 1 on atrial fibrillation. Overall, 8 studies found a positive association between patient preference and adherence to therapy, while the others found no association. In general, overall adherence was considered to be high in the published studies. The reasons for a positive association included reduced dosing frequency, route of administration, lower costs, and favorable safety profile, which is related to the diverse nature of the pathology and its type and duration of treatment. A literature review suggests that achieving good adherence and persistence to therapy requires evaluation of patient preferences. In a period of increasingly limited resources, more effort is warranted to promote better adherence to therapy, especially when patients must self-manage their disease in the long term. Our results further highlight that insufficient attention has been given to the relationship between patient preference and adherence and point out the complex nature of adherence and the need for adequate patient education. More efforts are also needed to better understand the entity of cost savings for payers for specific treatments and the link with patient preference.

Keywords: Adherence, Chronic disease, Preferences, Therapy

Introduction

Patient preference to any prescribed medication or therapy is assuming an increasingly important impact in achieving clinically relevant outcomes. For example, the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) 2018 consensus report for type 2 diabetes emphasizes that patient choices and preferences are of utmost importance when choosing a therapy (1). Adherence to prescribed medication is important to the management of all diseases, and especially those of a chronic nature such as diabetes, dyslipidemia, and hypertension since disease-related symptoms are often absent, and because long-term drug effectiveness is substantially compromised by nonadherence to therapy. According to a report from the World Health Organization (WHO), adherence to long-term therapy is a problem of global magnitude and averages about 50% in developed countries (2). Moreover, poor adherence is associated with poorer outcomes and increased costs of care (2).

It has also been hypothesized that increasing adherence to medications may have a greater impact on a population's health than further improvements in medical therapies (3). Poor adherence decreases the effectiveness of therapy and leads to suboptimal use of resources as undertreated patients tend to develop complications and comorbidities (4,5). In type 2 diabetes and hypertension, poor adherence rates may result in poorer health outcomes and increased mortality (4-8). Several studies in chronic conditions have also demonstrated that poor adherence is associated with adverse consequences in terms of risk of hospitalization and overall costs (9). For diabetes, hypercholesterolemia, and hypertension, savings in all-cause medical costs have been reported when levels of adherence are high (10).

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Lack of adherence is influenced by multiple factors that include psychological issues, polypharmacy, and ease of obtaining and administering the medication, as well as patient motivation and education (11). Several studies on patient preferences for a variety of therapies have suggested that increased patient preference for one therapy over another is likely to be associated with increased adherence to therapy (7,11,12). However, only a limited number of studies have measured the potential impact of patient preferences on adherence, meaning that both dimensions have been quantified in the same study.

With mounting attention to overall costs of healthcare, increasing weight is being given to cost-effectiveness of any given treatment. Indeed, it has been suggested that patient preferences for treatment may have significant implications for cost-effectiveness by affecting not only costs but also clinical outcomes (13). The impact of adherence and its potential importance in both clinical and economic terms can be highlighted by considering data from Italy where only 31% of patients with chronic diseases refer that they are completely adherent to therapy (14). Accordingly, payers should implement cost-effectiveness models that incorporate patient preferences (13).

Based on all these considerations, we performed a literature search and narrative review with the objective of overviewing the available evidence on the impact of patient preferences for therapy on adherence to a prescribed treatment in diseases requiring long-term treatment to help improve decision-making processes for physicians and payers. Focus was placed on chronic diseases, since outcomes are likely to be more linked to adherence to therapy in the long term.

Materials and methods

Overall strategy

The methods, including the search strategy, were developed based on relevant literature (15-18). A narrative literature review (19) of published studies assessing compliance, adherence, or persistence and treatment preferences from 2010 (inclusive) to April 20, 2021, was conducted using the PubMed-NCBI database. The following search string was used: ("Patient Preference"[mesh] OR Preferen*[tiab]) AND (adherence[tiab] OR compliance[tiab] OR persistence[tiab] OR concordance[tiab]) AND ("Chronic Disease"[mesh] OR diabetes OR copd OR asthma OR "chronic obstructive pulmonary disease" OR hypertension OR osteoporosis OR "Chronic Disease" OR "Chronic condition") With the exception of Medical Subject Headings (MeSH) terms associated with each article, to maximize the retrieval of relevant articles, keywords were searched only within the title and abstract. To increase sensitivity, the keyword "preference" was truncated to "preferen" and followed by the special operator "*" (e.g., to catch both preference and preferential). Further truncation did not improve sensitivity and was associated with a drastic deterioration in specificity of articles retrieved. Similar tests were carried out for all the other keywords. While the search priority was the association between patient preferences and medication adherence, associations between patient preferences and clinical efficacy were included if relevant.

Inclusion and exclusion criteria

All studies that involved human subjects of any age with one or more chronic diseases were considered. Focus was given to the Centers for Disease Control and Prevention (CDC) definition of chronic disease as those lasting more than 1 year and which require ongoing medical attention, limit activities of daily living, or both (20), with the exclusion of cancer. Interventions of interest included those related to pharmacological treatment, with or without associated use of medical devices. All studies published in English language including a clear method of how patient preference and medication adherence or persistence (or efficacy) were objectively measured were included. Editorials, review articles, surveys, opinion papers, letters to the editor, case reports or case studies, study protocols, and guidelines were excluded. Publications describing adherence only, preferences only, or those mentioning only self-management behaviors, but not medication adherence, were also excluded, as were speculative articles where patient preference was not directly assessed.

Search results were imported into Microsoft Excel. Two reviewers were responsible for data extraction. A two-part study selection process was used: title and abstract review followed by full-text review. In the first step, two reviewers separately review the title and abstract of citations from the search to determine the eligibility based on the inclusion and exclusion criteria. All articles considered relevant by either reviewer were included in the full-text evaluation where the two reviewers independently evaluated the full-text articles to determine if they met inclusion/exclusion criteria. In case of disagreement about inclusion, full-text articles were reviewed again by both reviewers and if agreement was not reached, this was resolved by consultation with an independent third reviewer. As this is a narrative review, no statistical analyses were performed.

Results

The initial literature search on PubMed retrieved 699 publications. Despite the structured query, the majority of extracted papers were not relevant to the topic of interest.

Analysis led to a selection of 12 publications. Overall, 275 papers were excluded because they did not consider patients' treatment or device preferences or a specific chronic disease involved; 265 due to article type (e.g., review/editorial); 71 investigated only dietary patterns, exercise, or nonpharmacological devices (e.g., mandibular advancement device or mobile apps); 56 because they explored only patient preferences without quantitative measurements of actual adherence; 12 investigated the treatment decision-making style adopted and not an actual preference for treatment; 6 because adherence of one treatment option was not assessed or close to 100% by study design (e.g., a single injection performed at study enrollment), making pointless any possible comparison with other treatments considered; and 2 because they were related to a study already included (two publications by Kendler (21,22) were excluded as they refer to the one by Freemantle with final results of the study (23) which was included).

The selected studies, listed in Table I, included 6 papers on osteoporosis, 2 on moderate-to-severe asthma, 1 on type

TABLE I - Studies included on patient preference and adherence to therapy

Author	Disease	Main aim	Study design	No. patients	Main finding
Eliasaf et al. 2016 (25)	Osteoporosis	Determine compliance and persistence with osteoporosis therapy among postmenopausal women and to assess attitudes regarding treatment resumption among patients on drug holiday.	Prospective observational study	150	Compliance was high overall (80%); there was not a preferred medication among patients on drug holiday.
Freemantle et al. 2012 (23)	Osteoporosis	Compare treatment adherence between subcutaneous denosumab every 6 months and oral alendronate once weekly.	2-year, randomized, crossover study	250	Of 198 subjects expressing treatment preference, 92.4% preferred injectable denosumab over oral alendronate. Denosumab was associated with less nonadherence than alendronate (first year, 11.9% vs. 23.4%; second year, 7.5% vs. 36.5%).
Jarab et al. 2020 (24)	Osteoporosis	Explore factors associated with medication nonadherence in Jordan.	Observational	296	72.3% were nonadherent; patients were less likely to adhere to the prescribed medications with each unit increase in the number of prescribed medications and if they did not have a trust in the efficacy of the medications.
Oral et al. 2015 (27)	Osteoporosis	Examine the level of compliance and persistence in patients with postmenopausal osteoporosis receiving daily risedronate with either fixed dosing of three different timing regimens (A: before breakfast; B: in-between meals; C: before bedtime) or with flexible dosing.	Randomized, crossover study	448	49.7% preferred flexible and 50.3% fixed timing; a significant difference between the flexible and fixed regimens was seen in persistence in favor of the flexible regimen. Persistence was defined as the continuation of treatment at Week 26.
Sakai et al. 2014 (26)	Osteoporosis	Evaluate the effects of once-monthly minodronate on treatment persistence and clinical parameters in outpatients previously treated with daily or weekly bisphosphonate products.	Multicenter, prospective, open-label, observational study	264 and 133 patients were allocated into the Switch and Continue groups (continue daily or weekly bisphos-phonates).	Approximately 65% of patients were willing to switch to minodronate, with the predominant reason being "less frequent dosing more convenient." Treatment persistence was significantly higher in the Switch group. Persistence was assessed through Kaplan-Meier curves and analyzed using the log-rank test.
Thomasius et al. 2016 (28)	Osteoporosis	Compared the preference, acceptability, and tolerability of a reformulation of Calcichew D31 500 mg/400 IU and Calcichew D3 500 mg/800 IU with Adcal-D32 500 mg/400 IU and Kalcipos-D 500 mg/800 IU.	Phase IV, randomized, open-label, two-period, cross-over study	276	Patients preferred Calcichew D3 500/400 and Calcichew D3 500/800 over comparators as it is significantly less chalky and sticky, and is easier to chew and swallow. Acceptability did not affect compliance.
Al Hayek et al. 2020 (31)	Type 1 diabetes	Compare preferences and adherence for 6-mm and 8-mm injection needles.	Prospective cohort study	62	6-mm needles were associated with lower pain score, higher patient adherence, greater patient satisfaction, and better glycemic control compared to 8-mm needles.

(*Continued*)

TABLE I - (*Continued*)

Author	Disease	Main aim	Study design	No. patients	Main finding
Ishii et al. 2018 (32)	Type 2 diabetes	Compare the treatment satisfaction of four classes of oral agents: DPP-4 inhibitors, α-glucosidase inhibitors, biguanides, and sulfonylureas.	12-week, randomized, controlled, open-label study	64	DPP-4 inhibitor was the most preferable option in terms of treatment satisfaction and had the highest adherence.
Plaza et al. 2018 (29)	Moderate to severe asthma	Assess the impact of patient satisfaction with an inhaler on adherence and health outcomes.	Cross-sectional, observational, multicenter study	778	High specific satisfaction with an inhaler was associated with younger age, male gender, controlled asthma, high general satisfaction with treatment, high adherence to inhaler, nonsevere asthma, and no trouble with inhaler use.
Valero et al. 2019 (30)	Moderate to severe asthma	Compare patient satisfaction of three dry powder inhalers.	Register of an observational, multicenter study	328	Specific satisfaction with inhaler was significantly higher with Easyhaler™, as well as the percentage of patients with high satisfaction with inhaler. Scores for Easyhaler™ were also significantly better for items such as learning how to use, inhaler preparation, inhaler use, weight and size, and portability. There were no significant differences in asthma control or adherence between inhalers.
Wu et al. 2019 (33)	Atrial fibrillation	Compare persistence and outcomes of non-vitamin K antagonist oral anticoagulants (NOACs) vs. warfarin.	Prospective cohort study	344	Persistence with anticoagulants was low and dropped sharply at the third month; patients on NOACs had worse persistence at 3, 6, and 12 months than those on warfarin; the main reason for anticoagulant cessation was patient preference (adverse events, costs).
Hugo et al. 2021 (34)	Kidney transplantation	Evaluate conversion from immediate-release tacrolimus (IR-T) to prolonged-release tacrolimus (PR-T) in stable kidney transplant recipients.	12-month, non-interventional study	183	Among patients reporting a preference, 78.4% preferred PR-T. Following conversion from IR-T to PR-T adherence was high and kidney function was stable over 12 months.

1 diabetes, 1 on type 2 diabetes, 1 on atrial fibrillation, and 1 in patients with a stable kidney transplant. Overall, 6 studies found a positive association between patient preference and adherence to therapy, while the others found no association.

In general, overall adherence was considered to be high in the published studies. An exception was the study by Jarab et al in which 72% of patients were nonadherent; patients were less likely to adhere to therapy with each increase in the number of medications and when they did not trust the efficacy (24). In the study by Eliasaf et al, 80% of patients took their medication as directed (64% were on an oral medication, mostly bisphosphonates). However, in comparing treatments for osteoporosis, there was no preferred medication among patients on drug holiday (25). Freemantle carried out a 2-year randomized, crossover trial comparing subcutaneous denosumab every 6 months to oral alendronate once weekly, and reported that denosumab was associated with less nonadherence than alendronate in both years of the trial (first year, 11.9% vs. 23.4%; second year, 7.5% vs. 36.5%) (23). This greater adherence to denosumab was likely related to the greater preference with injections every 6 months rather than an oral drug weekly. In considering dosing frequency, Sakai et al similarly reported that more patients were willing to switch to a weekly bisphosphonate, rather than continuing to receive daily administration, with the predominate reason that less frequent dosing is more convenient. Furthermore, they observed that treatment persistence was significantly higher in the Switch group than the Continue group (89.8%

vs. 78.9%; p<0.003) (26). Oral et al compared flexible to fixed dosing regimens in women receiving daily risedronate (27). While there was no difference in preference of the two regimens, a significant difference was seen, with treatment persistence favoring the flexible regimen. Lastly, the trial by Thomasius compared preferences of a reformulated vitamin D/calcium supplement (28). While patients preferred a formulation that was less chalky and sticky, and easier to chew and swallow, acceptability had no effect on compliance.

Two of the remaining studies investigated the use of inhalers in patients with moderate to severe asthma. The trial by Plaza et al found that high patient satisfaction with an inhaler, independently of the medication contained within, was associated with better adherence and, accordingly, better control of asthma (29). As in the study by Valero et al, patients preferred an inhaler that was easy to use and easy to learn to use (30).

The study in type 1 diabetes by Al Hayek et al evaluated 6-mm vs. 8-mm injection needles in terms of adherence, satisfaction, and glycemic control (31). It was reported that the narrower needle was associated with greater satisfaction, better adherence, and improved glycemic control compared to the high-gauge needle. The trial by Ishii et al examined treatment satisfaction of commonly used oral treatments, reporting that DPP-4 inhibitors were preferred over α-glucosidase inhibitors, biguanides, or sulfonylureas (32). DPP-4 inhibitors were also associated with better adherence to therapy vs. α-glucosidase inhibitors, biguanides, or sulfonylureas (93%, 87%, 64%, and 62%, respectively). The authors concluded that the higher treatment satisfaction of patients can motivate therapeutic adherence, likely resulting in better glycemic control (32). Wu et al compared persistence and outcomes of non-vitamin K antagonist oral anticoagulants (NOACs) to warfarin. Patients on NOACs were seen to have worse persistence at 3, 6, and 12 months than those on warfarin; the main reasons for anticoagulant discontinuation cited were related to patient preference such as adverse bleeding events and costs (33).

Lastly, Hugo et al examined the effects of converting patients with a stable kidney graft from immediate-release tacrolimus (IR-T) to prolonged-release tacrolimus (PR-T) (34). Over a period of 12 months, there was no change in renal function, adherence was high; 98% of patients referred that they were satisfied or very satisfied with the therapy, while 78% preferred PR-T.

Discussion

Herein, we performed a literature search yielding 12 publications in order to overview the available investigations on patient preferences and adherence to therapy for chronic diseases. Of note, there were more studies on osteoporosis (n = 6) compared to other chronic diseases, although this may possibly be explained that during the time of these studies more costly injection therapies were beginning to replace more consolidated treatments. The majority (8/12) of the studies in the present review reported a positive association between patient preference and adherence to therapy. The reasons for a positive association included reduced dosing frequency, route and means of administration, lower costs, and a more favorable safety profile. These factors may be related to the diverse nature of the pathology and its treatment.

Four of the studies did not report a direct association between patient preference and adherence to treatment, although this can likely be explained by factors related to the individual study. Considering the studies on osteoporosis, that by Eliasaf et al reported that their study included highly motivated patients, that compliance was higher than that previously reported in the literature, and that patients on drug holiday did not have a preference for medication (25). All these factors may have had a role in the lack of significant differences. The trial by Thomasius et al found that while patients clearly had a preferred formulation, acceptability did not influence compliance to therapy (28). This result could be attributed to the short-term nature of that study, which followed patients for only 30 days. Oral et al, instead, observed no difference in preference of the two regimens, and thus an association between preference and adherence cannot be assessed (31).

Moreover, regarding the two studies on inhaler preference for moderate to severe asthma, the study by Plaza et al found a positive association between inhaler satisfaction with adherence, while that by Valero et al found no such association (29,30). However, those authors commented that due to the sample size in the subanalysis performed, the difference in patient satisfaction was not adequate to properly reflect differences in adherence and control of asthma.

In the trial included in the present analysis on type 2 diabetes, DPP-4 inhibitors were considered to provide greater satisfaction with treatment, possibly because of the less frequent dosing and less concern over adverse events compared to other treatments; such factors likely motivate patients to better adherence to therapy and lead to superior glycemic control (32). Moreover, since drug reimbursements were not completely covered by the healthcare system in which the study was carried out, the cost of DPP-4 inhibitors appeared to be a concern for some patients since management of type 2 diabetes is lifelong, thus highlighting the important role of patient preference for therapy (32). Another recent study in patients with type 2 diabetes on oral treatment showed that the vast majority still prefer a daily oral simple therapy, but the second choice was for weekly injection with a ready to use device (35). In the study included in type 1 diabetes on needle preferences, a smaller gauge needle was associated with greater satisfaction in terms of injection comfort and pain as well as greater overall satisfaction. These preferences led not only to greater adherence to therapy but also to significantly fewer hypoglycemic episodes per month and to significantly lower glycated hemoglobin (7.9% vs. 8.3%) (31).

In the study on atrial fibrillation by Wu et al, persistence to therapy with anticoagulants was strongly influenced by costs, as well as with adverse events to treatment (33). Indeed, patients prescribed NOACs had worse persistence than those given warfarin and the study was carried out in China where, as noted by the authors, NOACs are approximately 80 times more expensive than warfarin, which influences not only preference, but the ability to acquire the drug. In that

investigation, there was no difference in adverse events between NOACs and warfarin.

Among the studies on osteoporosis, Freemantle et al reported greater adherence and preference with a single subcutaneous injection every 6 months vs. an oral treatment once weekly (23). Moreover, patients crossing over to weekly oral therapy had poorer adherence after the switch, suggesting a treatment sequence effect. The differences in adherence and preference are likely to be related to multiple factors such as frequency of administration, belief in the need for and efficacy of individual therapies, and duration of treatment. The impact of different dosing regimens in osteoporosis was explored by Oral et al who reported that while there was no actual preference for a fixed or flexible dosing regimen, the latter was associated with significantly higher persistence to therapy (27). Overall persistence levels with flexible dosing were 86.0% compared to 78.9% with a fixed regimen. It was speculated that alternate timing for administration of therapy might aid patients with difficulty following traditional before-breakfast dosing, thus offering an additional option that can be more easily incorporated into diverse lifestyles and needs. The importance of timing and frequency of administration can be further highlighted in the study by Hugo et al in kidney transplant patients who preferred prolonged-release tacrolimus over an immediate-release formulation (34). Moreover, PR-T was also easier to remember that IR-T, with the main reasons cited for preferring the prolonged-release formulation being no need to take it in the evening and reduced pill burden.

In the study by Jarab et al carried out in Jordan on osteoporosis, adherence was dismal, and 72% of women were nonadherent to therapy (24). Similarly low rates of adherence in osteoporosis were also reported in a study from the US where nonadherents were 70% at 1 year, and 84% at 3 years (36). Increased number of medications was a primary reason for nonadherence in the study by Jarab et al, although it should be mentioned that patients were taking an average of five medications, three of which were for osteoporosis. This highlights the need to simplify the overall therapeutic regimen. The study also reported that lack of trust in efficacy was a major motivator for nonadherence, which stresses the need for patient education and establish a good physician-patient relationship.

The manifestation of medical and psychological complications of any disease worsens the quality of life and leads to inefficient use of resources. Taken together, the consequences of poor adherence compromise the possibility that a healthcare system can fulfill the needs of patients. The problem of adherence to therapy occurs whenever self-administration of the treatment is required, regardless of the type and severity of the illness and the possibility of access to treatment. While the problem may seem simple, poor adherence is multifactorial and is related to factors related to the patient, its treatment, and the disease (2,11). For example, patients with diabetes generally have other comorbidities such as hypertension, obesity, and depression, which may contribute to a less than adequate response to therapy (37). Costs also increase 2.2-3.2 times when the patient develops micro- and macrovascular complications that could be prevented (38). The costs of hospitalization, which include treatment of long-term complications such as heart disease, can account for more than 50% of total costs (38). Thus, economic and social benefits will become substantial only if the healthcare system can achieve a greater level of efficiency in promoting adherence to self-management of chronic disease. The ongoing challenge is demonstrated from a study in Italy, wherein 45% of patients with a chronic disease indicated that they did not understand their disease and were not able to self-manage it; only 31% declared that they were completely adherent to therapy and over 50% of patients said they had thought about abandoning care for their disease (14).

This suggests that in order to achieve good adherence and persistence, evaluation of patient preferences is a crucial step. A study conducted in Italy on preference toward different therapeutic options in injection-naïve and -non-naïve patients with type 2 diabetes clearly showed preference for simple oral therapies and with a low risk of side effects to therapy in injection-naïve subjects (39). The situation dramatically changed in patients who had already experienced injection therapy, who preferred that their therapy be administered with a ready to use device over the possibility of going back to oral therapy. Moreover, when considering all the different therapeutic attributes, among all patients the most preferred option was for a weekly injectable therapy with a ready to use device, while the first oral daily therapy ranked fifth (39).

The WHO has classified barriers to adherence into five dimensions: healthcare team/system, therapy, condition, patient, and socioeconomic-related barriers (2). Better understanding of the barriers to adherence is needed to overcome them and increase therapeutic outcomes in chronic disease. In the past, less emphasis was placed on adherence, but this paradigm seems to have been gradually changing over the years; this may be related to the aging population and increased prevalence of chronic disease. Considering other dimensions of the WHO classification, individual patient characteristics such as age and level of education may be related to adherence, in addition to factors such as costs depending on the specific setting. Thus, despite the somewhat limited evidence to date, it should be assumed that patient preference has an impact on adherence.

The present analysis has some limitations. Firstly, we considered only a single database and it is possible that additional studies were not retrieved from the literature search. Secondly, the inclusion criteria were very strict, with the result that only a small number of publications were included. Lastly, four studies included considered patient satisfaction as a proxy for preference. Although a higher satisfaction among treatments will likely result in a preference, this was not directly assessed in those cases.

Conclusion

Our results highlight that insufficient attention has been given to studying the direct relationship between patient preference and adherence, but seem to confirm its existence. It is hoped that this review can serve as a stimulus for further research in this little explored area, which could

help to better understand patient needs and desires, with the overarching aim of improving adherence to treatment in chronic diseases, understand the impact on total costs of treatment, and therefore achieve better outcomes. This review also stresses the complex nature of adherence, and the need for adequate patient education so that they understand the benefits of therapy for their particular condition. Costs are undoubtedly important when considering any treatment for a chronic disease, and more efforts are needed to better understand the entity of cost savings for payers for specific treatments and the link with patient preference. If a patient prefers a certain treatment over another, adherence is likely to increase along with better allocation of resources.

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ORIGINAL RESEARCH ARTICLE

Clinical factors predictive of appropriate treatment in COPD: a community hospital setting

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ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is a common respiratory disease. The appropriate treatment according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guideline was 19-60%. However, there are limited data on predictors of appropriate treatment in patients with COPD. This study aimed to evaluate risk factors of appropriate treatment in patients with COPD according to the GOLD guideline in a real-world community setting.

Methods: This is a retrospective study conducted at a community hospital. Inclusion criteria were adult patients diagnosed as COPD treated at a COPD clinic. The primary outcome was the appropriate treatment, defined by correct pharmacological treatment by the GOLD guideline according to the ABCD severity assessment. Clinical predictors of appropriate treatment were executed by stepwise multivariate logistic regression analysis.

Results: 136 patients with COPD met the study criteria. Of those, 100 patients had inappropriate treatment according to the GOLD guideline. Three factors were independently associated with the appropriate treatment including number of admissions, modified Medical Research Council (mMRC) score, and CAT score. These factors had adjusted odds ratio of 3.11, 2.86, and 1.26, respectively. Causes of inappropriate treatment were unavailability of long-acting muscarinic antagonist (LAMA) (51 patients; 79.69%), treated by inhaled corticosteroid (ICS) alone (12 patients; 18.75%), and treated with only bronchodilator (1 patient; 1.56%).

Conclusions: Appropriate COPD patients' treatment according to the GOLD guideline was 26.47% in community setting. Factors associated with severity of COPD were associated with prescribing appropriate treatments.

Keywords: CAT, hospitalization, mMRC

Introduction

Chronic obstructive pulmonary disease (COPD) is a respiratory disease mainly caused by smoking. Patients with COPD suffer from several symptoms, exacerbations, or hospitalizations leading to 2.6% of disability-adjusted life years (DALYs) and at least 3.2 million deaths globally (1). Diagnosis of COPD can be confirmed by evidence of incomplete irreversible airflow limitation without other causes. Treatment of COPD comprises both pharmacological and nonpharmacological modalities such as smoking cessation. Uncontrolled COPD may lead to COPD exacerbations and mortality (2). A study of 73,106 patients with COPD found that the mortality rate was 50% at 3.6 years after hospitalization (3), while another study found that in-hospital mortality rate was 2.6% (4).

There are several factors associated with COPD control such as COPD severity, patient compliance, correct inhaler technique, or nonpharmacological treatment (5,6). Even though patients with COPD had medication adherence of 51.0%, 85 out of 549 patients or only 15.5% were under control (7). Another factor that may be associated with COPD symptom control is appropriately prescribed medication (6,8,9). An undertreatment according to the guideline increases risk of COPD exacerbation with a coefficient of –0.179 ($p < 0.001$) (9). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) guideline recommends

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various pharmacological regimens based on COPD severity (10). In real practice, the appropriate treatment according to the GOLD guideline was 19-60% (9,11,12). However, there are limited data on predictors of appropriate treatment in patients with COPD. This study aimed to evaluate risk factors of appropriate treatment in patients with COPD according to the GOLD guideline in a real-world community setting.

Methods

This study was a retrospective study conducted at Chumpae Hospital, the largest community hospital in Khon Kaen province, Khon Kaen, Thailand. The inclusion criteria were adult patients who were diagnosed with COPD and treated at the COPD clinic. The diagnosis of COPD was made according to the GOLD guideline (10). The study period was between May and November 2019. The study protocol was approved by the institutional review board, Ministry of Public Health, Khon Kaen Branch, Thailand (61165).

Eligible patients were enrolled from clinical charts and evaluated for baseline characteristics, smoking history, risk factor for COPD, symptoms, chest x-ray, pulmonary function test, COPD type, 6-minute walk test (6MW), history of exacerbations, history of admission, and COPD assessment. History of cough was defined by the presence of cough for more than 2 weeks, while productive sputum more than 2 months was recorded. COPD assessment was evaluated by using modified Medical Research Council (mMRC), COPD Assessment Test (CAT), and COPD classification by the GOLD guideline or ABCD assessment. The primary outcome of the study was the appropriate treatment, which was defined by correct pharmacological treatment by the GOLD guideline to category A to D: a bronchodilator for group A; a long-acting bronchodilator (long-acting beta2-agonists: LABA or long-acting muscarinic antagonist: LAMA) for group B; LAMA for group C; and LAMA or LAMA plus LABA or inhaled corticosteroid (ICS) plus LABA for group D. Treatment other than this recommendation in a particular category was defined as inappropriate treatment. The inappropriate treatment was also classified as under- and overtreatment according to the recommendation for each category. Note that information retrieved for the study was at the initial therapy of each patient.

Statistical analyses

Patients were categorized into two groups by appropriateness of treatment. The studied variables were compared between both groups by descriptive statistics. For numerical variables, mean and SD was reported and compared between groups by using independent t-test or Wilcoxon Rank Sum test where appropriate. Numbers and percentages of each categorical variable were reported and compared between groups by Chi Square test or Fisher Exact test where appropriate. Clinical predictors of appropriate treatment were executed by stepwise multivariate logistic regression analysis. Those factors with a p value of less than 0.20 by univariate logistic regression were put in the subsequent multivariate logistic regression analysis. The goodness of fit of the final model was tested by Hosmer-Lemeshow method. The statistical analyses were executed by the STATA software (College Station, Texas, USA).

Results

There were 136 patients with COPD who met the study criteria. Of those, 100 patients (73.53%) were with inappropriate treatment according to the GOLD guideline. Between those with appropriate and inappropriate treatment groups, there were two significant factors in terms of baseline characters including cough and sputum production (Tab. I). The appropriate treatment group had higher proportions of patients with cough and sputum production than the inappropriate treatment group (77.78% vs. 54.00%; and 80.56% vs. 60.00%, respectively).

TABLE I - Baseline characters of patients with chronic obstructive pulmonary diseases (COPD) categorized by receiving appropriate treatment

Factors	Inappropriate n = 100	Appropriate n = 36	p value
Mean (SD) age, years	64.51 (8.83)	63.47 (10.51)	0.566
Male sex, n (%)	94 (94.00)	35 (97.22)	0.675
Occupation: agricultural, n (%)	93 (93.00)	31 (86.11)	0.412
Diabetes mellitus, n (%)	8 (8.00)	7 (19.44)	0.060
Hypertension, n (%)	42 (42.00)	17 (47.22)	0.588
Dyspnea, n (%)	100 (100.00)	37 (100.00)	NA
Cough, n (%)	54 (54.00)	28 (77.78)	0.012
Sputum, n (%)	60 (60.00)	29 (80.56)	0.026
Smoking history, n (%)			0.992
None	9 (9.00)	3 (8.33)	
Ex-smoker	72 (72.00)	26 (72.22)	
Current smoker	19 (19.00)	7 (19.44)	
Mean (SD) pack-year of smoking	21.49 (15.40)	26.82 (29.24)	0.498
Exposure to noxious particles, n (%)	6 (6.00)	2 (5.56)	0.999
Mean (SD) BMI (kg/m^2)	21.17 (3.69)	21.90 (3.95)	0.446

BMI = body mass index; NA = not available.

Between these two groups, the appropriate treatment group had shorter 6MW test (328.05 vs. 353.49 m) and lower mMRC (1.83 vs. 0.96) than the inappropriate treatment group significantly (Tab. II). But the average CAT score (15.88 vs. 7.22), average number of exacerbation (2.83 vs. 1.13 times), and average number of admissions (2.83 vs. 1.13 times) were significantly higher in the appropriate treatment group than the inappropriate treatment group (Tab. II) while the post-bronchodilator FEV1/FVC was significantly lower in the appropriate treatment group than the inappropriate treatment group (53.19 vs. 57.32; p = 0.033). COPD class D

TABLE II - Laboratory results and disease status of patients with chronic obstructive pulmonary diseases (COPD) categorized by receiving appropriate treatment

Factors	Inappropriate n = 100	Appropriate n = 36	p value
CXR, n (%)			
Normal, n (%)	53 (53.00)	19 (52.78)	0.982
Hyperinflation, n (%)	36 (36.00)	13 (36.11)	0.990
Post-bronchodilator FEV1, mL	66.86 (17.40)	60.30 (19.11)	0.061
Post-bronchodilator FEV1, %	6.02 (7.00)	6.80 (7.21)	0.602
Post-bronchodilator FEV1/FVC	57.32 (9.17)	53.19 (9.99)	0.033
COPD type, n (%)			0.999
Chronic bronchitis	3 (3.00)	1 (2.78)	
Emphysema	5 (5.00)	1 (2.78)	
Mixed	92 (92.00)	34 (94.44)	
Mean (SD) 6MW, meters	353.49 (72.91)	328.05 (87.16)	0.222
mMRC, n (%)	0.96 (0.66)	1.83 (0.88)	<0.001
0	22 (22.00)	1 (2.78)	
1	62 (62.00)	13 (36.11)	
2	14 (14.00)	14 (38.89)	
3	2 (2.00)	7 (19.44)	
4	0	1 (2.78)	
Mean (SD) CAT	7.22 (5.31)	15.88 (5.04)	<0.001
Exacerbation, n (%)	1.13 (2.40)	2.83 (2.09)	<0.001
Admission, n (%)	0.26 (0.75)	1.22 (1.17)	<0.001
Category, n (%)			<0.001
A	42 (42.00)	0	
B	30 (30.00)	0	
C	25 (25.00)	0	
D	3 (3.00)	36 (100.00)	

6MW = 6-minute walk test; CAT = COPD Assessment Test; mMRC = modified Medical Research Council dyspnea questionnaire; COPD category by the GOLD guideline.

was also found more in the appropriate treatment group than the inappropriate treatment group (100.00% vs. 3.00%).

There were five factors remaining in the final model predictive of appropriate treatment in patients with COPD (Tab. III). Of those, three factors were independently associated with the appropriate treatment including number of admissions, mMRC score, and CAT score. These factors had adjusted odds ratio of 3.11, 2.86, and 1.26, respectively. The final model had the Hosmer-Lemeshow chi-square of 10.72 (p = 0.218), indicating goodness of fit of the model. Causes of inappropriate treatment were unavailability of LAMA

TABLE III - Factors predictive of appropriate treatment in chronic obstructive pulmonary diseases (COPD) treated at community hospital

Factors	Unadjusted odds ratio (95% confidence interval)	Adjusted odds ratio (95% confidence interval)
Age	0.99 (0.95, 1.03)	0.94 (0.89, 1.01)
Diabetes	2.77 (0.93, 8.31)	3.10 (0.46, 20.84)
Admission	3.73 (2.02, 6.88)	3.11 (1.39, 6.97)
mMRC	4.47 (2.41, 8.30)	2.86 (1.18, 6.94)
CAT	1.32 (1.19, 1.47)	1.26 (1.13, 1.42)

Factors in the model included sex, smoking, cough, sputum, body mass index, chest x ray, 6-minute walk test, post-bronchodilator FEV1, post-bronchodilator FEV1/FVC, and exacerbation.
CAT = COPD Assessment Test; mMRC = modified Medical Research Council dyspnea questionnaire.

(51 patients; 79.69%), treated by ICS alone (12 patients; 18.75%), and treated with only bronchodilator (1 patient; 1.56%). Categorized by COPD category, overtreatment was found in categories A, B, and C, while undertreatment was reported in categories B, C, and D (Tab. IV).

TABLE IV - Proportions of under- or overtreatment by chronic obstructive airway disease category (n = 100)

Treatment	A (n = 42)	B (n = 30)	C (n = 25)	D (n = 3)
Undertreatment	0	6 (20.00)	2 (8.00)	3 (100.00)
Overtreatment	42 (100.00)	24 (80.00)	23 (92.00)	0

Discussion

This study showed that the appropriate treatment for patients with COPD was 26.47%: in category D at 100.00% (Tab. II). Compared with other three previous studies, this study had appropriate treatment rate comparable with the study at VA hospital in the US (27.2% vs. 18.7%) and lower than two studies from tertiary hospitals. In this community hospital setting, patients with category D had highest appropriate treatment rate than others at 100.00% (Tab. II). This pattern was also found in other studies which may indicate that severe cases of COPD tend to follow the GOLD guideline as they may have severe symptoms and required appropriate and several pharmacological therapies (10,13).

This study also found another similar pattern on appropriate treatment: low appropriate treatment rate in categories A, B, and C. First, we found that inhaled corticosteroid alone was used in 12 patients or 18.75%. The study from Italy also found that inhaled corticosteroid was overused despite the GOLD guideline that does not recommend it as shown in Table V (11). But, the attending physicians believe that it is more effective. A study from Sweden also found that inhaled corticosteroid was used inappropriately in 45.5% of patients with COPD regardless of categories: A 33.6%; B 46.2%;

TABLE V - Appropriate treatment in ABCD severity assessment in patients with chronic obstructive pulmonary disease

Study, year	Country	Setting	Total	A	B	C	D
Palmiotti, 2018	Italy	Pulmonologists	419/693 (60.5%)	57/142 (40.1%)	110/238 (46.2%)	18/41 (43.9%)	234/272 (86.0%)
Foda, 2017	USA	VA and University Hospital	164/878 (18.7%)	30/86 (34.9%)	19/379 (5.0%)	73/292 (25.0%)	42/121 (34.7%)
Chan, 2017	Hong Kong	Tertiary Hospital	262/450 (58.2%)	1/5 (20.0%)	7/164 (1.6%)	0/8 (0%)	254/273 (56.4%)
This study	Thailand	Community Hospital	36/136 (26.47%)	0/42 (0%)	0/30 (0%)	0/25 (0%)	36/39 (92.31%)

C 54.8%; and D 71.0% (14). An inappropriate use of inhaled corticosteroid was also found in 50% of patients with COPD in the UK (15). Another limitation for community hospital in this study is lack of LAMA in 79.69%: it may be due to unavailability and cost of LAMA.

Not surprisingly, factors predictive for appropriate treatments were factors indicating severe COPD including hospital admissions, mMRC, and CAT score (Tabs. II and III). Among these three factors, admissions and mMRC had higher adjusted odds ratios than the CAT score. These may imply that the two factors are slightly stronger predictors for severe COPD than the CAT score (9,10,13). Additionally, hospitalizations may remind physicians to prescribe more proper medications for the patients as they may have more times to assess the patients than in the outpatient setting (16).

This study had some limitations. First, we did not evaluate association of COPD such as obstructive sleep apnea (OSA) or asthma which may result in overprescription of corticosteroids (17-21). Second, the study population was community hospital. The results of this study may not be applied for more complicated COPD patients. Second, there was no follow-up data on long-term outcomes. Finally, inappropriate treatment of not using LAMA was due to unavailability. Other causes of inappropriate treatment were treatment with only ICS (18.75%) or bronchodilator alone (1.56%).

Conclusion

Appropriate treatment of patients with COPD according to the GOLD guideline was 26.47% in community setting. Factors associated with severity of COPD were associated with prescribing of appropriate treatments.

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Disclosures

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ORIGINAL RESEARCH ARTICLE

Hexarelin modulates lung mechanics, inflammation, and fibrosis in acute lung injury

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ABSTRACT

Introduction: Acute respiratory distress syndrome (ARDS) is an acute form of diffuse lung injury characterized by (i) an intense inflammatory response, (ii) increased pulmonary vascular permeability, and (iii) the loss of respiratory pulmonary tissue. In this article we explore the therapeutic potential of hexarelin, a synthetic hexapeptide growth hormone secretagogue (GHS), in an experimental model of ARDS. Hexarelin has anti-inflammatory properties and demonstrates cardiovascular-protective activities including the inhibition of cardiomyocyte apoptosis and cardiac fibrosis, both of which may involve the angiotensin-converting enzyme (ACE) system.

Methods: In our experimental model, ARDS was induced by the instillation of 100 mM HCl into the right bronchus; these mice were treated with hexarelin (320 µg/kg, ip) before (Pre) or after (Post) HCl challenge, or with vehicle. Respiratory system compliance, blood gas analysis, and differential cell counts in a selective bronchoalveolar lavage (BAL) were determined 6 or 24 hours after HCl instillation. In an extended study, mice were observed for a subsequent 14 days in order to assess lung fibrosis.

Results: Hexarelin induced a significant improvement in lung compliance and a reduction of the number of total immune cells in BAL 24 hours after HCl instillation, accompanied with a lower recruitment of neutrophils compared with the vehicle group. At day 14, hexarelin-treated mice presented with less pulmonary collagen deposition compared with vehicle-treated controls.

Conclusions: Our data suggest that hexarelin can inhibit the early phase of the inflammatory response in a murine model of HCl-induced ARDS, thereby blunting lung remodeling processes and fibrotic development.

Keywords: ARDS, GHS (growth hormone secretagogues), Hexarelin, Inflammation, Lung fibrosis

Introduction

Acute respiratory distress syndrome (ARDS) is often underrecognized and undertreated, thereby contributing to a high mortality rate (1-3). ARDS constitutes an acute lung injury associated with (i) an intense inflammatory response, (ii) increased pulmonary vascular permeability, and (iii) the loss of aerated lung tissue (4). Diffuse alveolar damage is the most common morphological symptom of the acute phase of ARDS, and is characterized by the influx of neutrophils and macrophages into alveoli, and alteration of the alveolar epithelium (5). These alterations often progress to fibrotic development accompanied by a further decrease in pulmonary compliance (6). There is no documented and approved pharmacologic treatment for ARDS. Hexarelin is a synthetic hexapeptide that has already shown positive effects in experimental models of human pathologies such as epilepsy and cachexia (7-11). In particular, hexarelin reduced cardiac fibrosis in experimental models of myocardial infarction (12). Furthermore, we previously demonstrated that the protective effects of hexarelin on the cardiovascular system could be mediated by its interaction with the angiotensin-converting enzyme (ACE) system (13). Modulation of ACE activity associated with the reduction of angiotensin II synthesis may also be involved with reduced fibrosis development in the lung (14). In this study we have applied our validated experimental model of unilateral acid aspiration lung injury (16) to test specific growth hormone secretagogues (GHS) as potential

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adjunctive therapeutic tools for ARDS. Among GHS, ghrelin has demonstrated anti-inflammatory properties including inhibition of cardiac fibrosis (15). Consequently, this study examined the potential utility of hexarelin in the treatment of ARDS in our murine model.

The aim of this research was to ascertain whether hexarelin could have a potential therapeutic use to antagonize the inflammatory response and the lung fibrosis induced by unilateral acid aspiration in mice. The results of the present research demonstrate that hexarelin treatment reduces the development of lung fibrosis and further suggests that specific synthetic GHS could be developed in order to modulate lung and cardiac fibrosis such as those associated with COVID-19 infections.

Materials and methods

Animals

Male C57/BL6J mice (23-25 g; Harlan Laboratories, Udine, Italy) were used in this experimental study. Animals were housed five per cage in a limited access animal facility, with the room temperature at 20 ± 2°C and the relative humidity set at 55 ± 10%. Artificial lighting provided a 12 h light/12 h dark (7 am to 7 pm) cycle. The general condition of the animals before the experiment was assessed daily. The care and husbandry of animals were in conformity with the institutional guidelines in compliance with Italian and European laws and policies. The animal study was reviewed and approved by the Italian Ministry of Health (591/2017-PR) and by the Animal Care Unit of the University of Milano-Bicocca, Monza, Italy. In full respect of the Reduction principle of the 3Rs, the number of animals/groups selected was to obtain reliable results and enough biological samples to perform the analysis planned.

Chemicals

Hexarelin (His/D-2-Methyl-Trp/Ala/Trp/D-Phe/Lys-NH_2) (Sigma-Aldrich) was given at a dose of 320 µg/kg body weight, according to the assigned treatment group (see below).

Experimental protocol

Mice were anesthetized with ketamine (80 mg/kg, ip) (Ketavet 100; Intervet Productions) and xylazine (4 mg/kg, ip) (Rompun 2%; Bayer) and orotracheally intubated. Then, lung injury was induced in the right lung as described in Amigoni et al (16). The experimental protocol was divided into two time points including (i) the Acute ARDS study, in which animals were sacrificed after 6 or 24 hours following HCl instillation, and (ii) the Late ARDS study, with sacrifice performed 14 days after HCl instillation.

In the Acute ARDS study, mice were assorted into three treatment groups:

- Vehicle: mice received sterile physiological saline treatment (100 µL, ip), immediately after HCl challenge;
- Post-Hex: mice received hexarelin (320 µg/kg, ip, 100 µL), immediately after HCl challenge;
- Pre-Hex: mice received hexarelin (320 µg/kg ip, 100 µL) 2 and 1 day before and immediately after HCl challenge.

In the Late ARDS study, mice were assorted into two treatment groups:

- Vehicle: mice received sterile physiological saline (100 µL, ip), immediately after HCl challenge and twice daily in the following 4 days;
- Hexarelin: mice received hexarelin treatment immediately after HCl challenge (entire dose, 320 µg/kg ip, 100 µL) and twice daily in the following 4 days (two half doses, 160 µg/kg ip, 100 µL).

A group of healthy mice (n = 5) did not undergo any of the surgical interventions and was sacrificed (Healthy Mice). When, at the moment of the sacrifice, we identified that HCl instillation involved the contralateral lung (the left one) through a macroscopic lung evaluation, the animals were euthanized and excluded from analysis (approximately 2%). For some parameters (alveolar inflammatory cells count, alveolar protein content, and collagen deposition), the two lungs were analyzed separately, since the injury was induced only on the right lung. The contralateral (left) lung has been considered like an internal control.

Pulmonary function

At the time of sacrifice, mice were anesthetized with ketamine (100 mg/kg) and xylazine (4 mg/kg), and mechanically ventilated. In order to standardize lung recruitment, a maneuver (30 cm H_2O for 10 sec) was performed immediately after intubation. A pressure-volume (PV) curve was constructed by delivering five steps of inspiratory volume (200 µL) from functional residual capacity. For each step, the plateau pressure was recorded in order to calculate the static compliance by using a pressure transducer, which was interfaced to a PowerLab (AD Instruments) signal transduction unit. A mean value was calculated. After mechanical properties were measured, the chest was opened and a blood sample (0.1 mL) was withdrawn from the left ventricle and analyzed with an I-STAT 1 portable analyzer to analyze oxygenation value (PaO_2) (Oxford Instruments S.M., Burke e Burke) (16).

Inflammatory response

An aliquot of each blood sample was used to perform peripheral leukocyte (WBCs) counts. Subsequently, bronchoalveolar lavage (BAL) was performed separately for each lung, by clamping alternatively the left and right bronchus. Lavage was performed three times for each lung, with 600 or 400 µL of lavage solution (0.9% saline solution and protease inhibitor) respectively for the right and the left one. The BAL samples obtained were then centrifuged for 10 minutes, 1500 rpm, 4°C; the supernatant was then stored at –80°C for subsequent analyses. The cell pellet was resuspended in 500 µL PBS (Dulbecco's phosphate-buffered saline; GIBCO). Subsequently, a 100 µL aliquot was put in 200 µL of Turk (acetic acid gentian violet solution; Merck) for total leukocyte

count in a Burker chamber, while another 100 µL aliquot was centrifuged by a Cytospin (Centrifuge, MPW-351R, MPW) and then stained with a Diff-Quick kit (Medion Diagnostics) that differentially marks the nucleus and cytoplasm, thus facilitating a differential cell count (17).

Protein contents in BAL fluid were performed by the BCA (bicinchoninic acid) method at 24 hours and 14 days. Briefly, 200 µL of reagent (composed of 1:50 BCA and $CuSO_4$; Merck) was added to the samples. A standard curve was constructed with varied concentrations of bovine serum albumin; spectrophotometric measurement was performed at 570 nm with a multilabel spectrophotometer Victor³ (Perkin Elmer) (18).

Hydroxyproline (OH-Pro) assay

After exsanguination, a macroscopic observation of the lungs allowed identifying the localization of the acid injury; lungs were then excised and stored at −80°C. Collagen content was measured with the OH-Pro assay. We used the conventional method (19), which entails lung tissue homogenization and hydrolysis with 6N HCl at 120°C, followed by chloramine T and Ehrlich's solution (Merck) addition to samples for the OH-proline oxidation and a colorimetric reaction. Finally, absorbance was measured at 550 nm with a multilabel spectrophotometer Victor³.

Histological analysis

At sacrifice, some mice were devoted to histological analysis. Briefly, lung tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E) (automatic stainer Dako CoverStainer) and Masson's trichrome (automatic stainer Dako Artisan Link Pro Special Staining System). Morphological changes were analyzed by two experienced pathologists in a blinded fashion (Light Microscope Leica DM 2500 and Digital Microimaging Device Leica DMD108).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences in variances between treatment groups were assessed by one-way analysis of variance (ANOVA) and Tukey post hoc test. P values of less than 0.05 (two-tailed) were considered as statistically significant. In the Acute ARDS study, ANOVA was performed in experimental groups sacrificed at 6 hours and at 24 hours separately. Statistical analysis was performed by GraphPad Prism (version 8.4.2).

Results

Acute ARDS study

Pulmonary function

Acid instillation, as expected, reduced respiratory compliance (Fig. 1A); specifically, at 6 and 24 hours post-instillation respiratory compliance was significantly reduced by 30% ($p<0.01$) and 21% ($p<0.05$), respectively, compared with healthy mice. Mice presented no apparent adverse

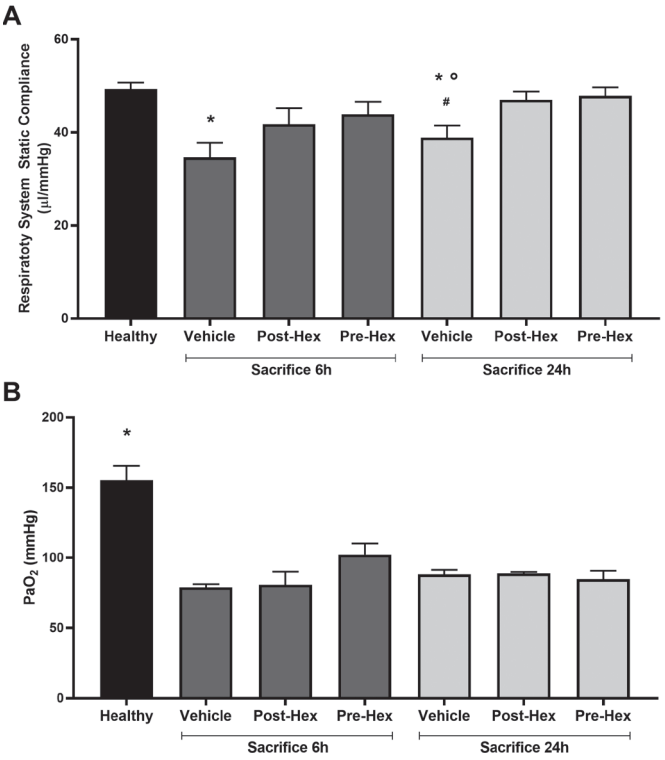

Fig. 1 - Respiratory system static compliance (panel A) and oxygenation (panel B). Respiratory system static compliance derived from the pressure-volume curve construction and oxygenation (PaO_2) was measured by arterial blood from the left ventricle.
A) Analysis of variance (ANOVA) in sacrifice 6 h experiment $p<0.05$, Tukey post hoc test; *$p<0.05$ vs. Healthy; ANOVA in Sacrifice 24 h experiment $p<0.01$, Tukey post hoc test; *$p<0.05$ vs. Healthy, °$p<0.05$ vs. Post-Hex, # $p<0.05$ vs. Pre-Hex.
B) ANOVA in Sacrifice 6 h experiment $p<0.01$, Tukey post hoc test; *$p<0.01$ vs. Vehicle, Post-Hex and Pre-Hex; ANOVA in Sacrifice 24 h experiment $p<0.01$, Tukey post hoc test; *$p<0.01$ vs. Vehicle, Post-Hex, and Pre-Hex.
Healthy: no surgical interventions or treatment (n = 5); Vehicle: HCl instillation + vehicle treatment (n = 8); Post-Hex: HCl instillation + Hexarelin treatment (n = 8); Pre-Hex: Hexarelin pretreatment + HCl instillation (n = 8).

responses to hexarelin pretreatment or treatment regimes; moreover, hexarelin administration, in both treatment regimes, modulated acid-reduced respiratory compliance. To illustrate, at 6 hours post-instillation the beneficial effects of hexarelin were slight but not significant; by contrast, hexarelin induced a significant ($p<0.05$) improvement in respiratory compliance 24 hours after lung injury. By comparison, arterial oxygen partial pressure (PaO_2) was severely and significantly decreased in all acid-treated animals ($p<0.01$) compared with Healthy mice (Fig. 1B).

Inflammatory response

Total numbers of peripheral white blood cells, an assumed index of systemic inflammation, increased significantly 6 hours after acid instillation (Fig. 2A) in both vehicle- ($p<0.01$) and hexarelin-treated ($p<0.05$) groups compared

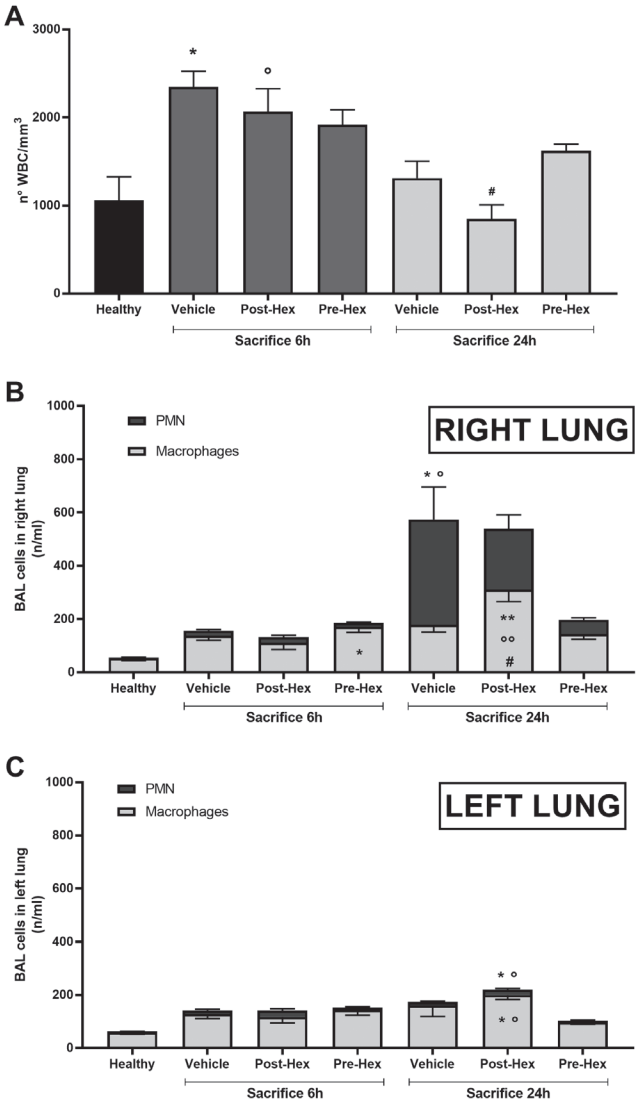

Fig. 2 - Peripheral and local inflammation: total white blood cells (A) and cell count in bronchoalveolar lavage (BAL) in right (panel B) and left lung (panel C). White blood cells were collected from the arterial blood and stained with Turk solution. Alveolar cells were collected by performing BAL and stained with Diff-Quik reagent solution.
A) Analysis of variance (ANOVA) in Sacrifice 6 h experiment p<0.01, Tukey post hoc test; *p<0.01 vs. Healthy, °p<0.05 vs. Healthy; ANOVA in Sacrifice 24 h experiment p<0.05, Tukey post hoc test; #p<0.05 vs. Pre-Hex.
B) Polymorphonuclear (PMN): ANOVA in Sacrifice 6 h experiment p = NS; ANOVA in Sacrifice 24 h experiment p<0.01, Tukey post hoc test; *p = 0.01 vs. Healthy, °p = 0.01 vs. Pre-Hex. Macrophages: ANOVA in Sacrifice 6 h experiment p = 0.02, Tukey post hoc test; *p = 0.01 vs. Healthy; ANOVA in Sacrifice 24 h experiment p<0.01, Tukey post hoc test; **p<0.01 vs. Healthy, °°p<0.05 vs. Vehicle, #p<0.01 vs. Pre-Hex.
C) PMN: ANOVA in Sacrifice 6 h experiment p = NS; ANOVA in Sacrifice 24 h experiment p = 0.01, Tukey post hoc test; *p<0.05 vs. Healthy, °p<0.01 vs. Pre-Hex. Macrophages: ANOVA in Sacrifice 6 h experiment p = NS; ANOVA in Sacrifice 24 h experiment p<0.01, Tukey post hoc test; *p = 0.01 vs. Healthy, °p<0.05 vs. Pre-Hex.
Healthy: no surgical interventions or treatment (n = 5); Vehicle: HCl instillation + vehicle treatment (n = 8); Post-Hex: HCl instillation + Hexarelin treatment (n = 8); Pre-Hex: Hexarelin pretreatment + HCl instillation (n = 8).

with healthy mice. At 24 hours post-instillation, total cell numbers decreased in all groups; in particular, cell numbers returned to normal (healthy) levels in hexarelin-treated mice.

Differential cell counts of BAL fluid were strongly influenced both by acid instillation and by hexarelin (Fig. 2B, C). In the BAL of healthy mice, polymorphonuclear (PMN) cells represented 4% of total white blood cells, the balance of which (96%) were macrophages. At 6 hours post-acid instillation, there was an important increase in BAL PMN; notably, PMN in the BAL of the pre-hexarelin-treated group was also elevated and significantly (p = 0.010) different from those in healthy mice. Acid instillation increased the differential cell count, which achieved maximum levels at 24 hours post-instillation (Fig. 2B, C). At this time, hexarelin treatment and especially pretreatment modulated PMN numbers to a lesser and greater extent. Curiously, macrophage numbers in hexarelin-treated mice were significantly greater compared with other groups in both the right (acid-instilled) as well as the left (acid-naive) lungs.

Indices of local inflammation included the total protein contents and differential cell counts of BAL. Acid instillation induced a very large protein extravasation into alveoli (Tab. I). Hexarelin treatment and pretreatment modulated acid-induced protein leakage; in fact, the BAL protein contents in both hexarelin-treated groups (6 hours post-instillation) were comparable with those of healthy mice and significantly smaller (p<0.01) than those in the acid-instilled-alone group.

TABLE I - Local inflammation: total protein BAL content in right and left lung by BCA method

	Groups	Right lung	Left lung
	Healthy	268 ± 24	423 ± 54
Sacrifice 6 h	Vehicle	2994 ± 496*,°	1148 ± 193
	Post-Hex	1585 ± 316	1380 ± 262
	Pre-Hex	1647 ±315	1013 ± 294
Sacrifice 24 h	Vehicle	2891 ± 289**	773 ± 103
	Post-Hex	2145 ± 266°°	616 ± 70
	Pre-Hex	2364 ± 296#	521 ± 90

In the right lung: ANOVA in Sacrifice 6 h experiment p<0.01, Tukey post hoc test; *p<0.01 vs. Healthy, °p<0.05 vs. Post-Hex; ANOVA in Sacrifice 24 h experiment p<0.01, Tukey post hoc test; **p<0.01 vs. Healthy, °°p<0.01 vs. Healthy, #p<0.01 vs. Healthy. In the left lung: ANOVA p = ns. Healthy: no surgical interventions or treatment (n = 5); Vehicle: HCl instillation + vehicle treatment (n = 8); Post-Hex: HCl instillation + Hexarelin treatment (n = 8); Pre-Hex: Hexarelin pretreatment + HCl instillation (n = 8).
ANOVA = analysis of variance; BAL = bronchoalveolar lavage; BCA = bicinchoninic acid.

Late ARDS study

Body weight was monitored throughout the experiment, since it is known that hexarelin stimulates food intake. As expected, mice belonging to acid-instilled groups showed a significant (p<0.01) loss of body weight 48 hours after HCl administration, compared with healthy mice (Fig. 3A).

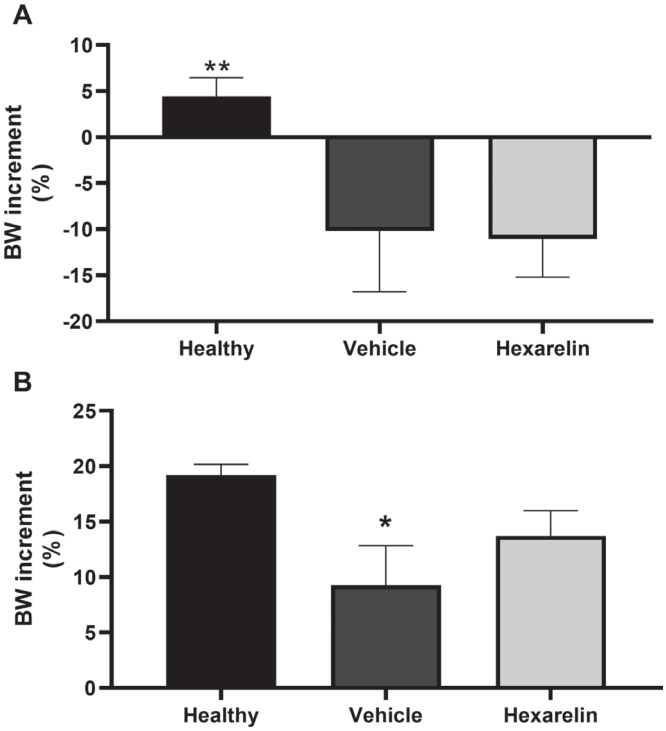

Fig. 3 - Body weight increment in 48 hours (panel A) and in 14 days (panel B).
A) Analysis of variance (ANOVA) p<0.0001, Tukey post hoc test; *p<0.01 vs. Vehicle and Hexarelin.
B) ANOVA p = 0.048, Tukey post hoc test; *p<0.05 vs. Healthy.
Healthy: no surgical interventions or treatment (n = 15); Vehicle: HCl instillation + Vehicle treatment (n = 18); Hexarelin: HCl instillation + Hexarelin treatment (n = 18).

Fourteen days post-instillation body weights of mice in the vehicle group were significantly (p<0.05) smaller than those of healthy mice; hexarelin treatment preserved body weight better than vehicle treatment alone (Fig. 3B).

Pulmonary function

By 14 days post-instillation, respiratory system static compliance did not differ significantly between groups. Acid-treated mice showed no different (ANOVA, p = ns) mechanical properties (51.6 ± 3.0 and 51.0 ± 3.4 µL/mm Hg in Vehicle and Hexarelin groups, respectively) compared with healthy mice (58.0 ± 4.5 µL/mm Hg).

Collagen deposition

In order to estimate fibrosis development at 14 days post-instillation, the collagen content of each lung was measured indirectly with the OH-Pro assay. The collagen contents of both right and left lungs of the hexarelin-treated group were smaller compared with those in the vehicle-treated group; however, only the right lungs showed a statistically significant difference (p<0.05) between the hexarelin- and vehicle-treated groups (Fig. 4A).

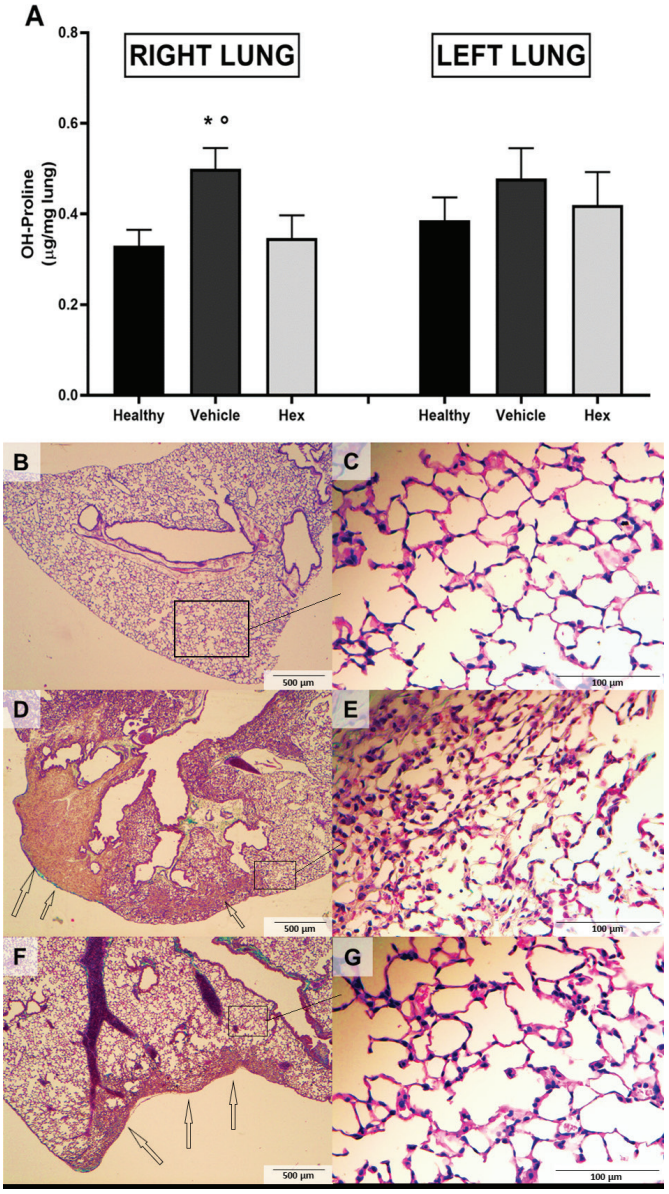

Fig. 4 - Lung fibrosis: OH-Proline lung content (A) and effects of hexarelin on lung histology (B) in acid-injured mice 14 days after lung injury induction.
The collagen deposition was evaluated by the assessment of OH-proline content in lung-homogenized tissue with chloramine T and Ehrlich's solutions.
In right lung: Analysis of variance (ANOVA) p<0.05, Tukey post hoc; *p<0.05 vs. Hexarelin, °p<0.05 vs. Healthy. In left lung: ANOVA p = NS. Healthy: no surgical interventions or treatment (n = 10); Vehicle: HCl instillation + vehicle treatment (n = 13); Hexarelin: HCl instillation + Hexarelin treatment (n = 13).
Histology: Representative images are shown.Tissues were prepared for Masson's trichrome staining. (B, D, F: 40× magnification; C, E, G: 400× magnification.) **(B, C)** Healthy: no surgical interventions or treatment (n = 4); **(D, E)** Vehicle: HCl instillation + vehicle treatment (n = 3); and **(F, G)** Hexarelin: HCl instillation + Hexarelin treatment (n = 5). Areas of fibrosis (stained in green) are indicated with arrows; boxes on the 40× images indicate the locations of the 400× images.

Histological examination

In healthy mice, lung parenchyma was substantially preserved with normal lobular structure, slight bronchial ectasia, normal alveoli, and alveolar ducts (Fig. 4B, C). In the vehicle-treated group, lung tissue was characterized by diffuse areas of fibrosis, especially subpleural with centrilobular extension. This fibrosis was of a "young" type, with several fibroblastic cells in deposits of collagen and with large subpleural nodules; as well, several small "fibroblastic foci" were found in adjacent parenchyma. There was also evidence of architectural distortion with marked bronchiolar dilatation forming cystic spaces resembling honeycombing-like features (Fig. 4D). In a vehicle-treated (acid-instilled) lung there was evidence of a slight alveolar distortion, accompanied by many alveolar macrophages and some PMN cells (Fig. 4E). In hexarelin-treated mice, by contrast, fibrosis was reduced compared to that of the vehicle-treated group; fibrotic distribution was patchy with subpleural localization prevalent. This fibrotic tissue was similar to that previously described; that is, appearing young and cellulated and accompanied by evidence of abrupt transitions from remodeled lung parenchyma to normal alveolar walls. At the center of a lobule, the presence of a fibroblastic focus is obvious (Fig. 4F). Almost normal alveolar walls and only some inflammatory cells were evident in lungs from hexarelin-treated mice (Fig. 4G).

Discussion

In this study, we demonstrate protective effects of hexarelin against ARDS in a unilateral acid aspiration lung injury model in mice. Our results show that hexarelin treatment significantly (i) ameliorated respiratory system compliance, (ii) reduced protein levels in the BAL, and (iii) blunted PMN infiltration compared with the vehicle-treated group; these effects eventually attenuated lung fibrosis and collagen content. To the best of our knowledge, this is the first study to evaluate the effects of hexarelin on ARDS in a mouse model of unilateral lung acid instillation. Hexarelin, a synthetic hexapeptide, is a powerful agonist of the ghrelin receptor and, consequently, manifests both endocrine and extra-endocrine activities. These activities include positive effects on gastrointestinal-, cardiovascular-, muscular-, and nervous systems, as well as participation in regulation of energy balance (7-11). In humans, hexarelin actions are not restricted to stimulating GH release; to illustrate, acute hexarelin administration markedly increased left ventricular function in (i) normal subjects, (ii) in patients with ischemic cardiomyopathy, as well as (iii) in patients with severe GH deficiency (20). Hexarelin significantly reduced indices of cardiac fibrosis in experimental models of myocardial infarction, likely through an underlying anti-inflammatory mechanism (12). The effects of subacute hexarelin treatments in rats (8) strongly suggested that hexarelin could interfere with the renin-angiotensin-aldosterone system (RAAS). We provided evidence in support of that hypothesis by showing that hexarelin and other synthetic GHS inhibited the activity of ACE (13). The somatic form of ACE is a type I membrane-anchored dipeptidyl carboxypeptidase consisting of two extracellular catalytic domains: the N- and C-domains specifically catalyze conversion of angiotensin I and bradykinin, respectively (21,22). Hexarelin and synthetic GHS selectively bind to and inhibit the activity of the C-domain, without being metabolized themselves (13). Hexarelin inhibition of ACE may account, in part, for its cardioprotective effects. To illustrate, binding sites for GHS are expressed in the heart; moreover, mRNA for the specific GHS receptor (GHS-R1a) has been detected by reverse transcriptase polymerase chain reaction (RT-PCR) in the rat aorta as well as the left ventricle and left atrium (23,24). Nonetheless, it is presumed that the levels of GHS-R1a expressed in these tissues are too small to be responsible for GHS cardioprotective effects. GHS cardioprotective effects are now being considered to be more related to inhibition of angiotensin II (AII) synthesis and/or the antagonism of its receptor (AT1). ACE synthesizes AII, which acts through the AT1 receptor to increase blood pressure and to promote fibrosis and inflammation. The vulnerability of AII to enzymatic conversion by ACE2 leads to reduction of AII levels and production of angiotensin (1-7) which selectively binds to the MAS receptor. The beneficial effects of ACE2 activity may be both systemic as well as localized in tissues, such as the heart, kidneys, and lungs, where it antagonizes pathological changes (25).

Our results that demonstrate hexarelin inhibition of lung fibrosis in acid-instilled mice are in agreement with our previous results demonstrating the cardioprotective and anti-inflammatory effects of hexarelin (8,26). Collectively, these findings strongly suggest that hexarelin may inhibit the development of pathological fibrosis in both these organs.

The lack of beneficial effect of hexarelin on oxygenation could depend on the short interval after treatment: it is possible that 24 hours is not enough to affect oxygen exchange. An impaired perfusion could also prevent an increase in oxygenation.

The precise pathogenesis of fibrotic pulmonary disorder is still unclear; it could result from an excessive host inflammatory response of the lung to an infectious or non-infectious insult. A common feature is collagen accumulation that leads to destruction of alveolar structures and promotes remodeling. This purported mechanism has been proposed to play a primary role in ARDS (27). In the lung and other tissues, inflammation correlates with the presence of macrophages and other immune cells that can be involved in the inflammatory process and thereafter its resolution and recovery from ARDS (28). It has been reported that ARDS occurs in some severe acute respiratory syndrome (SARS) patients despite a diminishing viral load, suggesting that the host immune response rather than viral infection itself could be responsible for lung damage (29). We have observed that hexarelin reduced the number of immune cells in the BAL, in particular PMN, suggesting that it could partially inhibit the recruitment of PMN into the alveolar space. This is consistent with the anti-inflammatory and antifibrotic effects reported for hexarelin in the heart (12). In this study, we observed that hexarelin significantly reduced OH-proline levels, a measure of collagen deposition, in the lung treated with acid instillation. This result is

consistent with the ability of hexarelin to reduce the fibrotic areas in lung histology.

In our histological observations, fibrosis features were similar in Vehicle- and Hexarelin-treated groups, either for cellular type or anatomical location (subpleural origin with following centrilobular involvement, like usual interstitial pneumonia pattern) (30). The smaller amounts of fibrosis in hexarelin-treated mice could be related to a lower collagen deposition, consistent with the reduced presence of inflammatory cells.

This study has some limitations that should be acknowledged. First, we did not confirm the findings in a different model of ARDS. Second, the possible mechanisms of action of hexarelin are only speculative. Third, the levels of inflammatory cytokines were not measured, which could support the results on inflammation obtained for local and peripheral inflammatory cell count. Fourth, as index of lung, edema, septal thickening, and hemorrhage were not assessed in this study.

In conclusion, our results suggest that hexarelin can ameliorate the static compliance of acid-injured lungs in mice, an experimental model of ARDS. Our results also show that hexarelin can reduce lung fibrosis, a complication often reported in patients with classical and COVID-19 ARDS. Collectively, these findings suggest that hexarelin and other synthetic GHS may be successfully co-opted for use in antagonizing lung and cardiac fibrosis.

Acknowledgments

Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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