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Menthacarin in functional gastrointestinal disorders: a pharmacy-based cohort study

Treatment of sore throat and hoarseness with Pelargonium sidoides extract EPs 7630: a meta-analysis

Controlling the activation of the prokineticin system as therapeutic approach to relief neuropathic pain and reduce neuroinflammation

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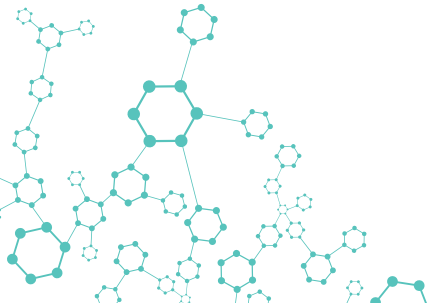

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EDITORIAL

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PHARMACOLOGY IN PEACE AND WAR TIMES

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This issue of *PharmAdvances* features a word that many of us repeat quite often: "stress". Dr. Brivio (who, with this paper, competes for the SIF/PharmAdvances PhD thesis award) clearly states that we do not know much about stress and that it is crucial to better understand the behavioral outcomes of stressful events as well as the molecular changes that may sustain them for the discovery of novel therapeutic targets and approaches to treat stress-related disorders and to promote resilience (1). Thanks to her and others' research we might better manage this condition that affects so many of us (1). Speaking of the SIF/PharmAdvances PhD thesis award, Dr. Lazzara (2) reviews the etiopathogenesis of diabetic retinopathy, addressing several hypotheses, trying to identify and validate novel and promising pathways implicated in this pathology that afflicts so many patients and causes irreversible vision loss.

In addition to stress (which is yet poorly defined), many of us often feel pain, which increases with age and that must be controlled by pharmacological means. Of all kinds of pain, neuropathic pain is a relevant clinical problem worldwide, and current therapeutic tools are unsatisfactory. The identification of novel therapeutic targets and the development of new pharmacological approaches remain a priority. Amodeo *et al.* (3) review the bases of neuropathic pain and state that the availability of specific receptor antagonists makes the prokineticin system a very interesting one to pharmacologically control prokineticin activity (3). We hope that future studies will finally nail the biochemistry behind prokineticin and pave the way to novel therapeutics.

In keeping with the above, Rullo *et al.* (4) review the pharmacological outcomes of opioids biased ligands by bringing together cellular results and the available data from clinical trials (4). Of note, the authors focus on biased μ-opioid receptor agonists given their therapeutic relevance. This is another example of the importance of basic research, to identify targets

for pharmacological interventions that should be as specific as possible to minimize side effects.

Therapy (= health care in general) is costly and this important issue is addressed in a paper coordinated by Professors Pani and Racagni (5). Where free healthcare is available to everyone, someone has to bear the costs of examining and treating patients. To optimize costs and reduce the tax burden on citizens, the experts started from analyzing the evidence on the positive impact of value-based differential prices on innovation and access to innovative medicines, then discussed and defined the specific features of payment models that could be implemented in the Italian context. Their conclusion is that it is essential to assess the value over the entire life cycle of drugs per every single indication, systematically collecting real-world evidence data to re-negotiate the value as these data are generated (5). Hopefully, the Italian scenario will be integrated into a broader one to give us a feeling of how future healthcare policies should be drafted.

We also feature two papers stemming from the recent "*1st Joint Meeting on Natural Products Pharmacology SIF-SIPHAR-IMGNPP*", held in Naples (Italy) in February 2022 and very well attended. Noé *et al.* (6) and Kamin *et al.* (7) provide interesting examples of how a well-developed and characterized improve gastrointestinal symptoms and dyspepsia of irritable bowel syndrome patients (the former) and reduce sore throat symptoms (the latter). These two are clear examples of how natural products should be investigated and exploited "the pharmacological way", *i.e.* by applying the same approach we use for allopathic medicine. The *IUPHAR Mediterranean Group of Natural Products Pharmacology* (https://www.imgnpp.org/about-us) collates many pharmacologists with interests in this and can only fledge with the contribution of the entire pharmacological community. In this respect, stay tuned for future publications and please contribute your own ones. The IMGNPP is taking the lead and will help you disseminate your findings in an international framework.

To add some final remarks, this issue of PharmAdvances is being produced during a war that nobody believed would be taking place again. Science in general, and pharmacology as integral part of it, have no borders, no nationality, no "backyards". Data should be free to circulate and this is exactly why we publish in PharmAdvances and in other scientific journals. Nearly all of us have worked abroad and/or hosted foreign investigators in our labs. Everyone contributes with its peculiarity and ethnic background, creating the magic mix that advances our knowledge and the well-being of humankind. As it is not acceptable to fight over scientific data that must benefit everyone, it is likewise not acceptable to kill human beings for purported national interests. PharmAdvances calls for peace in Ukraine and worldwide. Rather than spending money to kill each other, humans should invest resources in research, be it basic, applied, technological, *etc.* The current SARS-CoV-2 disaster is a reminder of how much progress is yet to be made and of how each and every one of us could and should contribute to the advancement of pharmacology.

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RESEARCH ARTICLE

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MENTHACARIN IN FUNCTIONAL GASTROINTESTINAL DISORDERS: A PHARMACY-BASED COHORT STUDY

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SUMMARY

Functional dyspepsia and irritable bowel syndrome (IBS) are among the most prevalent functional gastrointestinal disorders (FGID) and often treated by self-medication.

To gain insights into experienced effects and tolerability of pharmacy-supported self-medication with Menthacarin, a proprietary specified combination of essential oils from Mentha x piperita L. (90 mg WS® 1340) and Carum carvi L. (50 mg WS® 1520), in pharmacy customers suffering from FGID.

The study was designed as a prospective, observational, multicenter, pharmacy-based cohort study in pharmacy customers suffering from dyspeptic complaints, particularly with mild cramps in the gastrointestinal tract, bloating, and fullness, who were routinely recommended the commercially available preparation of Menthacarin. Occurrence and severity of 13 dyspeptic symptoms were assessed by the modified Gastrointestinal Symptoms Rating Scale (GSRS). Patient satisfaction and tolerability were evaluated.

50 customers (mean age 53.8 years) were recruited. After 3 weeks, GSRS total score was reduced from 48.6 ± 17.1 to 22.8 ± 12.3 points ($p < 0.001$). In 68.7% of the participating customers, an improvement occurred within the first week of Menthacarin administration. Greatest improvements were noted for abdominal pain, bloating, and impression of fullness. At study end, 83.3% reported general health state improvement. 44.9% rated the perceived effects as "very good" or "good", 30.6% as "satisfactory". Tolerability was rated as "very good" or "good" by 83.3% of customers and 87.7% of pharmacists.

These findings suggest the satisfactory and safe applicability of Menthacarin in pharmacy-supported self-medication of pharmacy customers suffering from dyspeptic and IBS-related complaints due to FGID.

Key words

Functional gastrointestinal disorder; Menthacarin; Peppermint oil; Caraway oil; Pharmacy-based cohort study.

Impact statement

The results of this pharmacy-based cohort study (a) show significant improvement in symptoms of functional gastrointestinal disorders, such as abdominal pain and bloating, and improved overall health status after 3 weeks of observational treatment, with improvement in pharmacy customers occurring already after one week of taking Menthacarin, and (b) suggest the satisfactory and safe applicability of Menthacarin in pharmacy-supported self-medication of pharmacy customers suffering from dyspeptic and irritable-bowel-syndrome-related complaints due to functional gastrointestinal disorders.

Abbreviations
CI: Confidence interval; FD: Functional dyspepsia; FGID: Functional gastrointestinal disorders; GSRS: Gastrointestinal Symptoms Rating Scale; IBS: Irritable bowel syndrome; NDI: Nepean Dyspepsia Index; SD: Standard deviation(s); VAS: Visual analogue scale.

INTRODUCTION

Functional gastrointestinal disorders (FGID) such as irritable bowel syndrome (IBS) and functional dyspepsia (FD) are highly prevalent and currently belong to the most common gastrointestinal syndromes (1, 2). For Southern Europe, pooled prevalences are estimated, for example, as 15.0% for IBS (3) and 24% for uninvestigated dyspepsia (4).

FGID is a diagnosis of exclusion and can solely be identified by associated symptoms, among which chronic abdominal pain and bloating belong to the most common (5, 6). The treatment is symptom-based and symptoms are usually chronic and not independent of each other, with considerable overlap between the symptoms of different FGID (5).

During the last decades, there has been a considerable effort among gastroenterologists to find ways to define, evaluate and - most importantly - appropriately treat the various forms of FGID. In the course of the revision of diagnostic algorithms, the current criteria, guidelines and recommendations of the Rome foundation, recently published as the Rome IV materials (7), describe FGID as disorders of gut brain interaction and introduce a number of diagnostic specifications that - among other issues - aim to address the high rate of concomitant IBS and FD occurrence (6, 8) and the frequent overlap and variability of symptoms in FGID in general. According to Rome IV, the main symptom occurring with IBS and FD is chronic or relapsing abdominal pain. In IBS, associated symptoms also comprise a change of bowel habit, stool consistency and/or frequency, and bloating: Symptoms must have existed for at least 6 months prior to diagnosis, with pain having occurred at least once a week for at least 3 months (9). Likewise associated with chronic or relapsing abdominal pain, FD presents with symptoms located in the upper abdomen, such as impression of fullness, early satiation and bloating (5, 7).

The chronicity or frequent relapsing of the symptoms over a long time can lead to serious reductions in quality of life as well as considerable impairment of social and professional functioning (6), let alone the socioeconomic burden implied (2, 10).

Given the long-term duration of the condition as well as the great variety and overlapping of symptoms, the need for a safe and efficacious therapeutic option applicable in FGID is obvious. Self-medication is a major issue here, which, besides broad-range efficacy, implies the particular importance of safe application and tolerability of the drug as perceived by the patient. It would therefore be helpful to complement medical data derived from clinical trials by data from self-medication. For this purpose, pharmacy-based observational studies - also called naturalistic cohort studies - are a valid and helpful research tool (11, 12). They are usually conducted by physicians. In Germany, they may also be carried out by other healthcare professionals, e.g., pharmacists, if the medicinal products are marketed as over the counter products (13).

Menthacarin[1] is a proprietary combination of peppermint oil (90 mg WS® 1340) and caraway oil (50 mg WS® 1520) with specified quality. One gastro-resistant capsule, soft, contains 90 mg *Mentha x piperita L., aetheroleum* (peppermint oil) and 50 mg *Carum carvi* L., *aetheroleum* (caraway oil). Menthacarin is available in Germany and other European countries in pharmacies without prescription. Herbal preparations such as peppermint (*Mentha piperita*) or caraway (*Carum Carvi*) have a centuries-long successful history in gastrointestinal conditions.

1 Menthacarin® is the active agent of the product Carmenthin® bei Verdauungsstörungen (Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe).

Whereas peppermint oil was proved to act as a spasmolytic in gastrointestinal disorders and thus be effective in reducing FGID-related abdominal pain (14, 15), caraway fruit and caraway oil are acknowledged to be effective in digestive problems with regard to bloating (16), to exert antispasmodic effects (17), and to act selectively growth-inhibiting on pathogenic intestinal bacteria (18). Four double-blind, randomized trials showed that treatment of patients suffering from functional dyspepsia with 2 x 1 or 3 x 1 capsules/day Menthacarin was significantly more effective than placebo and as effective as the prokinetic agent cisapride, respectively (19-22). Favorable treatment effects similar to those found for FD patients in the primary analyses were also shown for concomitant IBS-associated symptoms in FD patients (23).
The observational, prospective, pharmacy-based, cohort study presented here was conducted to gain insight into tolerability as a pharmacy-supported self-medication in patients with FGID as reported by pharmacy customers and pharmacists, as well as into patient compliance and experienced changes in symptom improvement.

MATERIALS AND METHODS

This study was conducted in 10 pharmacies in Germany. As this was a non-interventional, open, multi-center, pharmacy-based, post-authorization cohort study, this investigation was not subject to an ethics approval. In accordance with the national regulations, notification of the study was made to the Federal Institute for Drugs and Medical Devices (BfArM) and the National Association of Statutory Health Insurance Physicians prior to study start.
Pharmacy customers aged ≥ 18 years who were routinely recommended the commercially available preparation of Menthacarin due to the reason for the consultation at the pharmacy were asked whether they would participate in the cohort study. After verbal consent, they were asked about demographic data, existing diseases, medical treatment, and drug therapy in a baseline survey and the information was documented.

Pharmacy customers who reported to be suffering from dyspeptic complaints, particularly with mild cramps in the gastrointestinal tract, bloating, and fullness could be involved provided they had undergone a medical examination in the last 12 months to clarify the above-mentioned symptoms. Pharmacy customers with a medically confirmed organic cause for these symptoms, *e.g.*, gastric ulcer, short bowel syndrome or biliary tract disease were not included. The dyspeptic complaints had to be frequently relapsing during the last 3 months prior to participation. Study eligibility required the occurrence of either 3 out of 4 pre-defined symptoms related to the upper abdomen or 2 out of 3 pre-defined symptoms related to the bowel. Regarding the upper abdomen, pre-defined symptoms were the following symptoms occurring independent of meals: frequent eructation without acid taste, impression of fullness, frequently relapsing nausea and retching impulse, and the symptom early satiation already after small meals. Regarding the bowel, pre-defined symptoms required for study eligibility were increased borborygmi, increased bloating associated with altered frequency and consistency of stool.
Pharmacy customers participating in the study were asked to follow the instructions in the package leaflet to daily swallow 1 enteric-coated soft capsule in the morning and one at lunchtime, 30 minutes before meals, and to visit the pharmacy again after 3 weeks. Occurrence and severity of 13 dyspeptic symptoms were assessed by the pharmacist at baseline and after 3 weeks of administration of the herbal medicinal product.
Outcome measure was a modified version of the Gastrointestinal Symptoms Rating Scale (GSRS) (24). Symptom severity was evaluated by a visual analogue scale (VAS) covering degrees from 0 (not present) to 10 (severe). Also, drug tolerability as well as changes of symptoms accompanying treatment were evaluated by pharmacists and pharmacy customers using a scale from 1 (very good) to 5 (poor). Patient satisfaction with the treatment was assessed on a 5-point health-state scale ranging from "much better" to "much worse". In addition,

the pharmacy customers were asked about the convenience of dosage form, compliance, and willingness of future intake of Menthacarin.

The change in severity of present symptoms was evaluated by means of the intraindividual differences between the respective values of the final visit (after 3 weeks of drug intake) and the respective values at the baseline visit. Mean values, standard deviations (SD) and 95% confidence intervals (CI) were computed for symptom severity and change. Obtained data were analyzed for significance by application of the two-sided Wilcoxon signed-rank test. Due to the descriptive nature of the analyses, statistical significance was assumed for $p < 0.05$. Adverse events were documented.

RESULTS

A total of 50 customers (mean age 53.8 ± 13.6, 68.0% female) were recruited. A total of 48 questionnaires could be evaluated for effectiveness and 50 for safety.

The global FGID symptoms that were rated as "impairing" by most pharmacy customers at baseline were "bloating" (in 62% of the patients with a mean severity of 6.5±2.8 points as assessed on the GSRS), "impression of fullness" (50%, 6.1 ± 2.3), and "abdominal pain" (44%, 5.6 ± 2.6). The symptoms "early satiation" and "eructation" were initially noted in 34% and 40% of the patients, respectively. Symptoms related to defecation were reported by 26% ("liquid or soft stool") and 28% of the patients ("sudden urge to defecate"), respectively, at study start.

After 3 weeks of Menthacarin intake, the initially most impairing global FGID symptoms "abdominal pain" "bloating", and "impression of fullness" showed mean improvement rates of 3.2 ± 2.6, 3.2 ± 3.1, and 3.1 ± 2.6 points, respectively. Mean values of "early satiation" and "eructation" had decreased by 2.2 ± 2.5 points and 2.4 ± 2.8 points, respectively, whereas in the defecation-related symptoms "liquid or soft stool" and "sudden urge to defecate" an average decrease by 1.5 ± 1.9 points and 1.2 ± 2.0 points, respectively, was noted. The overall severity of the 13 symptoms assessed decreased during the study, which was statistically significant in 12 of the symptoms (**figure 1**). The total GSRS score significantly improved from 48.6 ± 17.1 points to 22.8 ± 12.3 points ($p < 0.0001$, two-sided, Wilcoxon signed-rank test).

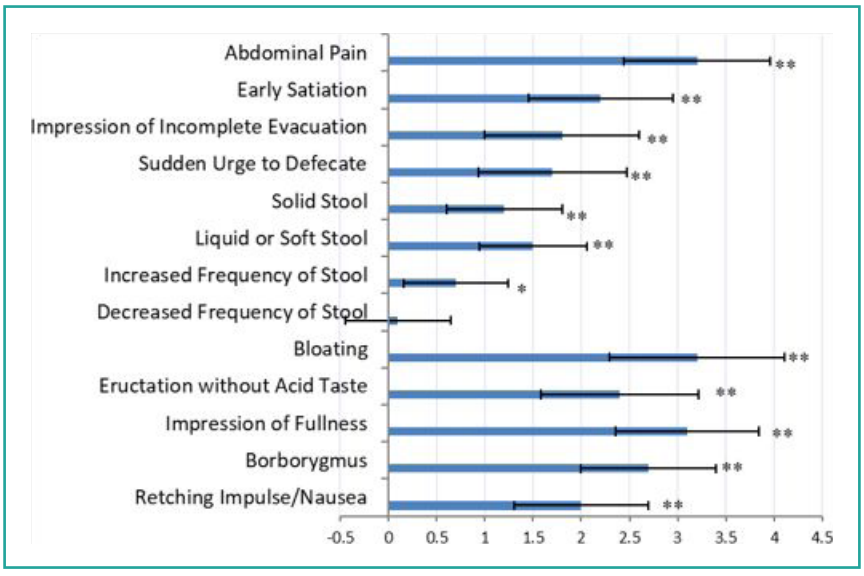

Figure 1. Improvement in gastrointestinal symptoms after 3 weeks of Menthacarin intake (mean GSRS score and 95% CI). *p = 0.0134, **p < 0.0001; two-sided, Wilcoxon signed-rank test.

At the end of the study, 83.3% of pharmacy customers reported an improvement in their general health state compared to two weeks earlier (**figure 2**). In 68.7% of all customers participating, an improvement occurred within the first week of Menthacarin intake.
10.2% of the participants rated the effectiveness of Menthacarin as "very good", 34.7% as "good", and 30.6% as "satisfactory". This was comparable to the pharmacists' rating (10.2% "very good", 42.9% "good", 10.2% "satisfactory") (**figure 3**).
83.3% of the pharmacy customers' and 87.7% of the pharmacists' ratings for tolerability of Menthacarin were "very good" or "good" (**figure 4**).
Nearly all participating customers (96%) rated the Menthacarin dosage form in capsules as "convenient". Almost 90% of the pharmacy customers took the drug as instructed over the complete study term. 22.9% of the pharmacy customers reported to be symptom-free after 3 weeks of Menthacarin intake. 73.5% of the participating customers stated their willingness to administer the drug again in the future, and 43.8% decided to continue Menthacarin intake after the end of the cohort study.
A total of 10 adverse events in 7 pharmacy customers were documented, for 3 out of which (stomach ache) a relation to Menthacarin could not be excluded.

DISCUSSION

Cohort studies in this over-the-counter pharmacy-based setting have the advantage of being able to assess tolerability and effectiveness of a medication by means of detailed questioning of the recipient under real-world conditions (25). In this context, the results presented here show that self-medication with Menthacarin carried out under the guidance of a pharmacist leads to a relevant improvement and alleviation of FGID-related symptoms. During the cohort study, considerable improvement in 13 pre-defined symptoms under Menthacarin intake was seen. Out of these symptoms, 12 improved significantly, especially abdominal pain, bloating, and impression of fullness.
These findings are in line with effects shown for Menthacarin reported earlier: Koch and colleagues (26) demonstrated the antifoaming and thus bloating-relieving effects of both essential oils, which are largely mediated by lowering the surface tension of the gastric or intestinal juices. In an investigation of the effects of an intraduodenal application of both essential oils on gastroduodenal motility in healthy volunteers, Micklefield and colleagues (27) demonstrated a significant decrease in frequency and duration of contractions for peppermint oil WS® 1340 and a significant reduction of the respective contraction amplitudes by caraway oil. These results therefore proved the contribution of both active ingredients to the spasmolytic effects of the combined preparation. A similarly relaxing effect of both peppermint oil and caraway oil on the gallbladder was shown by Goerg and Spilker (28), who suggested a synergistic action mode with both WS® 1340 and WS® 1520 contributing to the effectiveness of Menthacarin in an amplifying manner. The combination of peppermint oil and caraway oil as well as the individual substances were also tested in a model of post-inflammatory visceral hyperalgesia (29). For the combination, a significant effect on the sensitivity to pain was shown whereas neither peppermint oil nor caraway oil showed significant activity when administered individually.
The considerable reduction in abdominal pain during Menthacarin intake shown in the present cohort study is of particular relevance as this symptom is considered the most common symptom in nearly all types of FGID (7). The results also show that the FGID symptoms stated by the participating pharmacy customers as particularly impairing at the beginning of the study, namely abdominal pain, bloating and impression of fullness, were among the symptoms showing the greatest improvements. These findings are in line with results from a placebo-controlled clinical trial which

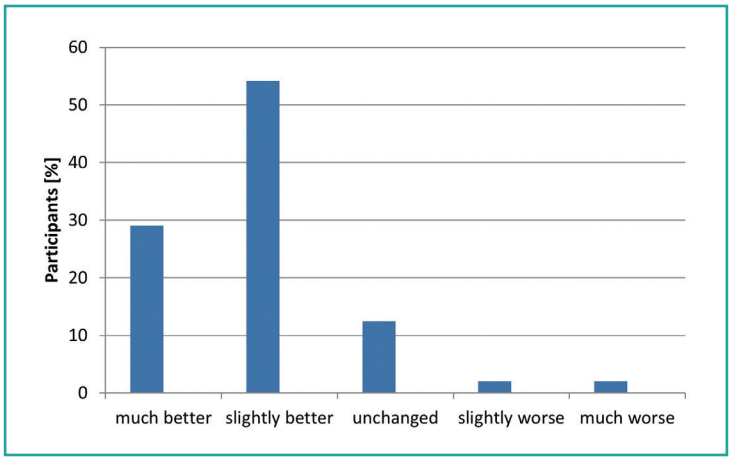

Figure 2. Change of individual health state during Menthacarin intake compared to two weeks previously as evaluated by pharmacy customers.

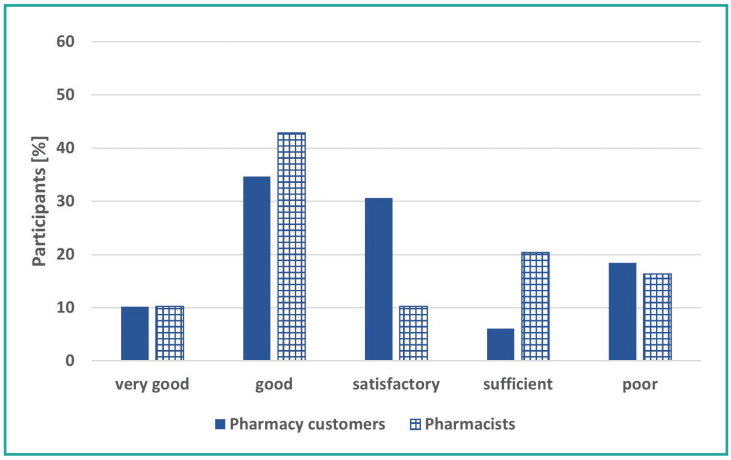

Figure 3. Effectiveness of Menthacarin as evaluated by pharmacy customers and pharmacists.

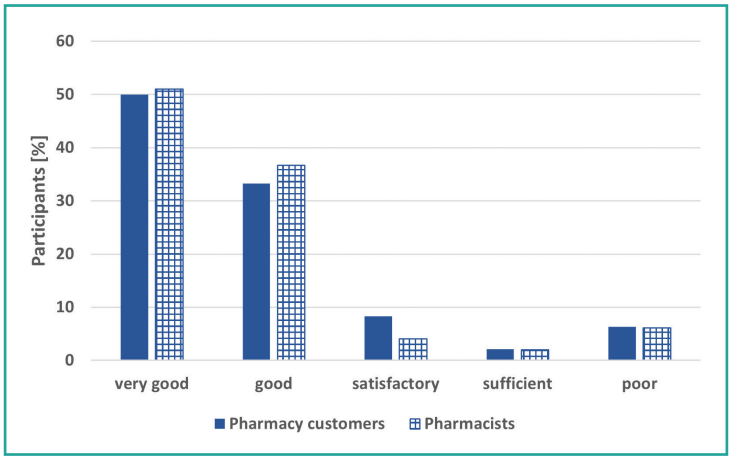

Figure 4. Tolerability of Menthacarin as evaluated by pharmacy customers and pharmacists.

show a significant reduction in FD-related pain and discomfort scores of the Nepean Dyspepsia Index (NDI) after a 4-week administration of Menthacarin (22). The findings are also consistent with results reported for a 4-week application of Menthacarin showing a reduction in pain which is comparable to that achieved under the prokinetic agent cisapride (20). Accordingly, the global effect of the reduction in FGID symptom severity as achieved by Menthacarin treatment was demonstrated to also have a considerable beneficial effect on the patients' quality of life (19, 22). These findings are supported by the results of the present study, in which nearly 85% of participating customers reported an improvement in their general health state.

An investigation of the long-term effects of Menthacarin was conducted as part of an open label study part over 11 months following up on a clinical trial by May and colleagues (19). During this active medication follow-up phase which followed the placebo-controlled trial phase, comparable treatment effects in both the former placebo and the Menthacarin groups after 4 months administration could be shown (30). The fact that treatment effects were comparable in both former randomization groups after 4 months suggests an overall regulating action of Menthacarin regarding FD-specific symptoms over the longer term.

Moreover, a clinical trial investigating the short-term effects of a combination preparation of caraway oil and L-menthol in FD patients (31) reported significant improvements in FD symptom severity within 24 hours as compared to placebo. These findings suggest an also short-term effectiveness of the peppermint oil-caraway oil combination which needs to be confirmed by further investigations.

With the prevailing majority of the pharmacy customers, and pharmacists' ratings for tolerability of Menthacarin being "very good" or "good" and an occurrence of only 3 adverse events for which a relation to the study medication could not be completely ruled out, the observed safety results also support former findings from randomized controlled trials (19-22).

In summary, the current study results derived from a "real-world" setting are in accordance with the current knowledge on safety and efficacy of Menthacarin from controlled clinical trials. Along with the results of the above-mentioned trials and literature reviews, the results of the present cohort study suggest Menthacarin to be a good alternative option to conventional management of abdominal pain in FGID. The high level of satisfaction with the dosage form of Menthacarin among the participating pharmacy customers, the favorable course of treatment, and the favorable tolerability ratings seen can be considered the main influential factors for the high compliance among the participating customers seen during the study. The fact that about half of the participating customers decided to continue Menthacarin intake after the end of the study and, moreover, nearly 75% of the customers considered a future use of the herbal drug is not only a statement in favor of the drug's convenience, tolerability, and safety but also for its applicability for self-medication in FGID.

CONCLUSIONS

The results of this pharmacy-based cohort study show a significant improvement in a broad range of FGID symptoms such as abdominal pain, impression of fullness and bloating, and improved overall health status after a 3-week observational treatment course with improvement onset in most participating pharmacy customers already after 1 week. Menthacarin was shown to be well applicable in pharmacy-supported self-medication of dyspeptic complaints due to FGID.

ETHICS

Fundings

The reported study and its publication were funded by Dr. Willmar Schwabe GmbH & Co.

KG. The funding source was involved in study design, data collection and interpretation. The final decision on content and submission was retained by the authors.

Conflict of interests

SN and AM have received honoraria from Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany. FAM, BS, and PF are employees of Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany.

Authors' contribution

SN was involved in interpretation of data, critical revisions related to important intellectual content, and gave final approval of the manuscript version to be published. FAM made substantial contributions to conception and design of the study, was involved in acquisition of data, analysis, and interpretation of data, critical revisions related to important intellectual content, and gave final approval of the manuscript version to be published. BS was involved in interpretation of data, drafting the article, critical revisions related to important intellectual content, and gave final approval of the manuscript version to be published. PF was involved in interpretation of data, drafting the article, critical revisions related to important intellectual content, and gave final approval of the manuscript version to be published. AM was involved in interpretation of data, critical revisions related to important intellectual content, and gave final approval of the manuscript version to be published.

Availability of data and materials

The datasets presented in this article are not readily available because raw data cannot be shared both due to ethical reasons and to data protection laws. All relevant data are within the paper. Requests to access the datasets should be directed to the corresponding author.

Ethical approval

As this was a non-interventional, open, multi-center, pharmacy-based, post-authorization cohort study, this investigation was not subject to an ethics approval. Prior to study start, notification was made to the National Association of Statutory Health Insurance Physicians and the Federal Institute for Drugs and Medical Devices (BfArM).

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RESEARCH ARTICLE

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TREATMENT OF SORE THROAT AND HOARSENESS WITH PELARGONIUM SIDOIDES EXTRACT EPS 7630: A META-ANALYSIS

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SUMMARY

The extract EPs 7630 from the roots of Pelargonium sidoides has been proven safe and effective in the treatment of acute respiratory tract infections (aRTI). The aim of this study was to perform a meta-analysis on the efficacy of EPs 7630 in patients suffering from acute non-streptococcal tonsillopharyngitis (ATP) or common cold (CC) regarding the symptoms sore throat and hoarseness.

This meta-analysis encompasses double-blind, placebo-controlled, randomized clinical trials investigating the efficacy of EPs 7630 in ATP or CC. Relevant publications were identified by searching PubMed and clinical trial registries (ISRCTN, ClinicalTrials.gov; search terms: acute tonsillopharyngitis, common cold, EPs 7630, Umckaloabo, hoarseness, and sore throat) and the assessment report on Pelargonium sidoides of the European Medicines Agency. Clinical study reports of unpublished trials were provided by the manufacturer of EPs 7630. Meta-analysis was performed separately for both indications, for formulations found, and for patient groups. Efficacy analyses were based on the change of symptom severity of 'sore throat' and 'hoarseness' as assessed by the respective indication-specific symptom scores, and on complete remission. Disease-related quality of life was also analyzed.

Seven trials with a total of 1,099 participants could be included into the meta-analysis. Clinical trials investigating EPs 7630 in CC comprised adults only, whereas those in ATP only included children. Results showed EPs 7630 to be superior to placebo in reducing both symptom severity and time until complete recovery for the symptoms 'sore throat' and 'hoarseness' in the indications investigated.

These findings suggest that EPs 7630 is effective in reducing severity and time to remission of the symptoms sore throat and hoarseness in ATP and CC, respectively.

Key words

Acute Tonsillopharyngitis; Common Cold; EPs 7630; Hoarseness; Sore Throat; Umckaloabo.

Impact statement

The provided evidence for superiority of EPs 7630 over placebo in reducing the symptom severity of sore throat and hoarseness in acute tonsillopharyngitis and common cold, respectively, and the fast symptom reduction seen suggest the herbal drug to

be a considerable alternative to therapy by analgesics or even antibiotics, which is a particularly important finding for the management of acute respiratory tract infections in children and adults.

Abbreviations

AB: Acute Bronchitis; aRTI: Acute respiratory tract infections; ATP: Acute Tonsillopharyngitis; CC: Common Cold; CI: confidence interval(s); CIS: Cold Intensity Score; FGK: Fragebogen zum Gesundheitszustand für Kinder; ICOS: inducible co-stimulator; ICOSL: inducible co stimulator ligand; IFN: interferon; IL: interleukin; QoL: quality of life; RCT: randomized controlled trial(s); RPS: Rhinopharyngitis-relevant Symptoms; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; TSS: Tonsillitis Severity Score.

INTRODUCTION

Acute respiratory tract infections (aRTI) are diseases that frequently affect otherwise healthy subjects of all ages (1-3). Whereas adults experience aRTI 2-3 times per year, children experience about twice as many (3, 4).

Of all aRTI, acute tonsillopharyngitis (ATP) is one of the most common conditions in children (5). Inflammations of pharynx, larynx, and neighboring areas appearing with symptoms of sore throat or hoarseness are also highly prevalent in common cold (CC), accompanied by acute cough and overall malaise (6, 7). These conditions are associated with a considerable degree of discomfort and pain and related interference with daily life activities (8-10). Therefore, in order to quickly reduce symptom severity, pharmacological therapy is often expected by patients consulting with primary care (11), which has currently led to excessive over-prescription and misuse of antibiotics (12), even though most cases have a viral origin (3, 5, 13, 14), in which no antibiotic treatment is needed (15, 16). This scenario has recently been subject to increasing criticism by the scientific community for reasons of medical consideration (low effectiveness in aRTI management (15)) and for economic aspects (unnecessary cost explosion (17)), as well as for reasons of sensible health care (increase in the risk of undesired side effects of antibiotics and development of antimicrobial resistance (18)), with the last aspect of sensible health-care being of particular interest for aRTI therapy in children, who are frequently affected.

Alternative and efficacious options for the treatment of aRTI exist and have gained increasing scientific interest. This group includes herbal medicines, some of which have a long-standing tradition of successful aRTI treatment (19), such as *Pelargonium sidoides*. EPs 7630[1], an extract from the roots of *Pelargonium* sidoides (1 : 8–10), extraction solvent: ethanol 11% (w/w), is used in children from the age of one year, adolescents and adults for the treatment of aRTI in several countries in Europe, Asia, Australia, as well as Central and South America. Tablet as well as liquid (syrup and drops) formulations are available.

EPs 7630 is classified by the European Pharmacopoeia as "other extract". It is therefore not adjusted to a specific content of constituents. To confirm the quality and identity of the herbal material, the dried material was tested in an array of biochemical and phytochemical methods. Approximately 80% m/m of the extract are assigned to six major groups of constituents, namely unsubstituted and substituted oligomeric prodelphinidins, monomeric and oligomeric carbohydrates, minerals, peptides, purine derivatives, and highly substituted benzopyranones (20). With a share of about 40%

1 EPs® 7630 is the active ingredient of the product Umckaloabo® (ISO Arzneimittel, Ettlingen, Germany).

of the dried extract, oligomeric prodelphinidins (in this context commonly designated as polyphenols) are the most significant of this groups.

Medicinal products with EPs 7630 as active substance are authorized by numerous authorities worldwide based on nonclinical studies as well as on their proven clinical safety (21-23). EPs 7630 contains a small amount of highly substituted coumarin derivatives that are exclusively 7-hydroxycoumarin derivatives (24) which do not possess the structural characteristics of the known anticoagulant coumarins (25-27). No influence of EPs 7630 on plasma coagulation, as well as possible pharmacokinetic or pharmacodynamic interactions with warfarin, was observed in animal experiments (24). On this ground, it appears unlikely that an increased tendency towards bleeding complications arises in patients due to intake of EPs 7630 (24). Furthermore, unlike other coumarins, 7-hydroxycoumarin derivatives contained in EPs 7630 do not possess hepatotoxic properties (28).

The proanthocyanidin-rich extract EPs 7630 displays immune-modulating as well as antiviral properties (29, 30). Several non-clinical studies demonstrated activity against a variety of respiratory viruses such as influenza A virus (H1N1, H3N2), respiratory syncytial virus, human coronavirus (HCoV) HCoV-229E, and parainfluenza virus (31, 32). Although the distinct contributions of the individual constituents of EPs 7630 are not fully defined yet, the polyphenolic compounds, in particular prodelphinidins, are thought to be responsible for the antiviral effects described (31, 32). EPs 7630 also inhibited the attachment of human immunodeficiency virus 1 to human immune cells and viral entry in cell culture experiments (33). Furthermore, the herbal extract inhibited replication of influenza A virus by inhibition of hemagglutinin and neuraminidase activity (31), which was at least partly mediated by the proanthocyanidin constituents. In human bronchial epithelial cells, EPs 7630 reduced replication of rhinovirus-16 by downregulating the expression of inducible co-stimulator (ICOS) and its ligand (ICOSL) as well as the surface calreticulin receptor, whereas levels of proteins supporting host defense were increased (34). Mechanistically, EPs 7630 significantly reduced host cell attachment of various viruses and prevented the release of viruses from infected cells. The extract was also shown to stimulate the release of nitric oxide, type I interferon (IFN), and different cytokines involved in host defense mechanisms (35-37). In athletes, EPs 7630 modulated the immune response during strenuous exercise by increasing the production of immunoglobulin α in saliva, decreasing serum interleukin (IL)-15 and IL-6 levels, and reducing IL-15 in the nasal mucosa (38). In children, the Pelargonium sidoides extract was also found to prevent asthma attacks provoked by rhinovirus, probably by interfering with IL-6-, IL-8-, and IL-16-mediated inflammation (39). Moreover, most recent results from in vitro experiments showed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cell entry inhibition and differential immunomodulatory functions of EPs 7630 against SARS-CoV-2 (30).

Over the past 25 years, more than 30 clinical trials were conducted investigating EPs 7630 for the treatment of acute respiratory tract infections (aRTI) (40). Several systematic reviews and meta-analyses of controlled, randomized clinical trials (RCT) investigating the efficacy and safety of EPs 7630 in aRTI (22, 23, 41-48) have been published, providing evidence for the efficacy and safety of the herbal medicinal drug in the treatment of acute bronchitis (AB), common cold, ATP, and acute rhinosinusitis (ARS). We now performed a meta-analysis of double-blind, placebo-controlled RCT in order to evaluate the efficacy of EPs 7630 compared to placebo regarding the symptoms sore throat and hoarseness, the most impairing symptoms that come with ATP and CC.

METHODS

Included trials

Double-blind, placebo-controlled RCT investigating the efficacy of EPs 7630 in the indications ATP or CC available until the end of the year 2021 were eligible. Relevant publications were identified by free-text searches of all fields of PubMed as well as of clinical trial registries (ISRCTN registry; ClinicalTrials.gov) using the search terms 'acute tonsillopharyngitis', 'common cold', 'hoarseness', and 'sore throat', each in combination with either 'EPs 7630' or 'Umckaloabo', and from the List of references supporting the assessment of Pelargonium sidoides DC and/or Pelargonium reniforme Curt., radix of the European Medicines Agency (EMA) (49). Moreover, clinical study reports of unpublished trials were provided by the manufacturer of EPs 7630 (Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany). The literature from the earliest record until 31 December 2021 was covered.

Outcome measures

Primary outcome measure of treatment effects was the change of the respective indication-specific symptom scores of 'sore throat' and 'hoarseness' between baseline and pre-defined day under EPs 7630 as compared to placebo, as well as the number of patients with partly or complete recovery from these symptoms. Response to therapy was also calculated (defined as the number of patients with a symptom reduction concerning 'sore throat' or 'hoarseness', respectively, by at least 50% between baseline and pre-defined day).

A further objective was to gain insight into data reflecting the change of disease-related quality of life (QoL) under EPs 7630 therapy as compared to placebo.

Only data of patients in whom the respective symptom ('sore throat', 'hoarseness') was present at baseline was included into the meta-analyses. Study selection and methods of the analysis were specified in advance and documented accordingly.

Ethics

All trials included into this meta-analysis were reported to be planned, conducted, and analyzed according to the principles of Good Clinical Practice and the Declaration of Helsinki. The trial protocols and other required trial documents were approved by the respective independent ethics committee and competent authorities. All participants in the studies gave their informed consent or informed consent was provided by their legal representative, respectively.

Statistics

All meta-analyses performed were based on the full analysis sets of the RCT included. Review Manager (RevMan) Version 5.4 software (50) developed by the Cochrane Collaboration was employed. Heterogeneity between the primary trials was assessed using the I2 statistic. Random effects models were computed in case of I2 > 5%, otherwise fixed effect models were used. Meta-analyses of treatment efficacy for continuous outcome parameters were based on the mean change in the corresponding symptoms between baseline and the pre-defined day for each treatment group in the single trials. The difference between mean values of the treatment groups and the associated 95% confidence intervals (CI) on the respective scales subsequently resulted from RevMan. Risk ratios and their 95% confidence intervals were the chosen estimates from the meta-analyses related to binary parameters such as the number of patients with partial or complete recovery. As we conducted descriptive analyses, the resulting 95% confidence intervals and p-values must be interpreted accordingly.

Results

A total of 9 eligible records (6 published (51-56), 3 unpublished (57-59)) were identified. For these records, the full text was assessed for further eligibility. One record reported a different dosing scheme compared to the other trials found and was therefore excluded to

ensure a better comparability of the studies (51). Thus, 8 records reporting on a total of 7 clinical trials (for one clinical trial, each of two trial parts was published separately (54, 55)) were included. Data in our meta-analysis were thus taken from publications and unpublished data of seven RCT investigating the efficacy and safety of EPs 7630 in the indications ATP and CC (**table I**).

Overall, the 7 RCT comprised 1,099 trial participants (549 randomized to EPs 7630, 550 randomized to placebo); of these, 267 were children (aged 6-10 years) and 832 adults (18 years or older). Mean demographic data are summarized in **table II**.

In those RCT investigating EP 7630 in ATP (Trials 1 (52) and 2 (53)), patients were children aged 6-10 years (EPs 7630: 133, placebo: 134).

In those RCT investigating EPs 7630 in CC (Trials 3-7 (54-59)), patients were 416 adults randomized to EPs 7630 and 416 adults randomized to placebo. Two of the trials investigated the efficacy of EPs 7630 film-coated tablets (Trials 4 (56) and 7 (59)), whereas the solution

Table I. Trials included.

Indication	Trial	Age range	Treatment duration/ dosage	Primary efficacy variable	Number of patients (FAS)	
					EPs 7630	Placebo
Acute tonsillo-pharyngitis	1 (52)	6-10 years	6 days: 20 drops t.i.d.	Change of TSS total score from baseline to day 4	60	64
	2 (53)	6-10 years	6 days: 20 drops t.i.d.	Change of TSS total score from baseline to day 4	73	70
Common Cold	3 (54, 55)	≥ 18 years	10 days 3a: 3*30 drops/d or 3b: 3*60 drops/d	Change of CIS total score from baseline to day 5	3*30 drops: 52	51
					3*60 drops: 52	52
	4 (56)	≥ 18 years	10 days 3*40 mg/d	Change of CIS total score from baseline to day 5	53	52
	5 (57)	≥ 18 years	10 days 3*30 drops/d	Change of RPS total score from baseline to day 5	99	101
	6 (58)	≥ 18 years	10 days 3*30 drops/d	Change of RPS total score from baseline to day 5	101	100
	7 (59)	≥ 18 years	10 days 3*20 mg/d	Change of RPS total score from baseline to day 5	59	60

Reference's number in parentheses.

Table II. Demographics.

Indication	Trial	Treatment group	Mean age (SD)	Male	Female	Total
ATP	1 (52)	EPs 7630	7.60 (1.09)	29	31	60
		Placebo	7.44 (1.19)	28	36	64
	2 (53)	EPs 7630	7.58 (1.26)	40	33	73
		Placebo	7.46 (1.13)	30	40	70
	EPs 7630		**7.59 (1.18)**	**69**	**64**	**133**
	Placebo		**7.45 (1.15)**	**58**	**76**	**134**
CC	3a* (standard dose) (54)	EPs 7630	34.52 (10.60)	16	36	52
		Placebo	37.35 (10.52)	16	35	51
	3b* (high dose) (55)	EPs 7630	36.81 (9.91)	14	38	52
		Placebo	33.75 (10.84)	12	40	52
	4 (56)	EPs 7630	34.98 (10.86)	13	40	53
		Placebo	37.69 (10.48)	11	41	52
	5 (57)	EPs 7630	37.11 (13.58)	33	66	99
		Placebo	37.13 (12.46)	35	66	101
	6 (58)	EPs 7630	44.76 (14.10)	37	64	101
		Placebo	46.18 (14.09)	30	70	100
	7 (59)	EPs 7630	32.63 (11.02)	33	26	59
		Placebo	33.33 (10.64)	31	29	60
	EPs 7630		**37.70 (12.93)**	**146**	**270**	**416**
	Placebo		**38.43 (12.80)**	**135**	**281**	**416**

*Trials 3a and 3b are two parts of the same clinical trial, each part was published separately (54, 55).
ATP = acute tonsillopharyngitis; CC = common cold.
Reference's number in parentheses.

formulation was investigated in the remaining three trials (54, 55, 57, 58).

As defined by the respective protocols, the trials investigating ATP (52, 53) employed a tonsillitis severity score (TSS) (60) for symptom severity rating, which comprises five subscores, namely 'dysphagia', 'sore throat', 'salivation', 'redness', and 'fever'. Each of these symptoms was rated by the responsible investigator on a four-point rating scale ranging from severe (= 3), moderate (= 2), mild (= 1) to not present (= 0).

Secondary efficacy variables of the ATP trials considered for the analysis were: Single items of the FGK Questionnaire (Fragebogen zum Gesundheitszustand für Kinder) (60) as supportive indicators of the children's health state and its change in relation to symptom severity.

The single items of FGK for children ≤ 18 years include 'Everything is too much for me', 'I am feeling ill', 'I am scared', 'I have trouble playing or learning', 'I sleep badly',

'I have problems getting into conversation with others'. Results for each item were documented on a 5-point scale (not at all (= 1), a little bit (= 2), moderate (= 3), distinctive (= 4), very distinctive (= 5)) in the patient diaries.
For those RCT investigating the efficacy of EPs 7630 in adult patients diagnosed with CC, two identical symptom severity rating tools named differently were employed, *i.e.* Rhinopharyngitis-relevant Symptoms (RPS) (57-59) and Cold Intensity Score (CIS) (54-56). Both rating tools include the CC symptoms 'nasal drainage', 'sore throat', 'nasal congestion', 'sneezing', 'scratchy throat', 'hoarseness', 'cough', 'headache', 'muscle aches', and 'fever', which had to be evaluated by the investigator on a five-point scale ranging from inexistent (= 0) to very severe (= 4).
Secondary efficacy variables of the CC trials considered for the analysis were the single items 'Mobility', 'Self-care', 'Usual activity', 'Pain/discomfort', and 'Anxiety/depression' of the EQ-5D Questionnaire (61) as further indicators for the disease-related QoL of patients. Each item was measured on a scale from 1 to 3 (1 = no problems, 2 = some problems, 3 = severe problems).

Participants

Patients included in the respective RCT had to be between 6 and 10 years old and to present with a diagnosis of non-streptococcal ATP and an overall symptom severity of at least 8 points as measured on the TSS.
Patients who participated in the CC trials employing the RPS (57-59) had to be 18 years or older and to present with both major rhinopharyngitis-relevant symptoms ('nasal discharge' and 'sore throat') rated with ≥ 2 points each and at least two minor rhinopharyngitis-relevant symptoms ('nasal congestion', 'sneezing', 'scratchy throat', 'hoarseness', 'cough', 'headache', 'muscle aches', or 'fever') rated with ≥ 2 points each or one major RPS rated with ≥ 2 points and at least three minor rhinopharyngitis-relevant symptoms rated with ≥ 2 points each on the RPS. Eligible patients who participated in the CC trials employing the CIS (54-56) were required to present with at least two major and one minor or with one major and three minor CC symptoms (maximum symptom score 40 points) present for no longer than 48 hours.

Interventions

In the clinical trials included in this meta-analysis, the solution (drops) and film-coated tablet formulations were investigated. In the ATP trials (Trials 1, 2 (52, 53)), children were administered 20 drops EPs 7630 t.i.d. over 6 days.
In the CC trials (Trials 3-7 (54-59)), patients were treated with EPs 7630 over 10 days. Medication formulation and dosage were usually 3 x 30 drops per day. In trial 3, a high-dose cohort received 3 x 60 drops per day (55). In Trial 4 (56), EPs 7630 was administered as 1 tablet t.i.d. each containing 40 mg of EPs 7630. In Trial 7 (59), participants received 3 x 20 mg tablets per day.

Outcome measures

The time point chosen for the assessment of change in symptom severity and in disease-related QoL was day 4 in the ATP trials, and day 5 in the CC trials.
The symptom 'sore throat' as rated by the investigator on the four-point TSS (ATP trials, trials including children) and on the five-point CIS/RPS (CC trials, trials including adults), respectively, was assessed in all eligible RCT. The symptom 'hoarseness' was rated by the investigator on the five-point CIS/RPS and was assessed in CC studies only.
In addition, the following responder criteria (number of patients) for the respective pre-defined days were calculated: Complete remission/recovery with respect to the symptom 'sore throat'/'hoarseness' ('sore throat'/'hoarseness' = 0 at pre-defined day); Reduction of 'sore throat'/'hoarseness' by at

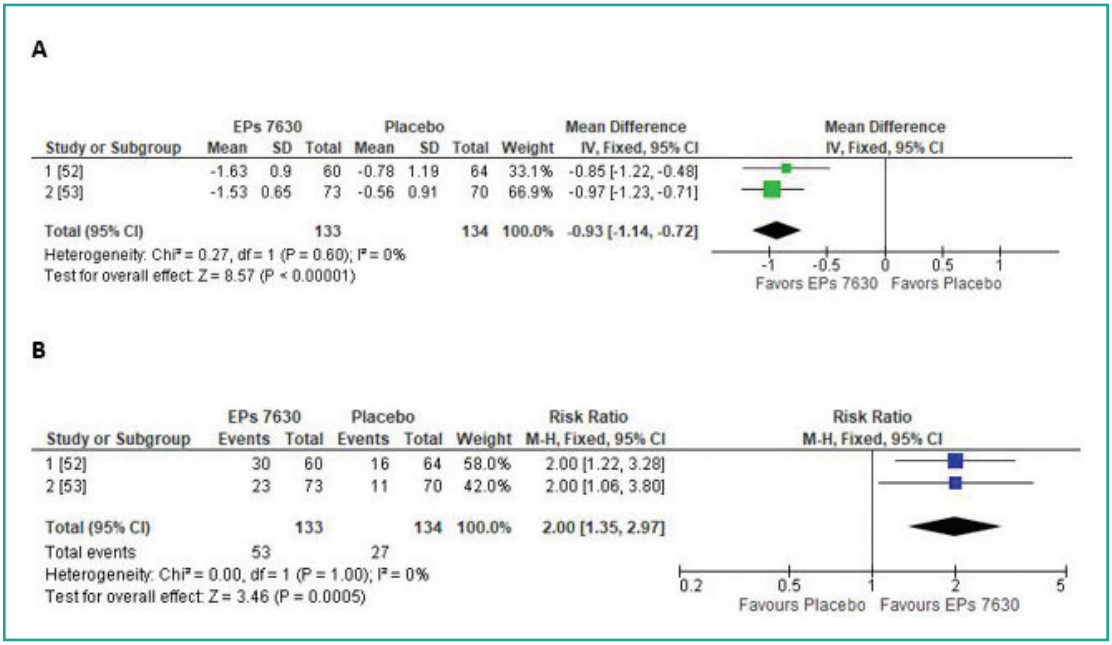
least 50% between baseline and pre-defined day (response to therapy); FGK questionnaire items (RCT including children): number of participants with remission as measured for single items at day 4; EQ-5D Questionnaire (RCT including adults): number of participants with remission as measured for single items at day 5.

Meta-analysis results

Sore throat

Meta-analysis results of the two RCT investigating the efficacy of EPs 7630 in ATP revealed that children treated with EPs 7630 showed a significantly pronounced improvement of the symptom 'sore throat' after four days compared to placebo treatment (- 0.93 ([- 1.14; - 0.72] 95% CI); (**figure 1 A**). Accordingly, a significant advantage of the herbal extract was shown for the number of patients with complete remission of the symptom 'sore throat' by Day 4, which resulted in a relative risk of 2.00 ([1.35; 2.97] 95% CI) favoring EPs 7630 (**figure 1 B**). Calculated responder rates (indicated by a ≥ 50% improvement of the symptom 'sore throat' by day 4 as rated on the TSS) were 88.0% for EPs 7630 and 41.8% for placebo, respectively. Therapy response rates under EPs 7630 by day 4 were therefore significantly higher than those achieved under placebo. Corresponding risk ratios calculated for therapy response in the indications and age groups under evaluation are shown in **table III**.

Meta-analysis results of the five RCT investigating the efficacy of EPs 7630 in adults with CC demonstrated a significant superiority of the herbal extract in reducing the severity of the symptom 'sore throat' by day 5 as assessed by CIS/RPS, resulting in a mean difference of -0.24 ([- 0.38; - 0.11] 95% CI) (**figure 2 A**). Similar results were obtained regarding the number of patients who showed an at least 50% reduction in symptom severity for 'sore throat' by day 5 - a goal that was achieved by 78.5% of the patients randomized to EPs 7630 and 65.2% of the patients randomized to placebo (**table III**). Calculations for patients who had completely



Figures 1. Children with acute tonsillopharyngitis: **(A)** change of symptom 'sore throat' until day 4 (patients with impairment at baseline); **(B)** complete remission of symptom 'sore throat' until day 4 (patients with impairment at baseline).

Table III. Meta-analysis results for responder rates (≥ 50% reduction in symptom severity at pre-defined day) in acute tonsillopharyngitis and common cold, based on Trials 1 and 2, and 3-7, respectively.

Outcome measure	Trial	EPs 7630			Placebo			Total relative risk [95% CI]† p-value
		n (participants impaired)	Responders		n (participants impaired)	Responders		
≥ 50% Reduction of symptom ´sore throat´ in children with ATP at day 4	1, 2 (52, 53)	133	117	88.0%	134	56	41.8%	2.11 [1.71;2.61] p < 0.01
≥ 50% Reduction of symptom ´sore throat´ in adults with CC at day 5	3-7 (54-59)	386	303	78.5%	374	244	65.2%	1.24 [1.05;1.46] p = 0.01
≥ 50% Reduction of symptom ´hoarseness´ in adults with CC at day 5	3-7 (54-59)	345	258	74.8%	339	212	62.5%	1.23 [1.02;1.47] p = 0.03

† values > 1 favor EPs 7630. ATP = acute tonsillopharyngitis; CC = common cold.
Reference's number in parentheses.

recovered from the symptom 'sore throat' by day 5 resulted in a relative risk of 1.13 ([0.87; 1.48] 95% CI) indicating no statistically significant differences between the treatment groups (**figure 2 B**).

Hoarseness

Meta-analysis results of the five RCT in adults with CC regarding symptom severity reduction by day 5 were also statistically significant, resulting in a mean difference of - 0.30 ([- 0.55; -0.04] 95% CI) favoring EPs 7630 (**figure 3 A**). Calculations regarding complete recovery from this symptom by day 5 resulted in a relative risk of 1.34 ([0.99; 1.81] 95% CI) favoring EPs 7630 (**figure 3 B**). The meta-analysis of response rate (indicated by a symptom severity improvement of ≥ 50% by day 5) showed statistically significant results in favor of EPs 7630, with response rates of 74.8% for EPs 7630 and 62.5% for placebo, respectively (**table III**).

Disease-related quality of life

Disease-related QoL in children with ATP was assessed by the FGK questionnaire. Regarding the change calculated for the single FGK items, children treated with EPs 7630 showed greater remission by day 4 than those treated with placebo. The meta-analysis of those FGK items that particularly reflect the immediate impact of ATP, namely item 1 ('Everything is too much'), item 3 ('Trouble playing/learning'), and item 5 ('Sleeping badly') resulted in considerable remission rates under EPs 7630 as compared to placebo (54.2% *vs* 17.8%, 44.0% *vs* 14.6%, and 74.4% *vs* 29.9%, respectively). All meta-analysis results regarding the change

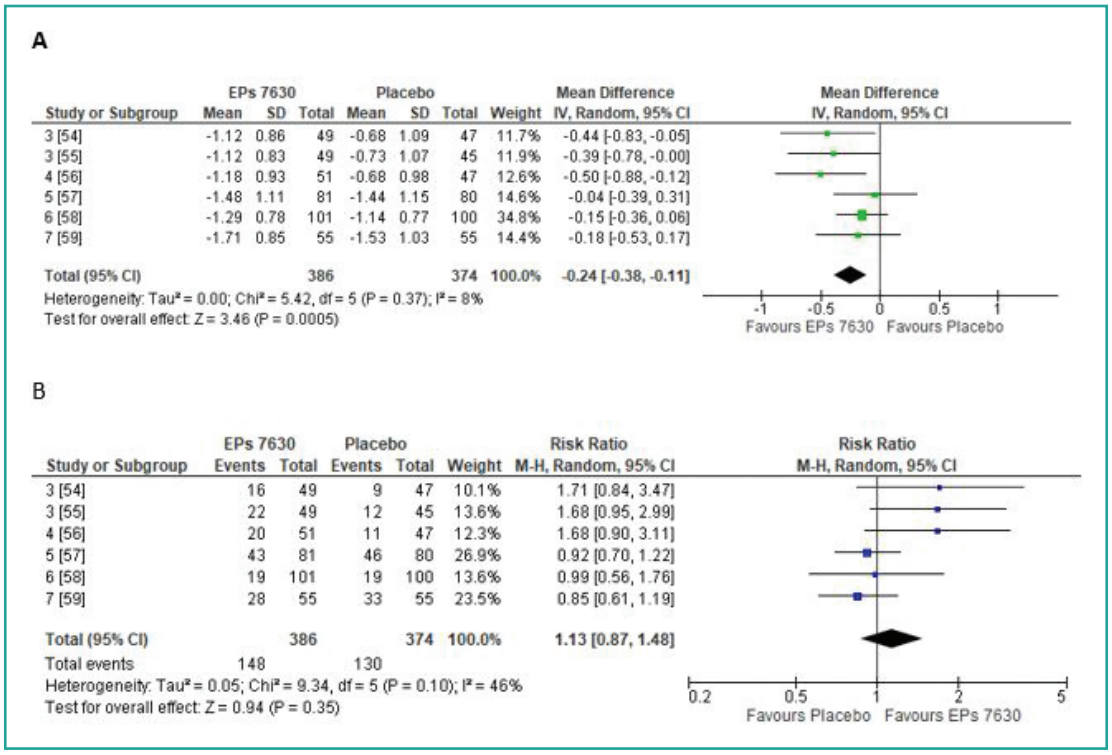


Figure 2. Adults with common cold: **(A)** change of symptom 'sore throat' until day 5 (patients with impairment at baseline); **(B)** complete remission of symptom 'sore throat' until day 5 (patients with impairment at baseline).

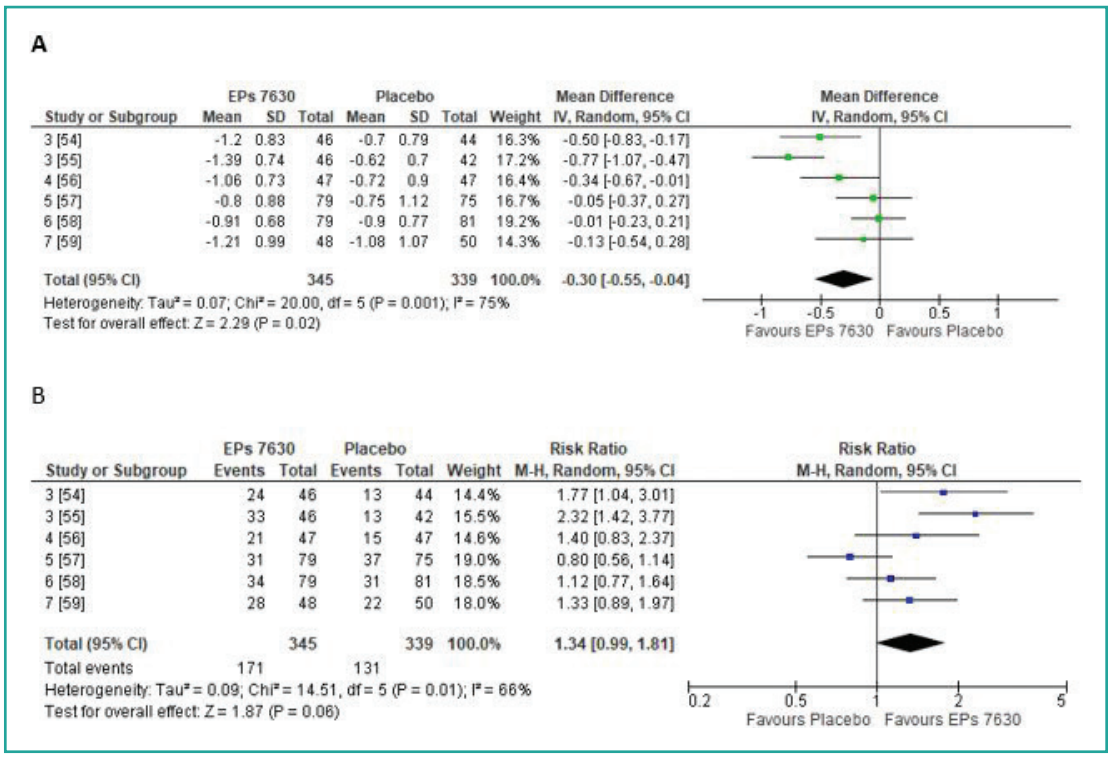


Figure 3. Adults with common cold: **(A)** change of symptom 'hoarseness' until day 5 (patients with impairment at baseline); **(B)** complete remission of symptom 'hoarseness' until day 5 (patients with impairment at baseline).

Table IV. Meta-analysis results regarding change of disease-related quality of life in children with ATP (Trials 1-2): full remission rates by day 4.

Indication	Outcome measure	Remission rates		Risk ratio [95% CI]
		EPs 7630	Placebo	
ATP in children	FGK item 1 (´everything is too much´)	54.2%	17.8%	3.06 [2.05;4.57]
	FGK item 2 (´feeling ill´)	28.6%	10.5%	2.79 [1.60;4.88]
	FGK item 3 (´scared´)	88.2%	51.6%	1.71 [1.39;2.11]
	FGK item 4 (´trouble playing/learning´)	44.0%	14.6%	3.02 [1.91;4.78]
	FGK item 5 (´sleeping badly´)	74.4%	29.9%	2.49 [1.87;3.31]
	FGK item 6 (´troubles getting into conversations´)	71.8%	39.3%	1.82 [1.42;2.35]

ATP = acute tonsillopharyngitis.

in disease-related QoL are summarized in **table IV**.

Disease-related QoL in adults with CC was assessed by the EQ-5D questionnaire. Data analysis related to QoL assessed at baseline and day 5 showed an advantage of EPs 7630 over placebo in numbers of patients who achieved full remission regarding the single EQ-5D categories assessed. Improvement in disease-related QoL in CC therefore corresponds to the likewise improvement in the symptoms 'hoarseness' and 'sore throat' at day 5.

DISCUSSION

Sore throat and hoarseness belong to the most bothering symptoms occurring in aRTI as they are associated with pain and difficulties in conducting daily life requirements such as work or school attendance (9, 10). The presented meta-analysis results demonstrate the superiority of EPs 7630 over placebo in reducing the severity of disease-related symptoms and in expediting the onset of symptom alleviation. Thus, the actual duration of perceived impairment as assessed by patient-reported data was reduced.

In the ATP trials**,** the symptom 'sore throat' was present in all patients at trial start and meta-analysis results showed a clear benefit with respect to the symptom relief until day 4 for EPs 7630 compared to placebo. This finding is supported by a significant advantage of the herbal extract when comparing the number of patients with complete symptom remission at day 4 between treatment groups (**figure 1 B**). Response to therapy regarding the symptom 'sore throat' by day 4 was significantly higher for children in the EPs 7630 group compared to the placebo group (**table III**), which also suggests an earlier onset of recovery under the herbal extract than under placebo. These findings are further supported by the results of FGK assessments at day 4: For any of the single FGK items, a significantly greater number of children with complete remission was reported in the EPs 7630 group compared with the placebo group. These findings are thus indicative of a beneficial impact of EPs 7630 on well-being and a fast return to daily-life activities.

For adults with CC, the meta-analysis results also demonstrated a statistically significant superiority of EPs 7630 over placebo in reducing the severity of the symptom 'sore throat' by day 5. These findings are also in line with results of a meta-analysis performed earlier (45), in which data from the whole study population of the same trials was analyzed with fo-

cus on CIS results. In this former investigation, findings for severity change of 'sore throat' were comparable to those found in the present analysis in which only patients presenting with the symptom 'sore throat' at baseline were evaluated. In a recently published secondary subgroup-analysis of an open-label, uncontrolled clinical trial in adults suffering from CC, it was also shown that the presence or absence of the CC-associated human corona viruses HCoV-HKU1, HCoV-OC43, HCoV-NL63, or HCoV-229E did not have an impact on treatment outcomes, with patients of both subsets showing comparable improvements in symptom severity of 'sore throat' (62). The analysis revealed a somewhat faster response during treatment with EPs 7630 in the HCoV subset. However, the group differences were not statistically significant.

Results of our analysis for the severity change of the symptom 'hoarseness' correspond with the findings on the symptom 'sore throat' favoring EPs 7630 over placebo. Again, results for the symptom severity reduction by Day 5 are in line with earlier results obtained for the complete study population (45).

Efficacy of EPs 7630 with respect to the analyzed symptoms could most evidently be shown in the studies with children suffering from ATP. Nevertheless, the efficacy of the herbal drug could be demonstrated for both indications and target populations. As both published and unpublished RCT could be assessed for inclusion in this meta-analysis, a publication bias, which is often associated with systematic reviews, can be excluded.

When performing a meta-analysis that aims at the achievement of credible results, the impact of spontaneous remission on efficacy assessment must be considered. The appropriateness of the time points for assessment of symptom severity change chosen in the RCT included in this meta-analysis (day 4 in ATP, day 5 in CC) is proven by the obtained results which in most cases show significant differences in effectiveness between EPs 7630 and placebo with regard to the reduction of the symptoms 'sore throat' and 'hoarseness', thus suggesting a minimal impact of spontaneous remission.

CONCLUSIONS

The meta-analysis presented provides evidence for superiority of EPs 7630 over placebo in reducing the severity of the symptoms 'sore throat' in children with ATP and adults with CC, respectively, as well as 'hoarseness' in adults suffering from CC. Furthermore, the results suggest an earlier onset of symptom remission as well as a shortened duration of the disease achieved by administration of the herbal drug. The fast reduction of these most impairing aRTI symptoms by EPs 7630 suggests the herbal drug to be a considerable alternative to therapy by analgesics or even antibiotics, which is a particularly important finding for aRTI management in children and adults.

ETHICS

Fundings

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The sponsor provided conceptualization of the study design, analysis of data as well as medical writing and gave editorial support. The final decision on content was retained by the authors.

Conflict of interests

WK, WL, GJS and PK have received honoraria from Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany for scientific services. WK and GJS also received research funds from Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany. PK also received lecture and advisory board honoraria from AstraZeneca, Chiesi, GSK, Menarini, Novartis, and Teva. WL also received personal fees from Bayer, Biotronik, Merz, Scope, Cassella, optima, Oxular, Fresenius, Hennig, Occlutech, CRM Biometrics, Clin-

Competence for Statistical Consulting and Analyses, and IDMCs, outside the submitted work. AZ, JBS and PF are employees of Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany.

Authors' contribution

All authors listed have significantly contributed to the development and the writing of this article.

Availability of data and material

Due to ethical reasons and in terms of data protection law, raw data cannot be shared. To the extent permitted by law, trial data required for validation purposes is already disclosed in result reports on corresponding databases. All relevant data are within the paper.

Ethical approval

All trials included into this meta-analysis were reported to be planned, conducted, and analyzed according to the principles of Good Clinical Practice and the Declaration of Helsinki. The trial protocols and other required trial documents were approved by the respective independent ethics committee and competent authorities. All participants in the studies gave their informed consent or informed consent was provided by their legal representative, respectively.

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REVIEW

pharmadvances

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CONTROLLING THE ACTIVATION OF THE PROKINETICIN SYSTEM AS THERAPEUTIC APPROACH TO RELIEF NEUROPATHIC PAIN AND REDUCE NEUROINFLAMMATION

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SUMMARY

Neuropathic pain is a relevant clinical problem worldwide, since current therapeutic treatments are unsatisfactory. The identification of novel therapeutic targets and the development of new pharmacological approaches remain a priority. This pathological condition is generally triggered by an injury at peripheral or central nervous system and it is characterized by pain exacerbation and neuronal hypersensitization, resulting in abnormal pain transmission. Neuroinflammation in the peripheral and central nervous system largely contributes to neuropathic pain onset, development and maintenance. In this scenario, the recently identified chemokine family, the prokineticin system (PKS), is a promising pharmacological target for the management of neuropathic pain, considering its pronociceptive and proinflammatory properties and its role in neuronal-glia interaction. Moreover, the availability of specific receptor antagonists makes this system even more interesting in order to control prokineticin activity. In this review we report all preclinical data available on the role of PKS in the physiopathology of neuropathic pain. The results clearly suggest that drugs which block the PKS may represent an innovative and efficacious pharmacological treatment to control neuropathic pain in patients.

Key words
Prokineticin system; neuropathic pain; neuroinflammation; animal models.

Impact statement

- PK2/PKRs play a pivotal role in pain transmission.
- Neuropathic pain state increases PK2/PKRs levels in the main pain stations.
- Blocking PKRs with specific antagonists reduces pain and neuroinflammation.
- Prokineticin system opens a new therapeutic avenue for neuropathic pain treatment.

Abbreviations

PNS: peripheral nervous system; CNS: central nervous system; PKS: prokineticin system; PK1: prokineticin 1; PK2: prokineticin 2; PKs: prokineticins; PKR1: prokineticin receptor 1; PKR2: prokineticin receptor 2; PKRs: prokineticin receptors; EG-VEGF: vascular endothelial factor of the endocrine gland; aa: amino acids; Trp: Tryptophan; NPY: Neu-

ropeptide Y; cAMP: Adenosine monophosphate cyclique; MAPK: Mitogen-activated protein kinase; Ala: Alanine; val: valine; TRPV1: transient receptor potential vanilloid receptor 1; CFA: Freund's Complete Adjuvant; hr: hour; DRG: dorsal root ganglia; WT: Wild Type; TRPA1: Transient receptor potential ankyrin 1; CGRP: calcitonin gene-related peptide; SP: Substance P; CCI: constriction nerve injury; SCI: spared nerve injury; CIBP: cancer-induced bone pain; STZ: streptozotocin; VCR: vincristine; BTZ: bortezomib; c.d.: cumulative dose; PWT: paw withdrawal threshold; PWL: paw withdrawal latency; sc: subcutaneous; iv: intravenous; PN: perineural; IT: intrathecal; min: minutes; PAG: periaqueductal gray; IL-1β: Interleukin 1 beta; IL-10: Interleukin 10; CD11b: Cluster Of Differentiation 11b; CD68: Cluster Of Differentiation 68; TLR4: Toll-like receptor 4; IL-6: Interleukin 6; TNFα: Tumour Necrosis Factor alpha; GFAP: Glial Fibrillary Acidic Protein; ATF3: Activating transcription factor 3; CD206: Cluster of Differentiation 206; iba-1: Allograft inflammatory factor 1; KDM6A: Lysine-specific demethylase 6A; PPAR: Peroxisome proliferator-activated receptor; DRG: dorsal root ganglia; PFC: prefrontal cortex; HPC: hippocampus; HPT: hypothalamus.

INTRODUCTION

As well known, acute pain has a physiologically protective role, since it warns body about an ongoing or impending tissue damage, in order to elicit appropriate behavioral responses to minimize it. When tissue damage occurs, there are changes in excitability of peripheral and central nervous system (PNS and CNS), which transmit nociceptive information from the site where the *noxa* is present up to the cortex. In the inflamed tissue sustained but reversible hypersensitivity may occur and the triggering of these mechanisms helps in the recovery process of wounds, thus avoiding any contact with the injured area until healing. In contrast, chronic pain does not offer biological or adaptive advantages, and becomes a disease itself. In particular, neuropathic pain is a highly debilitating form of chronic pain generally triggered by direct or indirect injury at PNS or CNS level and is one of the most important clinical problems worldwide (1). This pathological condition is characterized by pain exacerbation (in particular with allodynia and hyperalgesia development) and neuronal hypersensitization at spinal and supraspinal level, which lead to an abnormal pain transmission (2). The cause of neuropathic pain development cannot be always established or reversed (3). Indeed, its pathophysiology is very complex: imbalances between excitatory and inhibitory somatosensory signaling, ion channels alterations and abnormal immune reactions, associated with neuronal and synaptic plasticity, are all implicated in neuropathic pain states (4-6). Emerging evidence indicate that neuronal activity enhancement requires glial cells activation. These cells are physiologically involved in homeostasis maintaining, supporting and protecting neuronal cells (7), however, in pathological conditions, such as during a chronic pain condition, they become activated, proliferate, change their morphology and release pro-inflammatory mediators that promote neuronal sensitization (8-10).

It is now evident that pro- and anti- inflammatory cytokines produced by resident and infiltrating immune cells in the nervous system and by glial cells are common denominators in neuropathic pain (11). Indeed, cytokines start a cascade of events related to neuroinflammation which can maintain and/or worsen the original lesion, contributing to pain generation and its chronicization (12). In addition, current therapeutic tools are unsatisfactory since this type of pain is frequently resistant to available treatments (13, 14). For these reasons, the identification of novel therapeutic targets and the development of new pharmacological approaches for neuropathic pain remain a challenge. In this scenario, a novel class of chemokines and their receptors, the prokineticin system (PKS) have recently been demonstrated to have an important role in neuropathic pain, sustaining

pain and neuroinflammation and appear to be a promising pharmacological target for the management of this type of pain.

MATERIALS AND METHODS

The literature research was conducted between November and December 2021 via the PubMed, EMBASE and Cochrane Library databases. No filter time was used and only papers in English language were considered. Key terms used were 'neuropathic pain' OR 'neuropathy' AND 'prokineticin system' OR 'prokineticins' OR 'prokineticin antagonism' and were searched in paper title, abstract and keywords.

All titles and abstracts were independently revised by two authors (GA and DM) to assess their relevance for the inclusion in this review. In addition, some publications were searched in articles/ reviews reference lists on this topic and key publications were also identified through searches in the authors' files.

Full texts of manuscripts/reviews were analyzed by authors and 52 papers were included in this review.

PROKINETICIN SYSTEM

The prokineticin system, a new family of chemokines identified in 2001, includes two mammalian proteins, prokineticin 1 and prokineticin 2 (PK1 and PK2, respectively) and their receptors, PKR1 and PKR2. PK2 is also known as BV8 and was first isolated from the skin of the frog *Bombina variegata*, while PK1 is also known as *endocrine gland-derived vascular endothelial growth factor* (EG-VEGF). Homologous and orthologous of prokineticins (PKs) are highly conserved across species, indeed prokineticin-like peptides are present in invertebrates, *i.e.* shrimp and crayfish; vertebrates *i.e.* frog, black mamba snake, fugu and trout; and mammals *i.e.* bull, rodents, monkey and humans (15, 16). PK1 and PK2 are bioactive peptides of about 10 kDa with regulatory activity and consist of 86 and 81 amino acids, respectively. PKs share approximately 44% amino acid identity. Both chemokines have a structurally conserved motif characterized by a carboxyl-terminal cysteine-rich domain that forms five disulfide bridges with conserved spacing, a Trp residue in position 24 and an N-terminal AVITGA sequences, which is essential for the correct binding of receptors. These highly conserved homologies among species have been shown to be indispensable for the bioactivity of PKs (17, 18). The PK receptors (PKRs) have been identified in humans, rats and mice and are G protein coupled receptors (19-21). PKs can bind and activate both receptors. However, the signal transduction efficacy of PKR1 is slightly higher than the one of PKR2. It has been shown that activation of PKRs leads to accumulation of inositol phosphate and mobilization of intracellular Ca^{2+} via $G_{q/G11}$ proteins. In addition, PKRs may stimulate or inhibit cAMP accumulation through G_s or G_i proteins, respectively. Furthermore, PKRs can stimulate MAPK (mitogen-activated protein kinase) via G_o protein-mediated signaling (17, 22). PKs and their receptors are widely expressed in several organs and tissues. In particular PKs are co-expressed in brain, spinal cord, dorsal root ganglia, ovary, placenta, prostate, testis, adrenal cortex, peripheral blood cells, intestinal tract, spleen, pancreas, heart and bone marrow. However, there are also some differences; indeed, PK1 is predominantly expressed in steroidogenic organs, whereas PK2 is primarily, but not exclusively, expressed in the central nervous system and immune cells (23, 24). Besides, PKRs are co-expressed in certain tissues, but while PRR1 is mainly expressed in peripheral tissues, PKR2 results abundantly expressed in the brain (17, 25). Both receptors, however, are co-expressed also in small and medium-sized DRG cells as well as in the spinal cord. PKS has been linked to several biological effects like intestinal motility, neurogenesis, angiogenesis, circadian rhythms,

haematopoiesis and nociception. Emerging evidence have also indicated its involvement in pathologies which affect nervous and reproductive systems, myocardial infarction and tumorigenesis. Moreover, PKS is also involved in sensory processing and nociceptive signalling and is an important player in inflammation and pain pathophysiology (24).

PROKINETICIN SYSTEM IN NOCICEPTION REGULATION

The first evidence of a pronociceptive role of PKS was reported by Negri and colleagues (26). In rodents, the injection of Bv8/ PK2 induced mechanical and thermal hyperalgesia (26). The local injection of a very low dose of Bv8 (50 fmol) into the paw decreased the nociceptive threshold which reaches its maximum in 1 hr and disappears in 2-3 hrs. Systemic injection (subcutaneous, sc, and intravenous, iv) of higher doses induced hyperalgesia with a characteristic biphasic trend: the first peak occurs in 1 hr and the second peak in 4-5 hrs. This suggests that the first one depends on a direct action on nociceptors while the second may depend on central and/or peripheral sensitization. Indeed, subsequent studies supported the physiological role of PKS as peripheral and central pain modulator. Mice lacking PKR (pkr-/-) or PK2 (pk2-/-) are less sensitive to noxious stimuli than wild-type (WT), showing impaired hyperalgesia development after tissue damage (27-30). In particular pk2-/- mice showed a strong reduction in nociception induced by thermal and chemical stimuli, indicating an important role for endogenous PK2 in pain sensitization (27). Although both PKR1 and PKR2 are expressed in superficial layers of spinal cord, DRGs and peripheral terminus of nociceptors, and both mice lacking of PKR1 or PKR2 are less sensitive than WT-mice to Bv8-induced heat hyperalgesia, highlighting a positive interaction between PKR1 and TRPV1 channel, only Pkr1-/- mice showed also impaired responsiveness to tactile allodynia (28, 30), whereas pkr2-/- mice showed reduced nociceptive response to cold temperature (4 °C), suggesting a functional interaction between PKR2 and TRPA1 channels (30). Moreover, the molecular mechanisms of Bv8-induced hyperalgesia have also been studied *in vitro*, in neurons of DRG primary cultures (30, 31). It was observed that the number of neurons responding to Bv8 stimulus through an increase of intracellular calcium was five times lower in Pkr1-/- mice than in WT mice (28). Furthermore, it was also demonstrated that the percentage of DRGs neurons Bv8-responsive which were also responsive to mustard oil, was much higher in pkr1-/- mice than in pkr2-/- mice and a high degree of co-localization of PKR1 and of the vanilloid receptors TRPV1 and TRPA1, has been found. Therefore, taken together, these findings suggest a functional interaction between PKRs and TRP channels in the development of hyperalgesia. Additionally, half of neurons that responded to Bv8 stimulus also expressed/ released neuropeptides such as CGRP (calcitonin gene-related peptide) and SP (Substance P) (32, 33). In addition, Bv8 microinjection into the PAG exerted a pronociceptive effect by increasing the intrinsic GABAergic tone which is responsible for the inhibition of the antinociceptive output of the neurons of PAG (34).

These *in vivo* studies demonstrated the involvement and the ability of PKS to modulate the central pain pathways.

ANTAGONISTS OF PROKINETICIN SYSTEM

Being the PKS involved in the regulation of a wide spectrum of biological functions and pathological conditions, the development of effective PKRs antagonists may be useful in the treatment of different pathological conditions. The antagonism of PKS signalling emerges also as a new promising approach to control different types of pain. The identification of structural determinants, necessary for both receptor binding and PKs ac-

tivity, was fundamental to design functional PKR antagonists (35). Specifically, the highly conserved amino terminal sequence AVITGA and the Trp residue in position 24 are essential. As suggested by Miele and colleagues (36), AVIT proteins could interact with PKRs by orienting the protein region, including AVITGA sequence and the conserved Trp24. Moreover, it has also been demonstrated that deletions and/or substitutions in these conserved residues are able to produce antagonist molecules (37, 38). In addition, in Bv8 molecule the N-terminal deletion of the first two amino acids (Ala e Val) produces an analogue without biological activity but still capable to bind the receptors (named dAV-Bv8): in this way it acts as PKRs antagonist *in vitro* and *in vivo* (38). Even the substitution of Trp with Ala in position 24 produces antagonist-like protein (peptidic antagonist named A-24) (39). Unfortunately, the large size of these peptides makes their therapeutic use difficult and expensive. New promising non-peptidic PKR antagonists, triazine-guanidine derivates, have been synthetized and developed, *i.e.* PC1, PC7, PC10, PC18, PC25 and PC35 (**figure 1**) (35, 40). The different PC antagonists have been used in order to block the PKS activity in several pathological conditions. However only PC1 and PC7 were used in preclinical model of chronic pain. The "lead compound" is PC1. Indeed, PC1 mimics the structural features required for PKRs binding: the triazine-guanidine moiety of the molecule mimics the N-terminal AVIT sequence, whereas the methoxybenzyl moiety is oriented as the tryptophan residue in position 24 (35). Results from binding assay demonstrated that PC1 is a ligand that binds both PKR1 and PKR2, although it prefers PKR1. *In vitro* studies revealed a clear antagonist activity of PC1 that was able to block Bv8-induced intracellular calcium increase in CHO cells transfected with PKR1 or

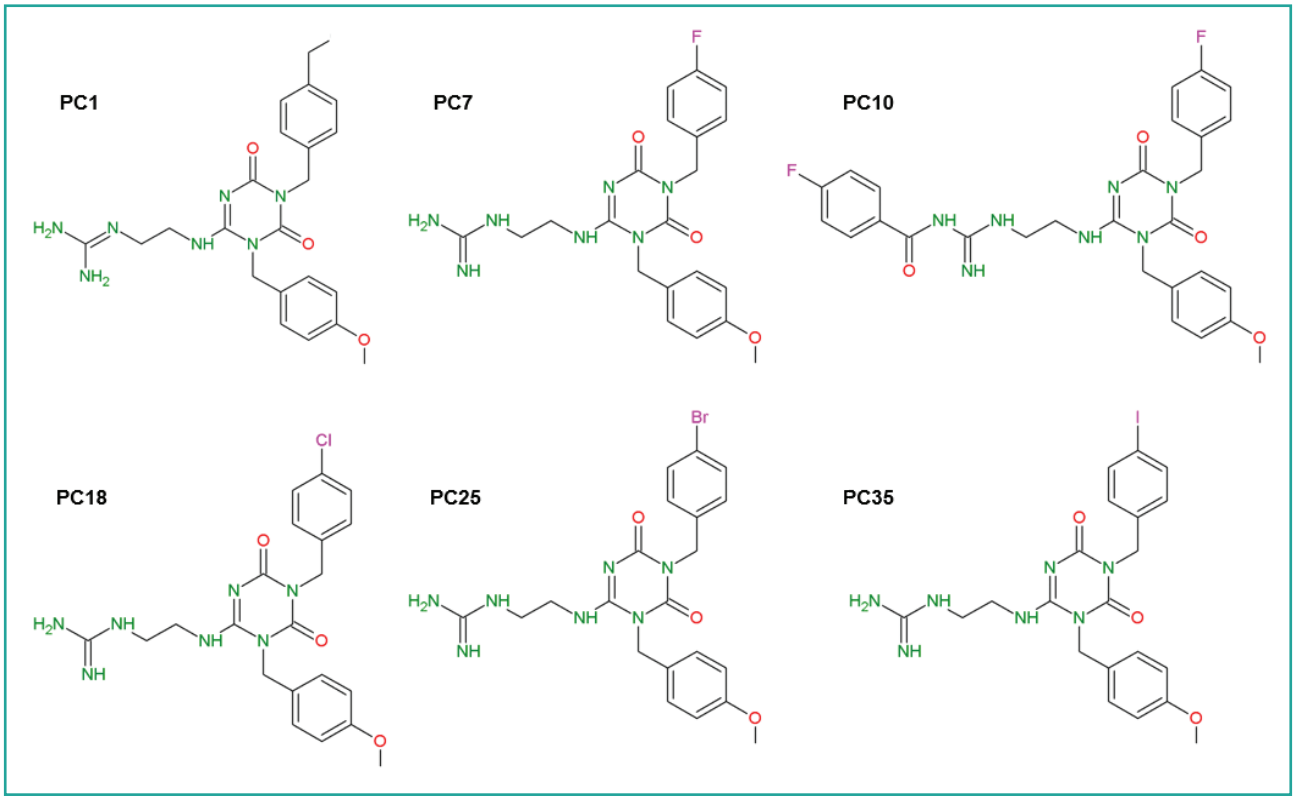

Figure 1. PK antagonists; triazine compounds.
2D chemical structure of synthetic organic compounds PC1, PC 7, PC10, PC18, PC25 and PC35.

PKR2 (35). Besides, *in vivo* studies demonstrated that both PC1 and PC7 were able to selectively antagonize Bv8-induced hyperalgesia, even if PC7 antagonizes it at doses ten times lower than PC1 (41). Moreover, PC1 also contrasts capsaicin-induced thermal hypersensitivity, suggesting that it may prevent the activation of PKRs and TRPV1 by their endogenous ligands (42, 43). In CFA-induced inflammatory pain model, systemic injections of PC1 (from 20 to 150 µg/kg, sc) reduced hyperalgesia in a dose-dependent manner, completely abolishing it at the dose of 150 µg/kg (44).

Besides these receptor antagonists, anti-Bv8 neutralizing antibodies are also commercially available, effectively capable of inhibiting PKS (45).

PROKINETICIN SYSTEM AND NEUROPATHIC PAIN

Studies aimed at identifying the link between PKS and neuropathic pain began in 2014. To date, 11 original manuscripts have been produced, and this review will illustrate the discoveries achieved so far. Neuropathic pain arises from both PNS and CNS lesions and many etiologies have been recognized in human. Several animal models of neuropathic pain, that mimic the different human conditions, are available and have been used to identify the role of PKs. Chronic constriction injury (CCI-model) (41, 46) and spared nerve injury (SCI-model) (47) mimic a direct nerve trauma and are the most frequently used. Painful neuropathy is a frequent complication of diabetes and STZ model (streptozotocin-induced diabetic neuropathy) represents the most commonly used model for the study of this type of pain (48). Peripheral neuropathy is a very frequent and severe side effect of chemotherapy and is often the limiting factor for achieving the effective dose; for this reason, a series of studies investigated the role of PKS in peripheral neuropathy induced by the chemotherapeutic vincristine (VCR-model) (49) and bortezomib (BTZ-model) (50-52). Moreover, a neuropathic pain component is often present also in cancer pain, and this aspect has been addressed in a model of cancer-induced bone pain (CIBP-model) (45). These studies were performed in male mice of the strain CD1 (41, 46, 47) or C57BL/6J (48-52) except the CIBP model which was induced in female Sprague-Dawley rats (45). All these models, develop a significant hypersensitivity to mechanical and/or thermal stimuli with a different temporal development. In particular, CCI-model is characterized by a decrease in paw withdrawal threshold and latency (PWT and PWL) as early as 3 days after sciatic nerve ligation (41, 46). After 5 days of induction, SCI model develops allodynia and hyperalgesia (47). The CIBP-model shows a gradual decrease in PWT from day 6 after tumour cell inoculation (45). Moderately low doses of STZ induced an evident mechanical allodynia starting from 14 days after treatment (48). Finally, both VCR and BTZ compounds induced a progressive development of mechanical and thermal allodynia, as well as of thermal hyperalgesia respectively 3 and 7 days after the first chemotherapic treatment (49-52).

Dose-finding experiments for PK antagonists in neuropathic pain

In a first series of studies, in order to identify the dose and the best route of administration for PKS antagonists, Negri's group performed a dose finding using PC1 (46) and PC7 (41) in the CCI-model and

data are reported in **figure 2**. Three days after CCI, in an evident state of hypersensitivity, mice were treated subcutaneously with 3 different doses of PC1 (30, 75 and 150 µg/kg) or PC7 (5, 15, 45 µg/kg). A single bolus of PC1 or PC7 reduced thermal hyperalgesia in a dose-dependent manner. The higher dose of both triazine compounds (PC1: 150 µg/kg and PC7: 45 µg/kg) restored thermal thresholds of pathological animals to basal level. This effect

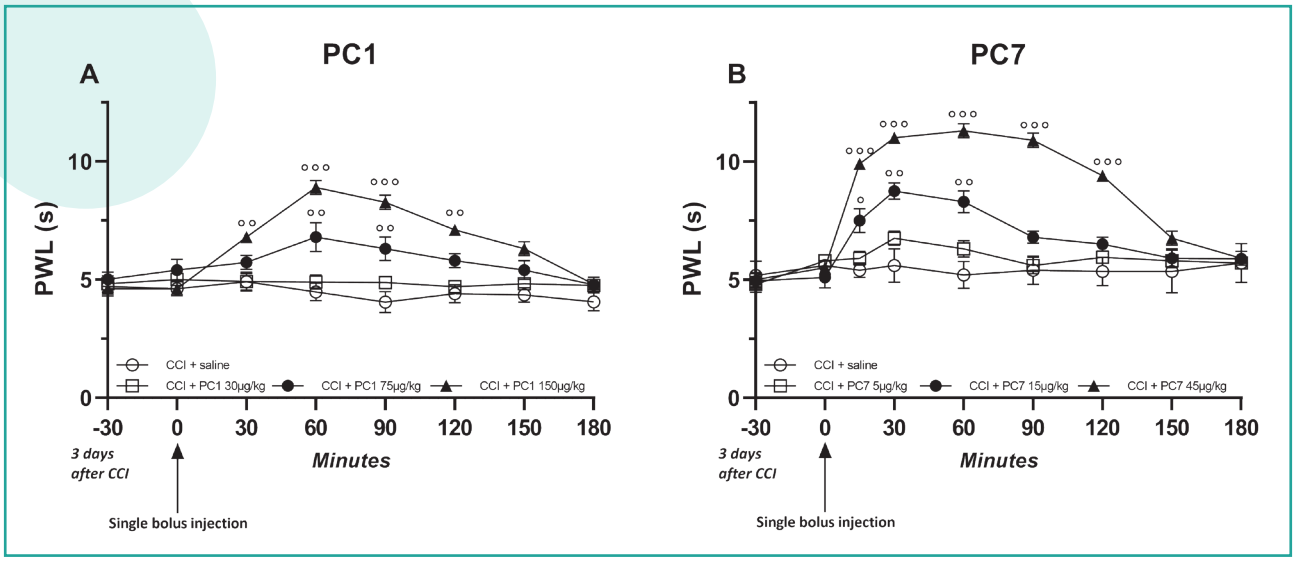

Figure 2. Dose-finding of PC1 and PC7.
A single bolus subcutaneous injection of PC1 (A, modified by Maftei *et al*., 2014) and PC7 (B, modified by Lattanzi *et al*., 2015) on day 3 after CCI restored the CCI-induced thermal hyperalgesia in a dose-dependent manner. PWL: paw withdrawal latency. The data represent the means ± SEM of 5 mice/group. Two-way ANOVA was used for statistical evaluation, followed by the Bonferroni's test. °p < 0.05; °°p < 0.01; °°°p < 0.001 *vs* CCI + saline mice.

remained statistically significant for 30-120 min after treatment.
Maftei and colleagues (46) also tested PC1 at 3 different doses (5, 15 and 50 ng) using two other different routes of administration, *i.e.* perineural (PN) and intrathecal (IT). Also in this case, regardless the route of administration, the higher dosage was the most effective, exerting an effect comparable to that of 150 µg/kg of PC1 injected subcutaneously. Since the subcutaneous route is faster, simpler and less stressful for animals than the perineural and intrathecal ones, in all subsequent studies where PKS was antagonized with PC1, the route of administration used was the subcutaneous one at the dose of 150 µg/kg. In all protocols PC1 was administered twice a day (41, 46-52).

PKS antagonism effect on hyperalgesia and allodynia

The acute effect of PKS antagonism was evaluated (**figure 3**) in CCI-, STZ- and BTZ-models of neuropathic pain (46, 48, 50). When hypersensitivity was well established (17 days for CCI, 21 days in STZ and 28 days in BTZ), a single bolus of PC1 (150 µg/kg, sc) was able to rapidly counteract the mechanical allodynia. In CCI mice, PC1 administration exerted an anti-allodynic effect in 30-120 min (46). In STZ mice the injection of PC1 produced a total recovery of PWT in 30 min, this anti-allodynic effect lasted for about 120 min and gradually disappeared within 240 min (48). In BTZ mice the effect of PC1 administration was maximal between 60 and 120 min and then progressively decreased, although it was still present after 240 min (50). In CIBP model, PKS was antagonized using neutralizing anti-Bv8 antibody (5 ng, IT) and a significant anti-hyperalgesic effect to mechanical stimuli was observed (45). In particular, this effect appeared at 15 min, peaked at 30 min and disappeared at 60 min after IT injection. Although in the different neuropathic models there are some differences in the rate and/or duration of the effect of acute PKS antagonism, it is possible to assert that the acute treatment with a PKS antagonist rapidly counteracts painful symptoms and its effect lasts for about

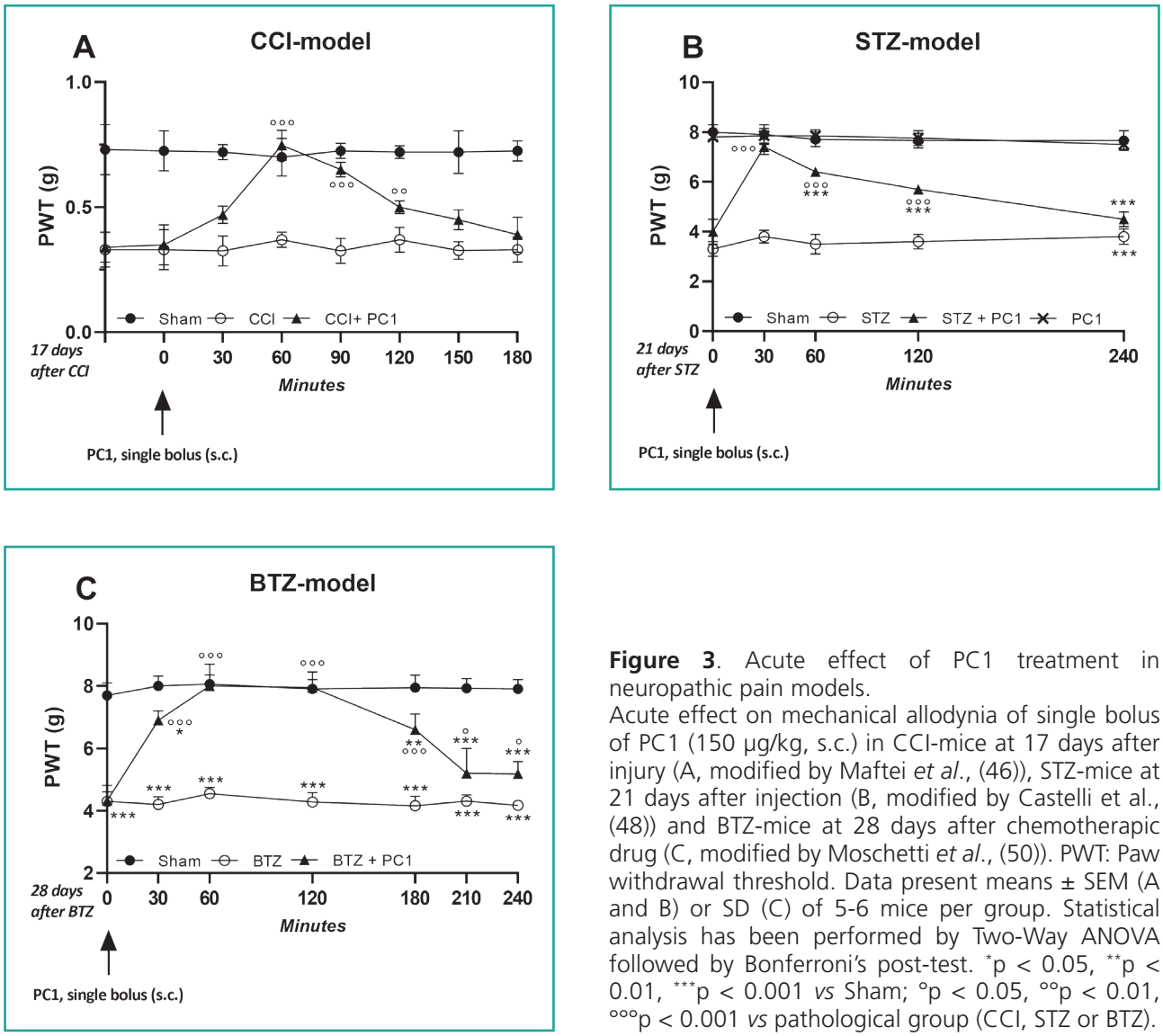

Figure 3. Acute effect of PC1 treatment in neuropathic pain models.
Acute effect on mechanical allodynia of single bolus of PC1 (150 µg/kg, s.c.) in CCI-mice at 17 days after injury (A, modified by Maftei *et al*., (46)), STZ-mice at 21 days after injection (B, modified by Castelli et al., (48)) and BTZ-mice at 28 days after chemotherapic drug (C, modified by Moschetti *et al*., (50)). PWT: Paw withdrawal threshold. Data present means ± SEM (A and B) or SD (C) of 5-6 mice per group. Statistical analysis has been performed by Two-Way ANOVA followed by Bonferroni's post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* Sham; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ *vs* pathological group (CCI, STZ or BTZ).

4 hrs. Chronic treatment with PC1 (**figure 4**) has been performed either with a therapeutic approach, starting when pain was fully established (41, 46, 48-52) or in a preventive way, before the induction of the pathology (47, 48). In all the neuropathic models used, the chronic therapeutic treatment with PC1 was able to counteract painful symptoms, reducing allodynia and/or hyperalgesia. Interestingly, pain relief is maintained for few days also after PC1 treatment interruption (41, 46-48, 50). Considering that chemotherapy treatment is often repeated, and pain always reappears, it was also demonstrated that animals treated with PC1 during the first cycle of BTZ showed lower allodynia during the second chemotherapic cycle (50). In addition, Castelli and colleagues (48) also showed how preventive treatment with PC1 completely prevented the development of painful symptoms in diabetic mice. This preventive effect of PC1 treatment was observed also in SNI-animals (47), suggesting that a selective PKS antagonism could be an effective preventive approach.

PK2 and neuroinflammation

Neuroinflammation plays a key role in the onset and maintenance of several types of chron-

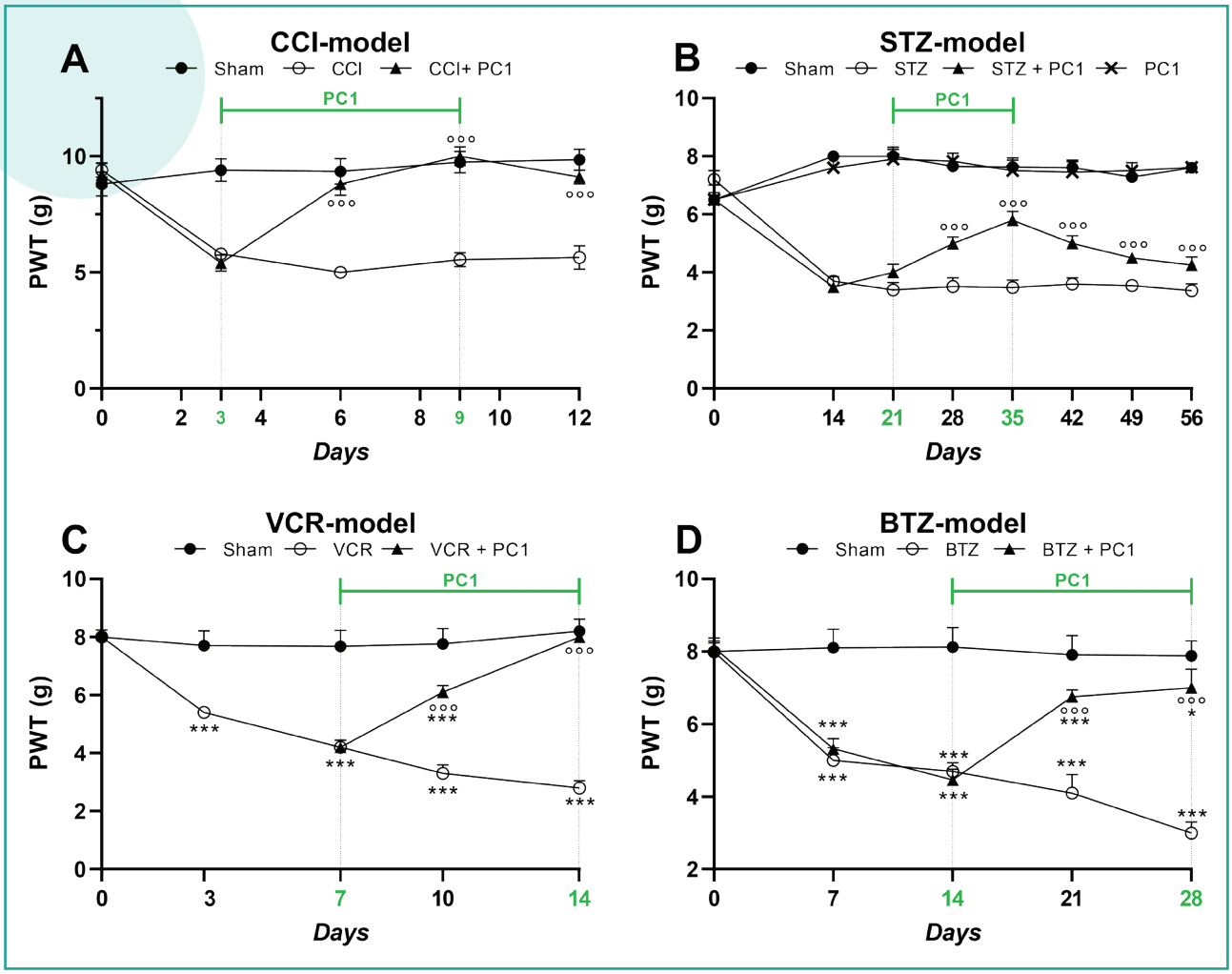

Figure 4. Effect of chronic PC1 treatment.
Therapeutic effect of chronic treatment by PC1 (150 µg/kg, s.c.) on mechanical allodynia in CCI-mice (A, modified by Lattanzi *et al*., (41)), STZ-mice (B, modified by Castelli *et al*., (48)), VCR-mice (C, modified by Moschetti *et al*., (49)) and BTZ-mice (D, modified by Moschetti *et al*., (50)). PWT: Paw withdrawal threshold. Data present means ± SEM (A-C) or SD (D) of 6-9 mice per group. Statistical analysis has been performed by Two-Way ANOVA followed by Bonferroni's post-test. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs* Sham; $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ *vs* pathological group (CCI, STZ, VCR or BTZ).

ic pain and has been clearly associated to neuropathic pain (53). Besides neurons, also glial and immune cells play an important role in this condition (54). Indeed, neuroinflammation is usually defined as a 'cytokine-mediated' inflammatory process (55). A peripheral damage to the nervous system induces the recruitment and activation of immune and glial cells in different anatomical sites (54). PK2 has a recognized pronociceptive and proinflammatory effect and is produced by immune cells, glial cells and neurons (41, 46-52, 56, 57). Its role in neuroinflammation has been studied in detail (**table I**). In all studies PKS members and other neuroinflammation markers were simultaneously evaluated in nervous stations involved in pain transmission and nociceptive processes, like sciatic nerve, dorsal root ganglia (DRGs), spinal cord and supraspinal areas.

Sciatic Nerve

In CCI animals, Lattanzi *et al*., (41) found an early upregulation of PK2 expression in ipsilat-

Table I Prokineticin system and neuroinflammatory markers in neuropathic pain models.

	CCI model [41,46]	SNI model [47]	STZ model [48]	VCR model [49]	BTZ model [50,52]	CIBP model [45]
Sciatic Nerve	↑ PK2(*) ↑ PKR1 and PKR2	↑ PK2(*) ↑ PKR2	↑ PKR1(*)	-	↑ PK2(*)	
	↑ IL-1β(*), TNFα(*), IL-6(*) and IL-17(*)	-	↑ IL-1β(*)	-	↑ IL-1β(*), TNFα(*) and IL-6(*)	
	↓ IL-10(*)	-	↓ IL-10(*)	-	↓ IL-10	
	↑ CD11b ↑ GFAP(*) and S100β	-	-	-	↑ CD68(*) ↑ TLR4(*)	
Dorsal Root Ganglia	↑ PK2(*) ↑ PKR2	-	-	↑ PK2(*) ↑ PKR1(*) and PKR2(*)	↑ PK2(*) ↑ PKR1(*) and PKR2	
	-	-	-	↑ IL-1β(*), TNFα(*) and IL-6(*)	↑ IL-1β(*), TNFα(*) and IL-6(*)	
	-	-	-	↓ IL-10(*)	↓ IL-10	
	-	-	-	↑ CD68(*) and CD11b(*) ↑ TLR4(*)	↑ CD68(*) ↑ TLR4(*)	
Spinal Cord	↑ PK2(*) ↑ PKR2	↑ PK2(*) ↑ PKR2	↑ PK2 ↑ PKR2	↑ PK2(*) ↑ PKR1(*) and PKR2(*)	↑ PK2(*) ↑ PKR1(*) and PKR2(*)	↑ PK2
	↑ IL-1β(*)	-	↑ IL-1β(*)	↑ IL-1β(*) and TNFα(*)	↑ IL-1β(*) and IL-6	↑ TNFα(*)
	= IL-10	-	= IL-10	= IL-10	↓ IL-10(*)	
	↑ CD11b(*) ↑ GFAP(*)	↑ iba1(*) and CD206 ↑ GFAP(*)	-	↑ CD68(*) and CD11b(*) ↑ TLR4(*) ↑ GFAP	↑ CD68(*) ↑ TLR4(*) ↑ GFAP	
	-	-	-	-	↑ KDM6A(*)	

(*) PKS antagonism (by PC1 in CCI, SNI, STZ, VCR and BTZ mice; or by BV8 neutralizing antibody in CIBP rats) countered neuroinflammation induced by neuropathic pain.

eral sciatic nerves (3 days after CCI) and this overexpression was maintained up to 17 days after CCI. In accordance with these observations, Maftei *et al.* (46) observed in CCI-mice at day 10 post-surgery a strong infiltration of PK2+ cells in the proximity of the nerve damage. In this model also PKRs were upregulated in comparison to sham mice. PK2 and PKR2 levels were higher also in the other model of direct nerve damage, the SNI (47). In BTZ animals, PK2 levels were upregulated at 28 days after chemotherapy treatment, corresponding to a high cumulative dose, but not earlier (14 days) and in these mice PKRs mRNA levels were never modulated by the chemotherapeutic drug (50).

It is interesting to note that PKS modulation was always associated with an increase of neuroinflammatory markers (41, 46, 50). In detail, in CCI-mice (day 10) a significant up-regulation of pro-inflammatory cytokines (IL-1β, TNFα, IL-6 and IL-17) and down-regulation of IL-10 levels were detected (41, 46). Also in BTZ mice, neuroinflammation was present and was more sustained at day 28 (maximal cumulative dose, c.d.) than day 14 (half c.d.), indeed, at day 14 only CD68, TLR4 and IL-6 mRNA levels were increased, while at day 28 also TNFα and IL-1β were upregulated and IL-10 expression levels were reduced (50). Moreover, in both models (CCI and BTZ) an increase of activated macrophages (identified by CD11b+ cells in CCI and CD68+ cells in BTZ) was observed, and PK2 co-localized with these cells. Furthermore, in CCI-mice (41) a strong Schwann cells activation (GFAP+ and S100+ cells) was detected and PK2 co-localized with these cells.
In all neuropathic models the PKS antagonism with PC1 was able to restore correct PK2 levels (41, 46, 47, 50). Conversely, PC1 treatment did not modulate PKRs levels altered by pathology. Moreover, PC1 treatment was able to contrast or prevent neuroinflammation. Indeed, in STZ mice IL-1β levels were decreased and IL-10 levels were increased by PC1 therapeutic treatment (48). In CCI and BTZ mice, where the panel of neuroinflammatory markers was more extensive, it was possible to observe that PC1 treatment restored almost all of the parameters analyzed (41, 50). Interestingly, in CCI mice treated with PC1, the decrease of PK2 was associated with a decrease of GFAP+ cells (41, 46), while in BTZ mice treated with PC1 was associated with a decrease of CD68+ cells (50). It could be hypothesized that the block of PK2 by PC1 treatment, counteracts neuroinflammation, macrophage infiltration and Schwann cell activation.

Dorsal Root Ganglia

The PK2 time-course expression was also evaluated in DRGs of CCI, BTZ- and VCR- models (41, 49, 50). In this station PK2 expression levels appeared up-regulated from 7 to 17 days post-surgery in CCI, (41, 46) and also PKR2 was increased as both mRNA and protein. In both chemotherapy models, VCR and BTZ, PK2 levels resulted significantly up-regulated at the maximal c.d. (49, 50). Interestingly, a clear up-regulation of both PKRs resulted early in VCR mice (49), whereas in BTZ mice it was only present in the later stage (50). In addition, the levels of pro-/anti-inflammatory cytokines were also modified, albeit with different timings in the two models. In VCR mice, IL-1β up-regulation is precociously present, followed by increase of TNFα and IL-6 and decrease of IL-10 (49). Instead in BTZ mice, IL-6 and TNFα levels were increased and IL-10 levels decreased at the half c.d. while IL-1β levels were upregulated at the maximal c.d. (50). In BTZ and VCR model, a clear up-regulation of macrophage markers (CD68 and CD11b) and of TLR4 mRNA levels was detected, suggesting the presence of macrophage infiltration and activation (49, 50). In CCI mice, chronic PC1 treatment was able to restore disease-altered PK2 levels, but an effect on PKR2 was not detected (41, 46), whereas in chemotherapeutic treated mice it prevented PK2 up-regulation and contrasted PKR1 and/or PKR2 overexpression (49, 50). Moreover, the treatment with the antagonist of PKS restored or maintained at physiological levels all the inflammatory markers modified by chemotherapic treatment (49, 50). A detailed immunohistochemistry analysis revealed that in CCI mice, PK2 and PKRs were expressed by neurons with a vesicular cytoplasmic pattern which is dense in proximity of the neuronal membrane. In addition, PKS was also expressed by satellite cells since a clear colocalization of both PK2 and PKRs with GFAP+ cells was detected (41, 46). Moreover, in BTZ-mice the PK2 colocalized mainly with CD68+ cells (macrophages) (50). It is clear that in DRGs several cell types represent a source of PK2 in pain conditions.
Moschetti *et al.* (56) used primary cultures of DRG neurons to further investigate the role of PKS in chemotherapy induced neurotoxicity. The authors observed that VCR (1 nM) or BTZ

(6 nM) has a strong impact on neurons, significantly reducing neurite growth and length. This effect in VCR cultures was also associated with an increase in PK2, PKR1, TLR4, IL-1β, IL-6 and IL-10 mRNA levels. In co-culture with the chemotherapy drug (BTZ or VCR), PC1 prevented the reduction of neurite length, and the upregulation of the neuroinflammatory markers, protecting neurons from chemotherapy-induced toxicity. Interestingly, a protective role of PC1 for DRG cells was also observed *in vivo* (50). In BTZ mice swollen mitochondria and enlarged endoplasmic reticulum cisternae scattered within the cytoplasm of both nerve cell bodies and satellite glial cells were present (50). *In vivo* PC1 treatment was able to partially preserve neurons and satellite glial cell structure (50). Thus, in this station a neuropathic pain conditions of different ethiology induced the activation of PKS which participated in the onset or maintenance of pain. Besides, a clear neuroinflammation with pro- and anti-inflammatory cytokine unbalance, macrophage infiltration and satellite glial activation/alteration was detected. PKS antagonism was able to counteract or prevent it, suggesting the role of the system in these processes.

Spinal Cord

In the spinal cord, PK2 expression levels were evaluated in CCI, SNI, STZ, BTZ, VCR and CIBP models (41, 46-50, 52). In diabetic and CIBP models, when painful symptomatology appeared, an evident up-regulation of PK2 levels (mRNA and/or protein) was already present (46, 48). Moreover, PK2 levels were over-expressed as long as the mice were in pain. This suggests that PKS activation is involved in chronic pain development and chronicization. In STZ model, 35 days after toxin injection, PK2 increase was associated with PKR2 overexpression (48). Consistently with these data, also in CCI- (46) and SNI-models (47) at 10 days post-injury increased levels of PK2 and PKR2, were detected. A different activation of PKS was observed in chemotherapy models. In BTZ and VCR mice pain is already developed at lower dose but a significant increase of PK2 levels were detected only at the end of the experimental protocol, when the animals received the maximal c.d. (49, 50, 52). These data suggest that in chronic pain induced by chemotherapy treatment, central activation of PK2 is more associated with the maintenance rather than with the onset of pain. In spinal cord, PKS activation is always associated with a pronounced neuroinflammation. Indeed, in STZ, CCI, VCR and BTZ models a significant increase of IL-1β levels was always present (46, 48-50). Additionally, in all neuropathic pain models, it was detected an overexpression of glial markers, indicating the important role of this cellular component in pain. However, no colocalization between microglia cells and PK2 was ever observed. In SNI, CCI and BTZ models an increase of GFAP+ cells was described and it was demonstrated a co-localization of PK2 with both GFAP+ cells (astrocytes) and synaptophysin+ cells (neurons) (41, 46, 47, 50). This suggests that microglia and/or infiltrating macrophages do not represent the main source of PK2 in the spinal cord, which could therefore be produced by the astrocytic and neural components. The PKS antagonism was able to reduce PK2 overexpression in all neuropathic pain models. Down-regulation of PKRs was also detected, although the main effect was observed on PKR2 levels. Moreover, in all models a general reduction of neuroinflammation was present both for pro-inflammatory cytokines and glial cell activation markers. Finally, in a very recent paper, the possible interplay between PKS and epigenetic mechanisms in BTZ mice was proposed (52). The histone demethylase KDM6A, that has a role in promoting IL-6 production was up-regulated in mice with chronic pain. The antagonism of PKS with PC1 was able to prevent KDM6A alteration, controlling epigenetic mechanisms involved in cytokine production. Moreover, the paper also showed that by blocking PKS, the anti-inflammatory response sustained by PPARs was enhanced (52). In the whole these results suggest that also in the spinal cord PKS

plays an important role in onset and/or maintenance of pain and neuroinflammation. PK2 produced by neurons and astrocytes induces the release of proinflammatory cytokines and epigenetic modifications that lead to microglia and astrocyte activation, triggering a proinflammatory loop that ends up with more PK2 production. The PKR antagonist interrupts this pathological loop that may be implicated in central sensitisation.

Supraspinal areas and mood alterations

In humans, the presence of chronic pain is frequently associated with mood alteration, such as depressive or anxious states (58). Also in experimental models of neuropathic pain, the development of anxious and/or depressive like behaviours has been reported (59). The effect of PKS antagonism on mood disorders in neuropathic mice was investigated in 2 papers (49, 51), which explored these aspects in chemotherapy-induced painful neuropathy. In BTZ-treated animals that had experienced chronic pain for 28 days, depressive and anxious behaviours were clearly present (51). The treatment with the PC1 antagonist, that as reported above, completely controlled painful symptoms, also counteracted mood alterations (51). Interestingly in VCR treated mice, who had been in a chronic pain condition only for 14 days, no mood alterations were recorded, suggesting that the duration of chronic pain may be important for the induction of neuropsychiatric alterations (49). In BTZ animals the presence of a neuroinflammatory condition in brain areas involved in anxiety and depression was also evaluated (51). A generalized neuroinflammation was observed with a significant mRNA level increase of CD11b in both prefrontal cortex and hypothalamus, of TRL4 in the prefrontal cortex and of GFAP in the hypothalamus. These results suggested the activation of both microglial and astrocytic components. Furthermore, the pro-inflammatory cytokines IL-6 and TNFα, that may be related to depressive condition, were up-regulated in prefrontal cortex, hippocampus and hypothalamus A drastic decrease in BDNF levels was also observed in the prefrontal cortex and hippocampus, condition widely correlated with depressive symptoms. Also PK2 was significantly increased in hypothalamus and hippocampus and an increment of PKR2 was observed in hippocampus. PKS antagonism with PC1 was able to prevent and/or counteract both neuroinflammation and BDNF decrease in these supraspinal areas. Consistently with the lack of mood alteration in VCR mice, no major alterations were observed in supraspinal areas in these animals (49).

CONCLUSIONS

From the evidence present in the literature we can affirm that PK2 overexpression is involved in the processes that underlie pain and neuroinflammation. An upregulation of this chemokine is consistently observed in nerves, DRG and spinal cords in models of peripheral neuropathic pain, independently of the causes (injury, diabetes, chemotherapic treatment) that induce pain. However, some differences in the time course of PK activation are present. For example, the system is immediately activated in models, such as CCI, where there is an immediate and strong local inflammatory response in the lesioned nerve with neurinoma formation, in comparison to chemotherapy induced neuropathy, where the PK system plays a delayed role that seems related to spinal sensitization. The results here summarized also demonstrate that both neurons and non-neuronal cells may express PK2. Besides neurons, infiltrating macrophages, DRG satellite cells and spinal astrocytes are important sources of the chemokine, that, on the contrary is not produced by spinal microglia. However, these cells express PK receptors and their activity is therefore modulated by prokineticins. The control of PK activation and of its effects with pharmacological antagonists, monoclonal antibodies or genetic strategies such as the generation of PK2 and PKR deficient animals, has proved to be a winning strategy to counteract pain and neuroinflammation. Interestingly an

effective control of neuropathic pain with PK antagonists can also prevent the development of pain related comorbidity such as depressive and anxious-like behaviours.
Although other studies are needed to better dissect and understand the downstream pathways of PKS effects, we can affirm that PKS is an emerging excellent therapeutic target for the resolution of chronic pain and its comorbidities.

ETHICS

Conflict of interests
The authors declare that they have no conflict of interests.

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N/A

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REVIEW

pharmadvances

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THE MULTIFACETED ASPECTS OF STRESS

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SUMMARY

The effects of stress depend on the nature and duration of the exposure, with the triggering of molecular mechanisms that allow individuals to react to the stressful context. In particular, while acute stress induces the activation of circuits to ensure normal homeostasis and mediates adaptive responses, chronic stress exposure has detrimental and long-lasting effects on brain functions. Indeed, chronic stressful life events act as precipitating factors for many psychiatric conditions, including major depressive disorders. In this context, it is worthy of mention that there are differences in individual susceptibility, with some people displaying vulnerability to stressful events and others being resilient to the same adversities. Moreover, exposure to chronic adverse situations may leave permanent 'scars' in the individual, which confer enhanced vulnerability for relapse since it is possible that not all the systems impaired by chronic stress are restored during the remission.

On these bases, it is fundamental to better understand the behavioral outcomes of stressful events as well as the molecular changes that may sustain them for the discovery of novel therapeutic targets and approaches to treat stress-related disorders and to promote resilience.

Key words

Acute stress; chronic stress; resilience; HPA axis; Bdnf.

INTRODUCTION

Every day we are exposed to different types of stress that lead to specific biological effects based on the type and duration of the exposure. Dhabhar and McEwen in 1997 gave an integrated definition of stress, which may recapitulate the large amount of information available in the literature: "stress is a constellation of events, consisting of a stimulus (stressor), that precipitates a reaction in the brain (stress perception), that activates physiological fight or flight systems in the body (stress response)" (1). The responses to behavioral and physiological stressors can be either adaptive or maladaptive (2): while exposure to acute stressors induces adaptive reactions that help the organism to adapt efficiently to experiences in daily life, extreme stress conditions may lead to maladaptive outcomes, including psychopathologies.

Stress is the major environmental factor for the etiology of depression (3), which causes stable changes in gene expression, neural circuit function, and behavior, which may be maintained by epigenetic modification. Actually, the interaction with the genetic background

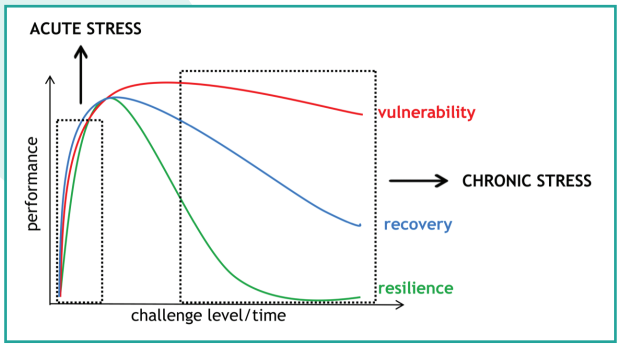

Figure 1. The different effects of stress exposure. Acute stress can have positive effects on the performance. Chronic stress can result in vulnerability (red line) or resilience (green line); moreover, the negative effects exerted by stress exposure may be recovered (blu line).

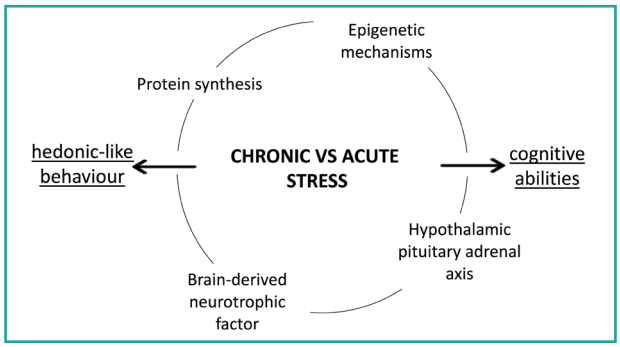

Figure 2. Molecular mechanisms investigated following chronic and acute stress exposure.

seems to be fundamental for the development of the disease, probably explaining the different responses to adverse events observed in humans, with some people displaying susceptibility and others resistance to maladaptive effects of stress. Indeed, by activating adaptive mechanisms, the brain has the ability to react to stressful changes, allowing continuous remodeling throughout the entire life (4) a concept known as resilience.

Moreover, the consequences of chronic stress exposure during adult life may have long-lasting effects or may be recovered, by the activation of dynamic processes to achieve a successful rescue (5). Nevertheless, a high percentage of depressed patients experience relapse after a period of recovery, suggesting that not all the systems impaired by stressful environmental factors are restored, thus representing scars of vulnerability, which in turn can promote the relapse to the pathology. On the opposite side, acute stress may have beneficial effects and increase the adaptive ability of the subjects to cope with stressors during life (**figure 1**).

To date, several attempts have been made to unravel the mechanisms underlying the different susceptibility to stress; however, we are still very far from fully clarifying the factors that draw the trajectory of the stress response. Accordingly, it is fundamental to characterize the molecular alterations associated with the development of a pathological phenotype through preclinical studies. In particular, great interest is turned to animal models based on chronic exposure to stress protocols that are widely used to study the behavioral and neurobiological modifications that develop under these conditions, in the assumption that these may be a correlate of the human disorder.

In this review, I will summarize the main outcome of my Ph.D. thesis centered on the role of stress as a risk factor for psychiatric disorders as well as of beneficial challenge (short stressor) in improving a general performance (6-10) with the focus on different potential molecular mechanisms that may be responsible for stress consequences (**figure 2**).

MOLECULAR MECHANISMS UNDERLYING THE BEHAVIOURAL ALTERATIONS EXERTED BY STRESS EXPOSURE

Stress and local protein synthesis (6)

Alteration of synaptic plasticity has been related to depression and psychiatric disorders and an impairment of the regulation of the *de-novo* protein synthesis at synaptic levels has been associated with memory deficits (11).

One of the main mechanisms in protein synthesis-dependent memory is the signaling cascade associated with *de novo* protein synthesis linked with the mammalian target of rapamycin (mTOR) complex 1 and the eukaryotic initiation factor 2 (eIF2) (12, 13). Moreover, mTOR activation through NMDA receptors is also involved in peptide elongation, with the involvement of the eukaryotic elongation factor 2 (eEF2) and the translation of specific mRNAs.

By exposing animals to the chronic mild stress (CMS) paradigm, one of the main animal models, in the field, used to recreate the depressive-like behaviour in rodents (14), we demonstrated that exposure to 7 weeks of CMS induced the development of the anhedonic-like behaviour in around the 80% of stressed animals, while the remaining were resilient to the negative effects of stress. By contrast, when we looked at cognitive functions by testing the animals in the novel object recognition (NOR) test, we observed that independently from vulnerability and resilience, all the stressed animals show cognitive deficits in the NOR task, indicating that the mechanisms responsible for anhedonia are different from those related to cognitive impairment. Given the association between local protein synthesis and synaptic plasticity and memory, we observed that NMDA and mTOR activation following the NOR test in control animals led to newly synthesized protein at synaptic levels through the enhancement of the elongation factor 2 (eEFF2), the factor that mediated the translation of specific mRNAs in the dorsal hippocampus (dHip), the subregion of the hippocampus mainly involved in cognition and spatial learning (15, 16). By contrast, the triggering of this intracellular signaling pathway was completely blunted in animals vulnerable to CMS, suggesting that deficits in these 'synaptic' mechanisms may indeed contribute to the cognitive impairment observed in stressed animals and highlighted a fundamental role of the elongation step of the protein synthesis in the correct cognitive performance (6).

Stress and the hypothalamic-pituitary-adrenal (HPA) axis (7)

Psychiatric diseases are characterized by an altered function of the HPA axis (17, 18) that is deregulated in stress-related disorders, with a disruption of the feedback that leads to excessive activation of the axis.

Moreover, since a correct hormonal response is essential for learning and memory processes (19) alterations of this system may contribute to the development of cognitive deficits, debilitating symptoms of depression. Indeed, cognitive impairment may persist even when patients are successfully treated with antidepressants and remission is achieved, representing residual symptom that reduces everyday performance.

Glucocorticoids (GCs) act via genomic mechanisms, involving nuclear receptors, as well as via non-genomic pathways that require membrane-associated receptors.

In particular, the genomic action of glucocorticoid receptors (GRs) regulates the transcription of target genes that contain in the promoter the glucocorticoid responsive element (GRE), including genes playing a key role in synaptic plasticity and memory (20-22). Furthermore, in the non-genomic pathways, GCs can directly stimulate the release of excitatory amino acids, via the synaptic membrane-associated receptors, and can regulate mitochondrial oxidation and free radical formation through the binding with GRs on the mitochondrial membranes (4).

Thus, it is fundamental to investigate the changes occurring in the HPA axis following chronic stress and their potential involvement in the anhedonic phenotype as well as in the cognitive impairment that develops in animals vulnerable to stress exposure. Moreover, in the field of discovering potential mechanisms that may be critical for the ability to modulate different pathologic domains associated with psychiatric disorders, it should be critical

to study the effects of pharmacological treatments in counteracting the behavioral impairment due to chronic stress exposure and the possible role of the GRs in its effect.
The chronic administration with the antipsychotic drug lurasidone (LUR), high affinity antagonist of dopamine D_2 receptor, serotonin 5-HT_{2A} and 5-HT_7 receptors, moderate affinity antagonist of adrenergic $_{\alpha 2A}$and $_{\alpha 2C}$ and partial agonist of the HT_{1A} receptors, approved by the Food and Drug Administration for the treatment of different psychiatric conditions, normalized the anhedonic phenotype and reverted the cognitive deficits in the NOR test due to 7 weeks of CMS.
At molecular level, while the correct cognitive performance of non-stressed animals was associated with the GRs nuclear translocation and the subsequent transcription of glucocorticoid responsive genes in the dHip, we found that this mechanism was impaired in animals exposed to CMS, which showed cognitive deficits in the NOR test. Interestingly, the chronic treatment with the multimodal receptor antagonist LUR was able to normalize the alteration of the GR genomic signalling during the ongoing cognitive activity.
Regarding the non-genomic pathway, the membrane-bound receptors were increased by chronic stress, effect that may suggest alterations in synaptic mechanisms and in mitochondria functionality. In line, this effect was paralleled by increased levels of the active form of SYNAPSIN I, marker of the activity of GR at synaptic level, and by the enhancement of the expression of *Cox3*, one of the catalytic subunits of cytochrome c oxidase, the last enzyme. Interestingly, chronic lurasidone treatment was able to normalize the stress-induced alterations of the non-genomic mechanisms of GR.
These findings suggest that the activation of the genomic pathway mediated by GR may contribute to the correct cognitive performance, while chronic stress exposure inhibits this mechanism. Moreover, CMS, increasing the availability of membrane GR, seems to direct preferentially the action of hormones more towards the non-genomic pathways, interfering with synaptic and mitochondrial signaling. At behavioral level, the anhedonic-like behavior and cognitive deficits may be related to both the altered genomic and non-genomic mechanism of GR and the dysregulations of these signaling in stressed rats might be indicative of the so-called "glucocorticoid resistant", a key feature of depressed patients.
Chronic lurasidone administration normalized the behavioral outcomes, induced by CMS exposure, by restoring the modification observed in the GR mediated effects, suggesting the potential ability of the drug in modulating dysfunction related to the HPA axis. These data provide new insights into the mechanism of action of lurasidone in modulating different pathologic domains associated with psychiatric disorders, as highlighted by the pro-cognitive effect we observed in the NOR task, which may be mediated by its intrinsic activity as antagonist of the serotoninergic receptor 5HT-7, important for learning and memory.

Stress and epigenetic mechanisms (9)

Epigenetics are mechanisms that in response to environmental stressors, both social and physical, can result in lasting changes that affect brain functions and neurobiological processes, including the neuroendocrine system (23), that in turn may contribute to develop psychiatric disorders.
They constitute important mechanisms by which transient stimuli can induce persistent changes in gene expression and in behavior (24, 25).
Moreover, many antidepressant drugs have been found to influence epigenetic processes, by acting as regulators of key mechanisms, thus exerting beneficial effects (26).
As mentioned, major depressive disorfer (MDD) is associated with functional alterations of the HPA axis and fundamental players of the axis undergo changes in the methylation in the context of environmental adversities. Hence, we studied whether chronic stress

may cause alterations in the transcription of genes associated with GR, that are sustained by epigenetic modification, DNA methylation and miRNA expression in the prefrontal cortex (PFC), a brain region tightly connected with stress. In particular, we focused on the methylation status of the CGs located in the proximity of the glucocorticoid responsive element (GRE) sequences of selected genes named *Gadd45β*, *Sgk1,* and *Gilz* to investigate the activity of GR as a transcription factor and the accessibility to its responsive element on the DNA. We explored the influence of the treatment with LUR in modulating the transcription of *Gadd45β*, *Sgk1,* and *Gilz* possibly by acting at the epigenetic level and we studied the methylation status of these genes and the potential involvement of miRNA in chronic stress effects following a period of rest to explore whether the effects exerted by CMS were long-lasting.

We found that chronic stress altered *Gadd45β* mRNA levels, and this transcriptional change was sustained by DNA methylation, effect still present after a period of rest from chronic stress, suggesting an enduring effect of the adverse manipulation. Interestingly, LUR administration reverted the stress-induced reduction of *Gadd45β* expression and the changes in the DNA methylation status due to chronic stress exposure. Moreover, stress also had enduring effect on Sgk1 methylation in the CGs of the GRE, independently from lurasidone administration and had negative effects on *Gilz* expression, both at the end of the stress procedure and following the rest period. Regarding miRNA, we observed that only the miR-143-3p of Gilz was still reduced after a period of washout from chronic stress.

All in all, these data highlight that chronic stress exposure results in persistent changes in DNA methylation in specific genes related to glucocorticoids signalling and that lurasidone act as a modifier of such mechanisms, suggesting its potential as modulator of the HPA axis that is compromised in different psychiatric disorders.

Stress and Brain derived neurotrophic factor (Bdnf): chronic (10) *vs* acute (8) exposure

As mentioned in the introduction, chronic stress exposure during adult life may have an adverse impact on the long-term course of MDD and it may increase the response to subsequent stressors, such as acute challenges. Indeed, it is possible that the impairment caused by stress exposure cannot be completely restored during the remission, thus leaving 'scars' of vulnerability that may facilitate the relapse to the pathology. Indeed, being depression considered a recurrent disorder, approximately 50% of patients affected by MDD experience relapse despite pharmacological treatments (27), indicating the importance of studying how long-term pharmacological treatments properly manage the chronic course of the pathology.

From the opposite side, acute stress may induce beneficial advantages, in a short-term, by activating protective functions or by preparing the organism to react with external demands. Furthermore, when the stress is short, it can have positive effects on memory and even be fundamental for good learning (28).

In the context of both negative and positive effects of exposure to chronic and acute stress exposure respectively, Bdnf plays a crucial role. Indeed, one of the main hypotheses regarding the pathogenesis of depression proposes a role for neurotrophic factors in the etiology of depression and its treatment, with Bdnf playing a pivotal role (29) and the detrimental effect of chronic stress on Bdnf in animal models of depression is well consolidated (30, 31).

Moreover, considering the positive effects of brief stressors in the activation of protective brain functions, the relationship between acute stress response and neuroplasticity is well-established (31).

In this context, starting from the negative effect of stress, despite the widely described outcomes of chronic stress exposure as main environmental factor able to induce depressive phenotype in rodents, limited information

is available on the long-lasting impact of stress as well as on the mechanisms that may promote or prevent relapse.
Hence, we investigated whether stress-induced changes may persist after a recovery period and if alterations in BDNF signalling may underlie the precipitation of a recurrent episode. To this purpose, after a period of rest from chronic stress, the animals were presented to an acute immobilization stress. We found that chronic stress induces prolonged molecular changes that impair the activation of BDNF signaling following a subsequent acute stressor and inhibit the ability of PFC to cope with an acute challenge. We observed an inhibition of the proper response of the HPA axis to the acute stressor in animals previously chronically stressed, whereas the activation of the immediate early genes *Arc* and C-*fos*, and of the early response gene *Gadd45ß* was preserved following the acute stress, despite a reduction of their expression following chronic stress exposure, suggesting that PFC preserves functional plasticity after the post-stress period.
With respect to neuroplastic mechanisms, we demonstrated that the acute challenge upregulated mBDNF and its receptor TRKB protein levels in non-stressed animals, whereas these modulations were completely blunted in rats previously exposed to chronic stress. The effects observed in stressed animals were reflected by the blunted induction of BDNF-TRKB intracellular-related pathways. The inability to activate BDNF cascades in response to acute stress in previously stressed rats suggested that the BDNF stress-induced changes were not completely recovered despite the period of washout and that some molecular scars, which inhibit the recruitment of the adaptive mechanisms activated in "healthy" subjects, are still present (10).
Concerning the adaptive effect of stress, we found that one hour of acute restraint stress led to an enhancement of total *Bdnf* mRNA levels and of its major transcript in the PFC at different time points following the challenge. Accordingly, we observed a positive effect of the acute stress also on markers of neuronal activity, *Arc, Gadd45b* and *Nr4a1*, genes rapidly activated following acute environmental stimulations (32) and involved in brain functions including learning and memory (33). These results indicated that short stressors may trigger the modulation of neuroplastic mechanisms mainly within the PFC, thus contributing to store information that could serve to set up a response to a new stimulus including a cognitive task.
Moreover, at behavioural level, we demonstrated that exposure to the acute restraint stress improved cognitive function in the NOR task with a specific temporal profile, in particular in the time frame of one hour following the acute challenge, indicating the positive and beneficial effect of short stressor on cognitive abilities. In line, several studies showed that stress, in close association with learning task, facilitated memory consolidation (34).
At molecular level, this enhancement in the cognitive performance was associated with an increased expression of *Bdnf* in the prefrontal cortex, suggesting that the acute stress may transiently affect neuroplastic mechanisms, in line with the notion that neurotrophic factors are implicated in long-term potentiation and that stress may modify cognitive function through the control of *Bdnf* (35).

CONCLUSIONS

Studying the multifaceted effects of stress at adulthood provide evidence about the consequences of both negative and positive outcome on the adult brain. The analysis of several molecular mechanisms that may contribute to different aspects of stress exposure, from vulnerability to resilience (6, 7, 36, 37), from the recovery to relapse (9, 10) following chronic stressors and about acute stressor (8), is crucial to increase the knowledge to the field of pharmacological research for searching novel targets and approaches for the treatment of depression and stress-related disorders as well as for the promotion of resilience.

ETHICS

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Conflict of interests
The authors declare that they have no conflict of interests.

Authors' contributions
PB prepared and edited the manuscript, which was an overview of the literature of the experimental data obtained during the PhD training (2015-2018).

Availability of data and materials
No new data were generated or analysed in this research.

Ethical approval
N/A

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REVIEW

pharmadvances

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NEW PHARMACOLOGICAL STRATEGIES FOR ANALGESIC DRUG DEVELOPMENT: FOCUS ON BIASED μ-OPIOID RECEPTOR AGONISTS

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SUMMARY

Chronic pain affects more than 30% of people worldwide and although mortality rates are highest for other pathologies, it is one of the main sources of human suffering and disability that profoundly impacts patients' quality of life. To date, opioids still represent the reference treatment for moderate to severe chronic pain, however their use is strongly limited by a plethora of unwanted side effects including analgesic tolerance and opioid induced hyperalgesia. In the last few years, numerous efforts have been made in order to develop new analgesic drugs characterized by reduced side effects and by a safer pharmacological profile. Molecules capable of modulating different downstream pathways, known as biased agonists, are one proposed strategy for the treatment of chronic pain due to their suggested ability to discriminate between analgesic and adverse effect. In this review, we discuss the pharmacological outcomes of opioid biased ligands by bringing together cellular results and the available data from clinical trials; in particular, we focus on biased μ-opioid receptor agonists given their therapeutic relevance.

Key words

Biased MOR agonists; chronic pain; opioids; analgesia.

Impact statement

The concept of MOR biased agonism has been used to identify new analgesic drugs. Although some criticism has been raised toward this strategy, it represents a useful step for the development of safer opioid analgesics.

INTRODUCTION

Chronic pain is one of the major pathological conditions that affect the general population with a prevalence ranging from 11% through 40% (1). This kind of pain that lacks the acute warning function of physiological nociception, lasts or recurs for more than 3 to 6 months (2). The occurrence of this condition is influenced by a wide range of factors that strongly affect its duration and intensity (3, 4). Thus, pain management, often requires a multidisciplinary and multimodal approach including pharmacological treatment, interventional therapies, and behavioral/physical therapy (5). Despite research advancement and the suggestions of new targets for moderate to severe chronic pain treatment, opioids still represent the gold standard analgesics due to their action on opioid receptors (6). However, prolonged opioid administration is often related to the development of several side effects including tolerance, physical dependence and opioid induced hyperalgesia (OIH) (7, 8). The appearance of these phenomena strongly limits opioid use and

could determine the reduction/cessation of opioid administration or the progressive dose increase in order to achieve adequate analgesia. Furthermore, respiratory depression, constipation, nausea and itching also represent common dose-limiting side effects (6, 9, 10). In addition, opiate effects on mood and reward behaviors highlights another major concern related to their possible misuse, abuse, and addiction state establishment. These latter could represent some of the main causes responsible for the opioid crisis outcome in US (6) and strongly impact their prescription as well as their usefulness for pain relief (11, 12).

Therefore, efforts are made nowadays to develop innovative analgesics characterized by similar potency and efficacy compared to the common opioid agonists (*i.e.*, morphine, oxycodone, fentanyl) and by a safer pharmacological profile (fewer side effects and lower abuse liability).

Given experimental evidence showing that opioid-induced analgesia and side effects could be processed by distinct cell signaling pathways (13, 16), and the attention dedicated to biased agonists, this review will discuss the molecular mechanisms of some biased μ-opioid receptor agonists and their preclinical and clinical relevance.

THE ENDOGENOUS OPIOID SYSTEM SIGNALING

The endogenous opioid system consists of four natural ligands represented by β-endorphins, enkephalins, dynorphins, and nociceptin/orphanin FQ and includes four cognate seven-transmembrane G protein-coupled receptors (GPCRs) which are μ, δ, κ, and the opioid receptor like-1 (MOR, DOR, KOR, NOP receptor, respectively). This system represents one of the main system involved in neurotransmission and neuromodulation and it is widely expressed across the neuraxis, particularly in pain pathways (17). Indeed, the activation of opioid receptors plays a crucial role in the modulation of pain transmission and therefore in analgesia. Following the activation by agonists, such as endogenous peptides or exogenous ligands like morphine and fentanyl, the receptor engages the GDP-bound Gαβγ complex that promotes the GDP dissociation from Gα thus leading to the separation of Gα and Gβγ subunits through conformational changes. In particular, opioid receptors activation reduces neuronal excitability and pronociceptive transmitters release through the modulation of calcium and potassium ion channels. Indeed, receptors activation is able to induce hyperpolarization by activating K^+ channels and by inhibiting Ca^{2+} channels (18). Moreover, agonist stimulation of these receptors inhibits adenylate cyclase (AC) thus reducing cyclic AMP (cAMP) production (17, 19, 20). The maintained G protein activation induced by opioids could lead to the receptors β-arrestin-mediated desensitization and internalization mainly due to the G protein-coupled receptor kinases (GRKs) phosphorylation at the intracellular C-terminus (18). The resulting phosphorylated arrestin-GPCR complex recruits downstream signaling cascades including ERK, JNK, and p38 mitogen-activated protein kinase (MAPK) which, in turn, leads to proliferation, differentiation, and apoptosis (**figure 1**) (17).

OPIOID BIASED AGONISM

The term "*functional selectivity*" was introduced for the first time in 1998 and was initially referred to dopamine receptors (21). Theoretically, functional selectivity is due to different agonists' interaction with specific residues at the orthosteric binding site of a GPCR, thereby inducing different conformational changes of the intracellular loops that eventually result in different signaling outputs (22). This phenomenon, also known as "biased agonism" defines the capability of a specific ligand to activate different cellular pathways.

Within the opioid receptor subfamily, G protein signaling is accountable for opioid-induced analgesia whereas the β-arrestin pathway determines the occurrence of side effects (16). Thus, the functional selectivity of G-protein pathway

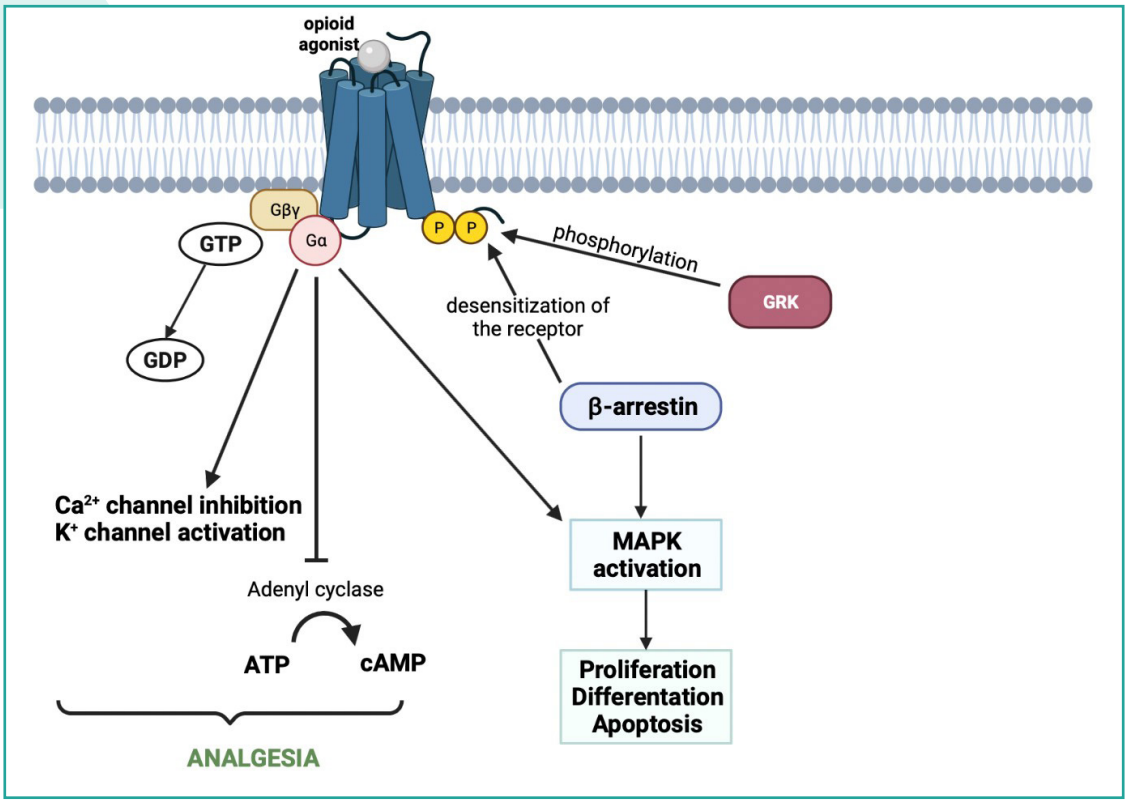

Figure 1. Intracellular pathways following opioid receptors activation.
After the agonist binding to an opioid receptor, conformational changes lead to the activation (via GDP e GTP exchange at the G-protein) of different signaling pathways. G-protein signaling pathways, enabled after the dissociation of G-protein into α and β/γ subunits following agonist binding, are accountable for the inhibition of calcium channels, activation of potassium channels (reducing the excitability of the cell membrane), inhibition of adenylate cyclase and stimulation of mitogen associated protein kinases (MAPKs) cascades. Bias towards G-protein signaling has been associated with analgesia. On the other hand, β-arrestin recruitment and its signaling pathways activation are due to G-protein receptor kinases (GRK) phosphorylation of the active G-protein coupled receptors, thus leading to the internalization/desensitization of opioid receptors and the activation of mitogen associated protein kinases (MAPKs) cascades. Bias towards β-arrestin has been linked to side effect profile.

could be relevant to avoid undesirable opioids effects (**figure 2**). The first suggestion of targeting a specific signaling pathway following MOR-agonist activation came from a study of Bohn and colleagues in 1999 demonstrating that mice lacking of β-arrestin-2 show a more potent and prolonged analgesic effect after morphine administration together with less tolerance, constipation, and ventilatory depression, compared to wild-type mice (13, 23, 24). It is relevant to note that different outcomes regarding tolerance development may occur depending on the specific neuronal system. In fact, some evidence shows that β-arrestin-2 knockout (KO) mice do not develop tolerance in the hot plate test, which is related to supraspinal pain responsiveness. Instead, the warm water tail immersion assay, linked to spinal reflexes activated by painful thermal stimuli, highlights the development of morphine tolerance, even if to a lesser degree than the wild-type (WP). Of note, low doses of protein kinase C (PKC) inhibitor (*i.e.*, chelerythrine) is able to completely reverse morphine tolerance in β-arrestin-2 KO mice (25). This evidence suggest that β-arrestin-2 regulates MOR sensitivity to morphine, although other regulatory proteins can also impact receptor function at spinal

cord level. Thus, it is clear that β-arrestins are critical in understanding mechanisms responsible for tolerance development. In this regard, *in vitro* studies show that both β-arrestin-1 and β-arrestin-2 differently regulate MOR signaling and that β-arrestin-2 only can rescue morphine-induced MOR internalization (26).

Over the years, the concept of biased agonism has been extended to the other opioids receptors. Indeed, also DOR activation, known to produce analgesia, anxiolytic- and antidepressant-like effects, is linked to the development of some side effects such as tolerance and convulsions that seems mainly due to arrestin-mediated internalization (15, 19, 27). Moreover, recent evidence also demonstrates that KOR activation in β-arrestin KO mice induces a potent antinociceptive and antipruritic effects, thus suggesting that biased KOR agonists are able to provide analgesia without producing dysphoria, sedation, abuse potential, anxiety, stress, and depression that are common side effects related to the use of KOP agonist (15, 28). To date, only few studies explored biased NOP receptor agonists. Pacifico *et al.* found that lipidation of N/OFQ(1−13)-NH2, a potent NOP receptor agonist, could be a valuable strategy for developing G-protein biased NOP receptor agonists (29). On the other hand, it seems that NOP receptor ligands are capable of fostering the interaction between the receptor and β-arrestin-2 leading to anxiolytic-like effects. At the same time, molecules that inhibit this interaction are responsible for antidepressant-like effects in mice (19, 29, 30).

BIASED MOR AGONIST MOLECULES

The above findings highlight the relevance of functional selectivity in intracellular GPCR sig-

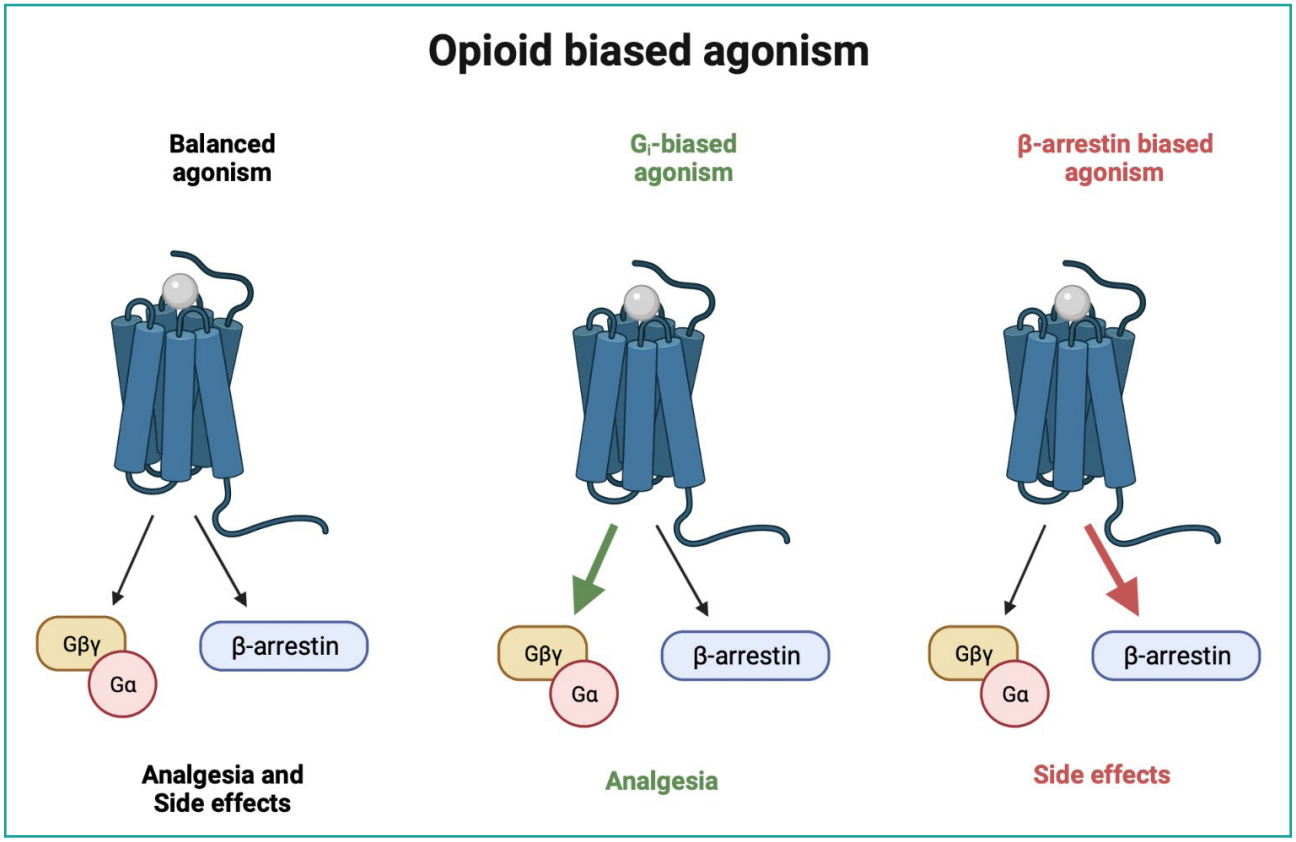

Figure 2. Opioid biased mechanism. In regard to opioid receptor subfamily, ligands that are able to selectively activate G-protein signaling lead to analgesia whereas side effects occurrence is mainly a consequence of the selective β-arrestin-2 recruitment.

naling and suggest that the development of newer MOR agonists able to discriminate antinociception from adverse effects could have an important therapeutic relevance. Although the concept of biased agonist is extended to all four opioid receptors it is important to emphasize that the most satisfactory results have been obtained from the research on μ-opioid receptors. Therefore, by bringing together cellular results and the available data from clinical trials, in this section biased-MOR agonists will be mainly discussed.

Herkinorin

Herkinorin represents the first μ-opioid receptor ligand derived from the selective KOR salvinorin A (31). This molecule acts as an agonist of both MOR and KOR showing high binding selectivity for μ-opioid receptor *in vitro*; a lesser selectivity has been also reported for KOR and DOR (32). Due to its ability to activate G protein coupling and ERK1/2 without inducing receptor-β-arrestin interactions or receptor internalization in MOR-expressing HEK-293 cells (26), herkinorin was initially defined as a biased MOR agonist. However, a more recent study using enzyme complementation assay to assess β-arrestin-2 recruitment, reveals that this molecule seems able to recruit this protein (33). *In vivo* studies demonstrate that herkinorin is able to induce low tolerance development coupled to potent antinociceptive effect at peripheral level only. Although the reduced CNS activity (32) strongly limits its use, herkinorin represents a starting point for the development of analogs.

Mitragynine and 7-hydroxymitragynine (7-HMG)

Mitragynine has been isolated from *Mitragyna speciosa* (traditionally known as kratom), a medicinal plant used for its analgesic properties. This molecule demonstrates mixed MOR agonist and DOR/KOR antagonist activity (34). In murine models, mitragynine does not recruit β-arrestin-2 and induces a lower tolerance as well as physical dependence than MOR opioid agonists (15). It is also capable to produce a lower respiratory depression compared to codeine (35). The second most plentiful alkaloid with a bias towards G-protein signaling extracted from kratom is 7-hydroxymitragynine, a full MOR agonist showing a binding affinity 5-fold higher than mitragynine (34, 36). However, both these molecules are able to induce opioid-like adverse effects (*e.g.*, withdrawal, constipation) even if to a lesser extent than morphine (19).

Although mitragynine and its derivatives seem to show an interesting pharmacological profile, the concerns related to their toxicity mainly due to their ability to interact with different cellular pathways, strongly limited their use. However, these compounds could be useful for the development of new effective molecules characterized by lower side effect (37).

Oliceridine

Oliceridine (TRV130) is a novel intravenous G protein-selective MOR agonist developed by Trevena, Inc. and it is the first biased agonist approved by US FDA in 2020 (Olinvyk™). It has been authorized for moderate-to-severe acute pain management and for whom alternative treatments are inadequate and it is currently tested for clinical use in Europe and Asia (**figure 3**). Oliceridine exhibits nearly a 3-fold preference for G-protein signaling over β-arrestin, thus providing potent analgesia with reduced adverse events generally related to opioids (38-40).

Although there are no structure similarities between oliceridine and morphine, the former has higher selectivity for MOR than the latter (41). In particular, this molecule is able to preferentially activate MOR, leading to a decreased cAMP activity which, in turn, generates analgesia. At the same time, oliceridine reduces MOR activation of β-arrestin which is related to respiratory depression, opioid tolerance, OIH, and feedback inhibition of the G-protein

pathway (38). Nonetheless, Araldi and coworkers reported that peripheral injection in the dorsum of rats' hind paw of both oliceridine and PZM-21 elicit OIH and hyperalgesic priming; this latter procedure represents a model commonly used to investigate the transition to chronic pain induced by the administration of potent MOR agonists (*e.g.*, DAMGO, fentanyl) (42). Overall, results show that after continuous infusion of oliceridine in rats (28 days) and in monkeys (14 days) there are no peculiar noxious effects compared with those associated with chronic opioid administration and that oliceridine shows an advantageous risk-benefit profile when compared to equianalgesic doses of morphine (43, 44). Indeed, a randomized phase II study suggests that oliceridine may enhance the analgesic efficacy with acceptable tolerability when compared to that of classical opioids (45).

Different clinical trials on oliceridine are in agreement on its improved pharmacological profile. ATHENA was a phase III, open-label, multicentric trial conducted from 2015 to 2017 in order to assess the safety and tolerability of oliceridine in a wide setting of patients with moderate-to-severe acute pain and painful conditions for which intravenous opioid would be warranted. In this study, among patients that experienced adverse events (64%) nausea, vomiting, and constipation were the most frequent "probably" or "possibly" related to oliceridine that were reported in 33% of patients although with lower incidence (46, 47).

When compared to morphine, oliceridine displays several differences including β-arrestin recruitment, receptor phosphorylation, and receptor internalization. In addition, DeWire and coworkers demonstrated that oliceridine administration in mice results in less gastrointestinal dysfunction as well as in an increased therapeutic index for analgesia versus respiratory suppression and sedation in rats, compared to morphine. The enhanced respiratory safety profile has been confirmed also by ATHENA (47, 48). Likewise, APOLLO-1 (a phase III, multicenter, randomized, double-blind, placebo- and active-controlled study for the management of moderate-to-severe acute pain following bunionectomy) and then APOLLO-2 (a phase III, multicenter, randomized, placebo- and active-controlled study for the management of moderate-to-severe acute pain following abdominoplasty) have demonstrated the reduced incidence of adverse events regarding the gastrointestinal and respiratory functions, confirming previous phase Ib and phase II studies (41, 49-51).

Several investigations show that oliceridine could have reduced analgesic tolerance (52-55). However, other investigations reported that oliceridine, despite biased agonism on MOR, maintains an abuse potential similar to that of conventional opioids. In fact, a study from Altarifi *et al.* shows that repeated administration of oliceridine in rodents leads to effects comparable with morphine intracranial self-stimulation (ICSS), while Austin Zamarripa and coworkers find that oliceridine and oxycodone exhibit equipotent reinforcement effects in rats. Nevertheless, when compared to morphine, oliceridine displays less tolerance, which occurs after 4 days of treatment, and OIH event though there are evidence for physical dependence development and conditioned place preference (CPP) (55-57).

PZM-21

Despite the poor structure analogy between PZM-21 and opioids, computational docking and structure-based optimization led to the identification of this molecule as a non-prototypical G_i activator of MOR with minimal β-arrestin-2 recruitment (19, 58). Some evidence showed that systemically administration of PZM-21 is able to produce hyperalgesia at low doses and analgesia at high doses as well as hyperalgesic priming at both tested doses (42). Moreover, it has been recently demonstrated that PZM-21 causes long-lasting potent antinociception in the hot plate and formalin tests in rodents and in non-human primates (NHPs). However, its potency is lower than that of oth-

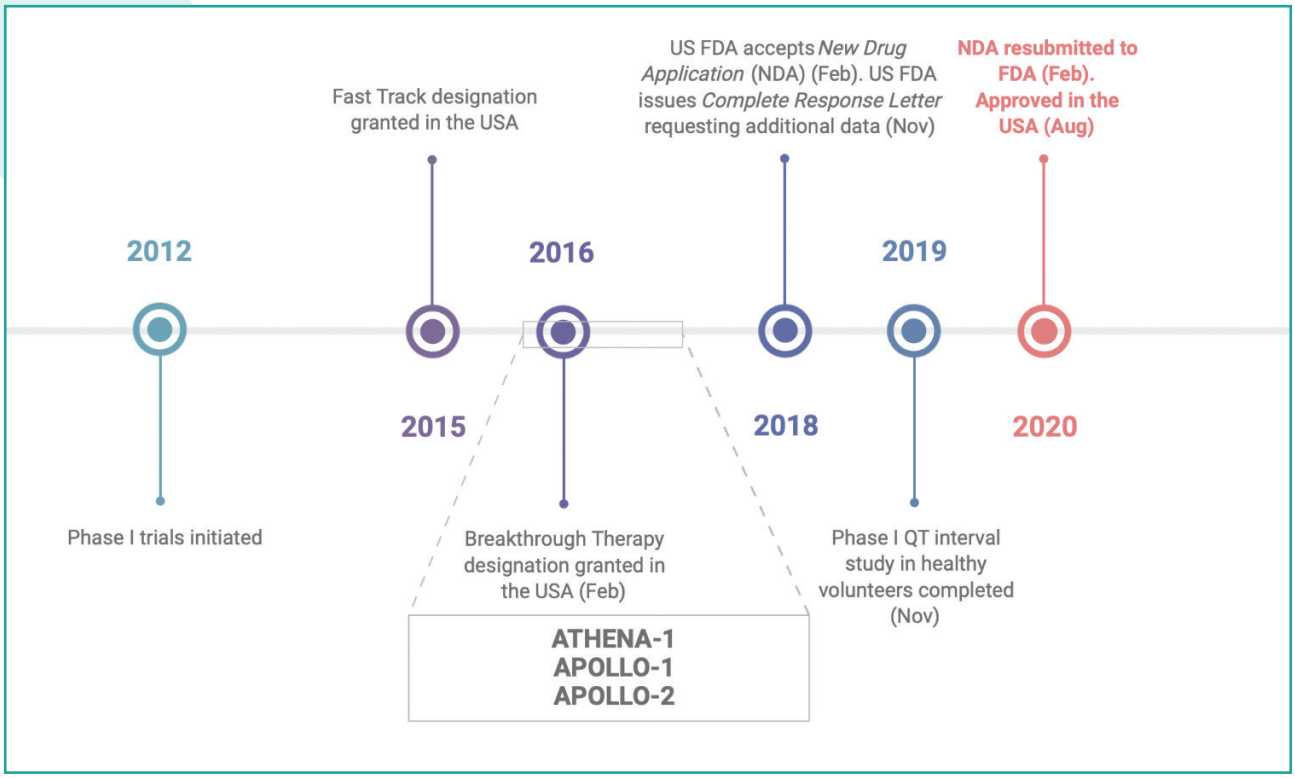

Figure 3. Key milestones in clinical investigation for the development of oliceridine.

ers commonly used MOR agonists; indeed, PZM-21 is about 10-fold less potent than oxycodone (16, 19, 59). Furthermore, Kudla and colleagues have also shown that the pretreatment with PZM-21 is able to enhance morphine-induced analgesia and to attenuate the expression of morphine reward (16, 60). However, even though PZM-21 does not induce conditioned place preference (CPP) in rodents, in a NHPs model of intravenous drug-self administration, it seems to induce reinforcing and pruritic effects similar to clinically used MOR receptor agonists (59). In addition, repeated administration of PZM-21 leads to the development of antinociceptive tolerance (61).

In regard to respiratory depression, there are controversial data. Manglik *et al.* (2016) reported that PZM-21 causes a reduced development of respiratory depression but, from a study of Hill and colleagues, it seems that this drug is able to depress respiratory function in mice with a profile comparable to that of morphine. These data could be partially explained by evidence highlighting that this side effect results also from G_i/G_o signaling (33, 61).

Piperidine benzimidazoles

SR-17018, SR-15098, and SR-15099 are some of substituted piperidine benzimidazoles with high affinity for MOR. Structure-activity studies have identified halogens and a central piperidine as important structural features for bias (19, 62). In particular, SR-17018, SR-15098, and SR-15099 are partial agonists with a G-protein signaling preference as well as the full agonist SR-14968 whereas SR-11501 is a biased partial agonist for the β-arrestin recruitment (63). These drugs have a long-lasting effect and seem to promote potent antinociception similar to morphine and fentanyl in hot plate and warm water tail withdrawal tests (19). Moreover, SR-17018, SR-15099, and SR-14968 produce in mice a lower respiratory depression than morphine (14, 64).

SR-17018, is able to suppress signs induced by morphine withdrawal but like, but differently

from buprenorphine, substitution of SR-17018 in morphine-tolerant mice is also able to restore morphine sensitivity (14). In addition, it has been showed that SR-17018 induces a higher allodynia suppression in comparison with morphine or oxycodone, in a paclitaxel-induced neuropathic pain model (65); finally, its chronic administration in mice produces less tolerance to the analgesic effect than morphine in the hot plate test (14). However, it is interesting to note that SR-17018 produces antinociceptive tolerance in the warm water tail immersion test, in the same animal species (65). The ability of SR-14968 and SR-17018 to produce antinociceptive effect and limited respiratory suppression (66) has bee also demonstrated in NHPs, thus corroborating their interesting pharmacological profile. However, additional studies are needed to evaluate the future development and potential application of these molecules in humans.

CONCLUSIONS AND FUTURE PERSPECTIVE

This review focused its attention mainly on biased μ-opioid receptor agonists. However, as above mentioned, it is important to underline that the functional selectivity concept involves also the other opioid receptors and it seems to be relevant for developing new pharmacological strategies for the management of chronic pain and for preventing the common side effects related to opioids (*i.e.*, tolerance, itching). Some criticism has been raised concerning biased agonism (67, 68) and it has also been suggested that the peculiar pharmacological profile of selected ligands could be mostly related to their low efficacy/partial agonism rather than to G-protein bias (69, 70). However, given pre-clinical and clinical results, biased agonists can represent a useful step in research strategy for the development of safer opioid analgesics. In this context, also dual agonism shown by selected molecules acting as MOR/NOP receptor agonists (*e.g.*, BU08028, BU10038, and AT-121) as well as MOR/KOR agonists, KOR/DOR agonists, and MOR agonists/KOR antagonists could be considered a promising approach in this field (71).

ETHICS

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Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

LR, LML and CM wrote the draft. All authors revised the final manuscript.

Availability of data and materials

The data underlying this manuscript are available in the article.

Ethical approval

N/A

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REVIEW

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DIABETIC RETINOPATHY: NEW PHARMACOLOGICAL TARGETS

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SUMMARY

Diabetic retinopathy (DR) is a secondary complication of diabetes mellitus and represents the most common cause of irreversible vision loss in working people of industrialized countries. DR is generally considered a microvascular complication of diabetes, although the inflammatory component plays a key role. The main cause of vision loss in diabetic patients with proliferative diabetic retinopathy is the diabetic macular edema (DME), that is responsible to the retinal detachment. DME is mainly caused by new angiogenesis, which is a hallmark of the advanced stage of DR (proliferative diabetic retinopathy, PDR). Retinal neovascularization is principally driven by pro-angiogenic factors (*e.g.*, VEGF-A, PlGF), inflammatory mediators (TNF-α, interleukins, chemokines) and oxidative stress-related elements. Chronic hyperglycemia is the primary causative factor of DR, however, several points of DR etiopathogenesis are still unclear. Many other factors are involved during the early stages of DR such as the retinal hypoxia, that is a trigger of VEGF release in the back of the eye. Up to now, the pharmacological approaches for DR are intravitreal anti-VEGF agents and corticosteroids. However, some patients can be refractory to anti-VEGF therapy, therefore, efforts must be carried out to discover novel pharmacological targets to handle DR. Hereby, the current literature will be revised about novel potential pharmacological targets, with a focus on PlGF, miRNAs and purinergic P2X7 receptor. Future drug development campaigns on these targets might lead to better clinical outcomes, possibly in the early phase of the disease.

Key words

Diabetic retinopathy; angiogenesis; hypoxia; anti-VEGF; inflammation.

Impact statement

Currently, management and treatment of diabetic retinopathy (DR) are characterized by several unmet medical needs. Particularly, early-stage DR lacks of approved therapeutical intervention, and its pathogenesis is multifactorial. Pharmacological research should focus on novel pharmacological targets, that address pathogenetic factors of DR, such as inflammation, oxidative stress and angiogenesis.

INTRODUCTION

Diabetic retinopathy (DR) represents a major public health concern, and it is the leading cause of vision loss in working age (1). The prevalence of DR among diabetic patients is about 40%, and approximately 5-10% of these individuals have vision threatening conditions (2, 3). Chronic hyperglycemia is the primary causative factor of diabetic retinopathy, however, etiopathogenesis of DR is still unclear (4-7). Ophthalmologists classify diabetic retinopathy mainly into two stages, the non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) (8). The NPDR is characterized by lesions due to chronic hyperglycemia, that can lead to microaneurysms due to instability of

capillary walls. As soon as microaneurysms start leaking, NPDR can develop in macular edema and consequent impaired vision, due to deposition of fluid under the macula. The presence of this fluid, composed by lipids, leads to the formation of yellow deposits, called hard exudates. Moreover, with the progression of the disease, the affected vessels can be obstructed, then leading to impaired retinal perfusion. Retinal ischemia can cause the infarction of the nerve fiber layer, resulting in fluffy and white patches, called cotton wool spots (CWS). Main cause of NPDR to PDR progression is represented by an extensive retinal ischemia (9), which promotes vitreoretinal neovascularization. In fact, the retina is a high oxygen demanding tissue; under ischemia, cells release angiogenic factors, like vascular endothelial growth factor-A (VEGF-A) and placental growth factor (PlGF), which promote neovascularization. These vessels are typically fragile, fenestrated, brittle and leaky. Leaking vessels can cause vitreous hemorrhages, which are associated with gliosis and fibrovascular scar formation. Moreover, contraction of fibrous tissue can result in tractional retinal detachment and sudden loss of vision (10, 11), along with further activation of pro-fibrotic pathways. As soon as extensive vitreous hemorrhage occurs, the PDR patient is considered at high-risk of vision loss due to retinal detachment (4, 12). In case diabetic retinopathy affects the macula, the disease is also termed 'diabetic maculopathy'. Vascular leakage at the macula leads to macula swelling (diabetic macular edema, DME), which is the most common cause of blindness in diabetic patients (13, 14). DME is most prevalent during PDR, following progressive vascular and neural damage (15). Diabetic DME can be classified as 'ischemic' or 'non-ischemic', based on the involvement or preservation of the perifoveal capillary network, respectively (10). Several causative factors contribute to the pathogenesis of DME, including hypoxia and oxidative stress, upregulation of VEGF-A, alteration of the blood-retinal barrier (BRB), retinal vessel leukostasis, pericyte loss, and vascular hyperpermeability (16, 17).

THE RETINAL NEUROVASCULAR SYSTEM AS A BASIS TO UNDERSTAND DIABETIC RETINOPATHY

The retina is the innermost light-sensitive tissue of the eye, able to convert light to electrochemical signals, at first through photoreceptors, that transmit electrochemical signals to retinal neuronal circuitry (bipolar, amacrine cells). Neuroretinal electrochemical stimuli are thereafter processed and collected by retinal ganglion cells (RGCs), that transmit signals to the visual cortex by means of the optic nerve, that is constituted by RGCs axons (18, 19). The retina is characterized by a complex vascular system, whose integrity is necessary for the correct retinal function, providing nutrients and oxygen to the inner and outer retina (1). The retinal vascular system, similarly to central nervous system, is characterized by blood-retinal barrier (BRB), which maintains the right *milieu*. The BRB includes the inner and outer components. Inner BRB (iBRB) is characterized by junctions between endothelial cells (ECs) and supporting pericytes and astrocytes; while in the outer BRB (oBRB), junctions are between retinal pigmented epithelial cells (RPEs) (20, 21) (**figure 1**). Diabetes can affect both iBRB and oBRB before and after neovascular events, involving endothelial cells, pericytes (at the capillary level), vascular smooth muscle cells (arteriolar/arterial level), glia, neuronal processes, associated immune cells, and if choroid is affected, also RPEs (22).

Pericytes, endothelial cells and iBRB

Depletion of pericytes is a hallmark of DR. Pericytes wrap capillary walls and share basal lamina with endothelial cells, with which they directly interact through N-cadherin and connexin-43 hemi channels (19, 23, 24). Pericytes wrap around retinal capillaries providing structural support, modulation of endothelial cell function and homeostasis. In the inner BRB, retinal endothelial cells form the physical barriers between vascular lumen and the retina. Retinal endothelial cell-cell junctions include tight-, adherens- and gap-junction, that regulate sev-

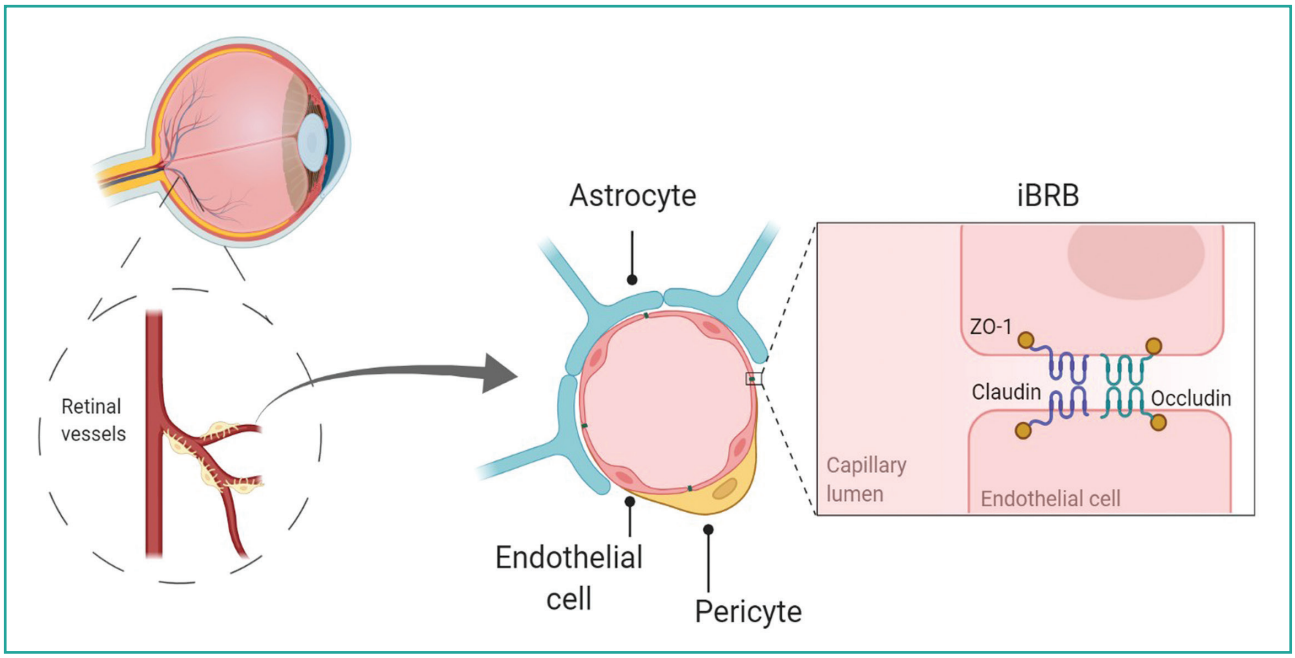

Figure 1. The neovascular unit.
In a healthy retina, vessels are structured by endothelial cells closely associated with pericytes, maintaining the function of the inner blood retina barrier (iBRB). In particular, adjacent endothelial cells are connected by tight junctions and adherens junctions.

eral cell functions such as migration, growth, protection from cell death (necrosis, apoptosis) and damage (inflammation, ischemia) (19, 25).

Retinal glia and neurons

Retinal glial cells, including Müller cells and astrocytes, provide metabolic support to neurons and play a critical role in iBRB homeostasis and integrity (26, 27). Moreover, Müller cells regulate glucose flux between the circulation and retinal neurons and have a role in providing substrates for aerobic metabolism in neurons by gluconeogenesis (28).

Immune cells

The development of new blood vessel is also supported by microglia, monocyte-derived tissue macrophages of the central nervous system. Interestingly, retinal microglia is present in the retina before development of vascularization (29). In the adult retina, ramified microglia cells were found in the inner and outer plexiform layers, and are able to produce factors that support neuronal survival. Several types of trauma or insults lead to microglia activation, characterized by amoeboid morphology transition and production of pro-inflammatory cytokines (30).
Nowadays, the improvement of the research methodologies allows us to mimic, *in vitro*, the real complexity of the ocular structures and barriers. Recently, two labs (Wisniewska-Kruk *et al.* and Fresta *et al.*,) set up two different *in vitro* BRB models based on a triple co-culture of retinal cells, the first research's group used bovine cells, the second research's group used human cells. Fresta *et al.* used human retinal pericytes, astrocytes and endothelial cells to mimic the human BRB with the same cellular layer order and the same numerical ratio. This *in vitro* paradigm is useful to study and investigate the molecular mechanism related to DR, and to test new pharmacological molecules (31, 32).

PATHOPHYSIOLOGY OF DIABETIC RETINOPATHY

The pathogenesis of DR is complex and involves multiple interlinked mechanisms, in-

cluding metabolic modifications, mitochondrial dysfunction, vascular damage, apoptosis, inflammation, and oxidative stress (33-36). Several pathways have been proposed to better understand microvascular complications during DR along with sustained hyperglycemia: *e.g.*, accumulation of advanced glycation end-products (AGEs), inflammation, activation of protein kinase C and neuronal dysfunction (19, 37). All these pathological modifications lead to increased vascular permeability and capillary depletion, resulting in macular edema and retinal neovascularization.

Hyperglycemia and Retinal Microvasculopathy

One of the earliest abnormalities observed in DR is related to retinal blood vessels, with the constriction of arteries and arterioles and blood flow anomalies (38-40). Vessel abnormalities result in a series of metabolic and biochemical alterations, like: (i) induction of activation of several PKC isoforms (*e.g.*, PKC-α, -β, -δ and –ε; in particular PKCβII isoform (41, 42); (ii) altered function of ionic channels in smooth muscle cells (BK channels) present in the retinal arteriolar vasculature (43-45). As mentioned before, retinal pericytes loss is another hallmark of the early events of DR. Several *in vitro* and *in vivo* studies report that hyperglycaemia leads to pericyte loss (34, 46, 47) or degenerated pericytes, also called "ghost cells". Therefore, pericytes loss leads to endothelial cells degeneration, microvascular destabilization and perfusion alterations with consequent ischemic events due to capillary occlusion (38, 48-50). On this regard, pericyte-like differentiation of human adipose-derived mesenchymal stem cells (hASCs) has been recently proposed as putative therapeutic tool for restoring damaged BRB (51).

Retinal inflammation

Several studies were focused on the role of inflammatory processes in early stages of DR, although, inflammatory mechanisms are still poorly understood. Chronic low-grade inflammation has been detected in different stages of DR, both in diabetic animal models and in patients (52, 53), along with increased systemic and local expression of proinflammatory cytokines (54). In particular, microvascular endothelium, activated by these cytokines and angiogenic growth factors, expresses pro-inflammatory molecules (*e.g.*, IL-1β, IL-6, TNF-α, high-mobility group box-1 (HMGB1) and chemokines (MCP-1), involved in leukocyte recruitment and activation (55-57). Leukocyte-endothelium adhesion, mediated by adhesion molecules, has been implicated in leukostasis during diabetes. Sequential adhesive interactions between endothelial cells and leukocytes, are modulated by adhesion molecules (*e.g.*, ICAM-1) present in the surface of endothelial cells, which interact with the leukocyte counter-receptor CD18 (58, 59). All these inflammatory responses may contribute to neovascularization in the retina during DR, especially under hypoxic conditions. Furthermore, increasing data suggest a crucial role of tool like receptors (TLRs) in the pathogenesis of DR; indeed, TLR4 expression is significantly increased in diabetic retinas, activating the linked inflammatory pathways (60). As regards the biochemical pathways involved in DR, expression of inflammatory cytokines might be mediated by activation of mitogen-activated protein kinases (MAPKs) (61), as well as ERKs, normally involved in several cellular processes (62). ERK pathway can influence NF-κB activation, by the regulation of NF-κB-dependent genes expression, *e.g.* inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and tumor necrosis factor-alpha (TNF-α) (63).

Retinal hypoxia

The retina is one of the most oxygen and glucose demanding tissue (64). Retinal hypoxia represents an important causative factor for DR development, and plays a central role in progression of NPDR to PDR, due to the release of some soluble mediators such as cytokines, chemokine and growth factors, which promote the growth of extraretinal neovascularization (65). Ocular ischemic events are

considered crucial for promotion of vascular abnormalities, due to endothelial cells adaptation to stress, which upregulate several genes, like VEGF-A (66). Furthermore, VEGF-A and other hypoxia-regulated growth factors, are controlled by hypoxia-inducible factor (HIF) (67). HIF is a heterodimer, HIF-1α (inducible subunit) and HIF-1β (constitutively expressed). Oxygen deprivation, induces HIF-1α to translocate into the nucleus and to bind the hypoxia-response elements (HREs) in DNA, leading to expression of inflammatory and pro-angiogenic genes, promoting inflammation and angiogenesis, respectively (68, 69).

Retinal angiogenesis

Angiogenesis is a crucial mechanism in physiological vascular development and during pathological conditions. Angiogenesis is related to ECs that, stimulated by some angiogenic factors, generate new blood vessels (70). Indeed, this process is characterized by the angiogenic growth factors, which activate the receptors present on resident ECs; then, endothelial cells begin to release specific enzymes such as matrix metalloproteinases (MMPs) which degrade the basement membrane, leading ECs to leave the original vessel wall. After that, endothelial cells start to proliferate into the surrounding matrix, thanks to the adhesion molecules (**figure 2**).

The main regulators of angiogenesis are the vascular endothelial growth factors (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) and the placental growth factor (PlGF) (71-76). VEGFs can bind to three tyrosine kinase receptors:

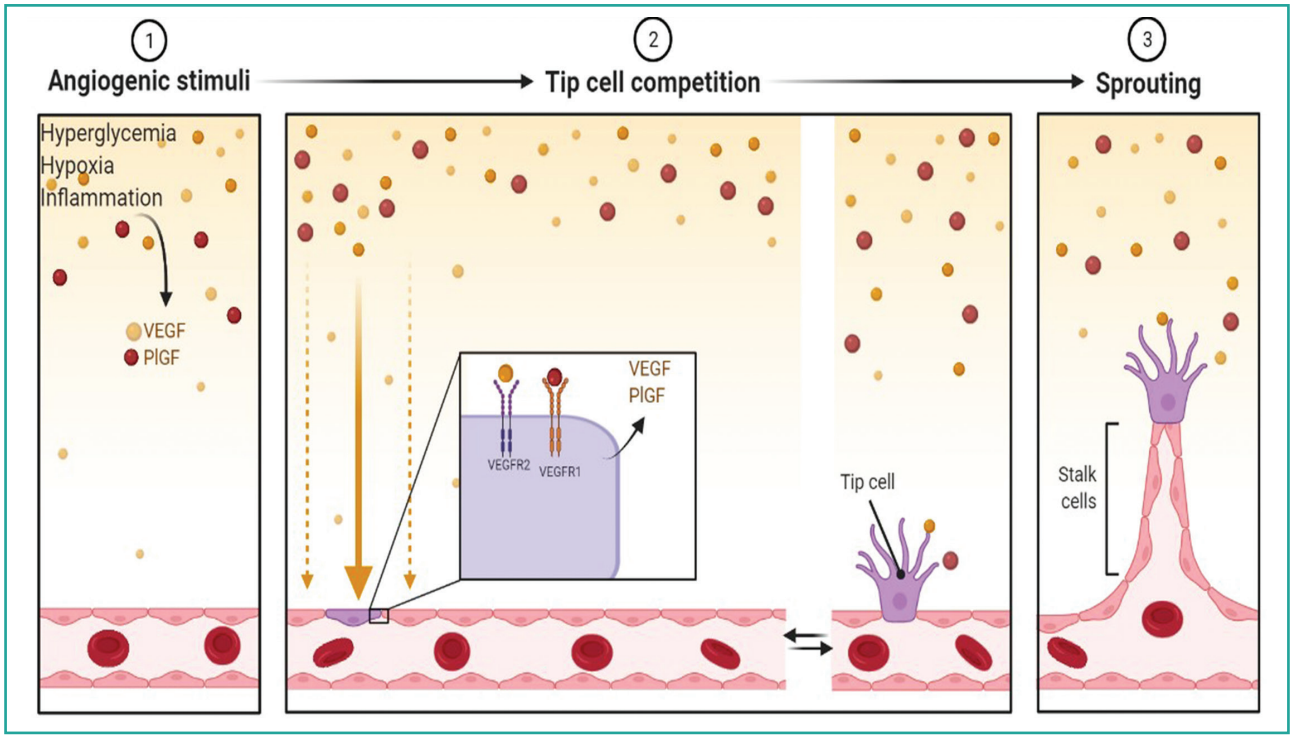

Figure 2. Retinal angiogenesis in diabetic retinopathy.
Angiogenic factors (*i.e.*, VEGF-A and PlGF) stimulate angiogenesis in tissues. VEGF-A/PlGF bind to VEGFR-2/VEGFR-1 on the surface of endothelial cells (ECs), triggering competition between neighboring cells as they differentiate. In normal intact retinal vessels, blood flow is regular and the vascular configuration is stable. However, in diabetic retinopathy, this process is strongly exacerbated by several phenomena, such as hyperglycemia, hypoxia and inflammation. The expression of angiogenic factors is increased and the growth of new blood vessels is uncontrolled. The neovascularization is typical of the later stages of diabetic retinopathy, with the formation of new unstable vessels. This leads to vascular damage, loss of endothelial tight junctions, pericytes detachment and basement membrane thickening (iBRB breakdown).

VEGFR-1 (Flt-1), -2 (KDR), and -3 (Flt-4) (77). VEGFR-1 (78) and VEGFR-2 (79–81) are the main receptors involved in angiogenesis. VEGFR-2 (also known as Flk1) is expressed on endothelial cells. Binding to VEGFR-1 (also known as Flt1) leads to the activation of quiescent endothelial cells and promote vascular permeability (82-85). VEGF-A is significantly increased in ocular tissues from patients with diabetes (86). All the mechanisms linked to the progression of DR, are responsible of the overproduction of VEGF-A, including hypoxic events. Besides stimulation of endothelial cell growth, VEGF-A can also promote the disassembly of junctions between endothelial cells, leading to vascular permeability (BRB breakdown).

Fibrosis

Angiogenesis and subsequent fibrotic events occur with progression of PDR. Fibrosis can cause the formation of fibrovascular epiretinal membranes, which lead to retinal complications such as tractional retinal detachment and, at last, vision loss (87-89). Fibrosis is a complex reparative process that is activated to restore damaged tissue, by means of remodelling extracellular matrix (ECM). Cell proliferation, ECM deposition and neovascularization are key mechanisms during PDR, usually stimulated by pathological conditions like hypoxia or inflammation, promoting formation of fibrotic tissue (90, 91). Along with microglia and astrocytes, Muller cells in response to retinal injury, participate to fibrotic events, through production of inflammatory and angiogenetic mediators (92, 93). Fibrosis can also be promoted by retinal hypoxia, leading to a consequent overproduction of VEGF-A (94-96). Several growth factors play a role in fibrosis, such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and the pro-fibrotic connective tissue growth factor (CTGF) (97-99). Precisely, increased levels of CTGF were found in the vitreous of patient with PDR (100, 101) and it has been supposed that CTGF could be a downstream mediator of TGF-β, the main regulator of pro-fibrotic effects.

PHARMACOLOGICAL TREATMENT OF DR

Currently, only PDR can be pharmacologically treated, and no approved treatments are available for NPDR. As mentioned above, the hallmarks of this disease are the abnormal vessel growth in retinal area, up-regulation of inflammatory factors, and the breakdown of the blood-retinal barrier (**figure 3 A**). Clinical history of PDR has been revolutionized with anti-angiogenic treatments, that are invasive and expansive. Along with the anti-VEGF agents (34), anti-inflammatory drugs are also used (102). Steroids are potent drugs to quench inflammation and reduce edema, fibrin deposition, capillary hyperpermeability and phagocytic migration typical of the inflammatory response (103-105). Furthermore, they also counteract the action of VEGF-A (106). Three corticosteroids are actually approved to handle diabetic macular edema (DME): dexamethasone (DEX), fluocinolone acetonide (FA) and triamcinolone acetonide (TA). The limitation of these drugs is related to the side effects such as cataract and rise in intraocular pressure (102).

Anti-VEGF therapy

The anti-VEGF therapies have revolutionized the treatment of DR. These medications, such as ranibizumab (Lucentis, Genentech) and aflibercept (Eylea, Regeneron), called vascular endothelial growth factor inhibitors (anti-VEGF), have a consolidate history in terms of efficacy and safety for the treatment of DME.
Ranibizumab is a 48 kDa antigen-binding fragment (Fab) of a humanized monoclonal antibody with high affinity for VEGF-A (**figure 3 B**) (107); it binds with high affinity all the VEGF-A isoforms (such as VEGF-A_{165}, VEGF-A_{110} and VEGF-A_{121}) reducing the activation of VEGFR-1 and VEGFR-2 receptors. The small size of this fragment enhances its diffusion from the vitreous to the retina and the choroid, improving

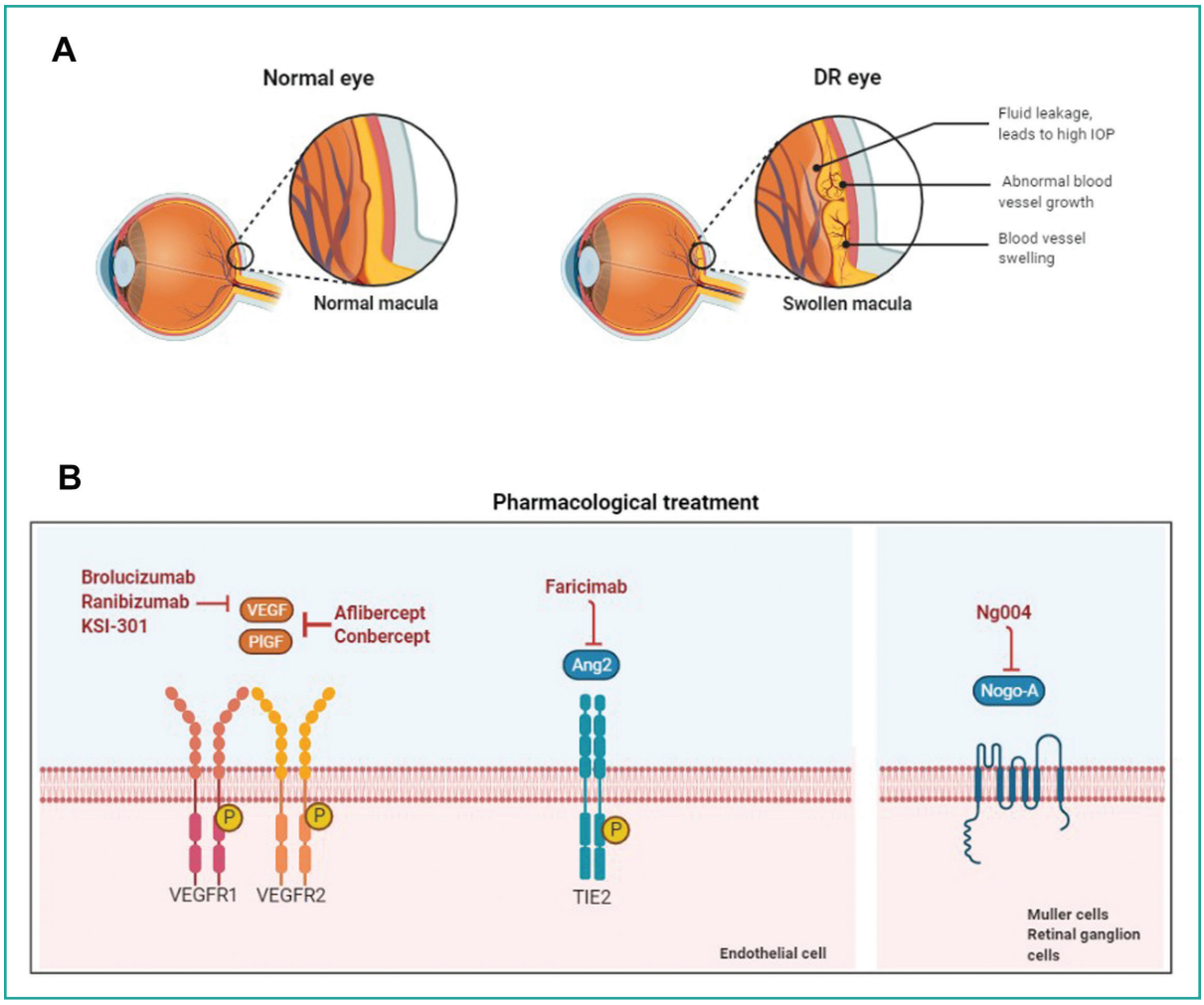

Figure 3. Diabetic retinopathy clinical hallmarks and treatments. **(A)** Diabetic retinopathy is the leading cause of vision loss in diabetic patients and is characterized by abnormal vessel growth in retinal area, inflammation, breakdown of the blood-retinal barrier and fluid accumulation. **(B)** Therapeutic strategies targeting different signalling pathways involved in the pathogenesis of proliferative DR and diabetic macular edema.

the pharmacokinetic profile, compared to bevacizumab (108).

Aflibercept has been approved by Food and Drug Administration (FDA) in 2011 for the treatment of age-related macular degeneration (AMD), for impaired vision due to secondary macular edema, caused by retinal vein occlusion (Branch RVO or central RVO) and for the treatment of visual impairment due to myopic choroidal neovascularization (CNV). Recently, aflibercept was also approved for the treatment of diabetic macular edema. Aflibercept (VEGF-trap) is a fusion protein (115 kDa) bearing two binding domains of VEGF receptors (**figure 3 B**) (109). Moreover, aflibercept's binding affinity to VEGF-A_{165} is almost 100-fold greater than ranibizumab and bevacizumab (110-112), and is the only anti-VEGF agents that binds PlGF, although with lower affinity (38.9 nM dissociation constant – KD), compared to VEGF-A (0.49 nM dissociation constant – KD) (113).

Conbercept (Lumitin) is a 141 kDa recombinant fusion protein composed of the second Ig domain of VEGFR-1 and the third and fourth Ig domains of VEGFR-2, fused to the constant region (Fc) of human IgG1 (**figure 3 B**). Considering the increasing need for less frequent intravitreal injections of anti- VEGF, conbercept

has designed to improve dose regimens and compliance. Similarly to aflibercept, conbercept has multiple targets (114).
In 2021 FDA approved the faricimab (Roche) with the following indications: wet AMD and DME. This antibody targets two different pathways involved in progression of these retinal diseases: angiopoietin-2 (Ang-2) and vascular endothelial growth factor-A (VEGF-A) (**figure 3 B**). Faricimab showed positive results across four phase III studies in AMD and DME. Faricimab clinical trials proved a non-inferiority efficacy evidence, compared to aflibercept (115, 116).
Brolucizumab (Beovu, Novartis), recently approved in US and EU, is a humanized single-chain antibody fragment (scFv) targeting three major isoforms of VEGF-A (*e.g.*, $VEGF_{110}$, $VEGF_{121}$, and $VEGF_{165}$) (**figure 3 B**). Compared with other VEGF-A inhibitors, brolucizumab is smaller (26 kDa). In 2021 Novartis announced the positive results of the Phase III KESTREL study. This study assessed safety and efficacy of 6 mg brolucizumab in patients with DME.
Although most of patients show beneficial effect by the approved anti-VEGF agents, a significant percentage of people are poor responders. To overcome this unmet medical need, NovaGo Therapeutics is developing a first-in-class fully human antibody therapy with a novel mechanism of action (NG004) targeting the protein Nogo-A. This latter is endogenously expressed by Müller cells and retinal ganglion cells, and it represents one of the most up-regulated protein during DR (117, 118). It has been demonstrated that the block of this protein could lead to the reduction of angiogenesis and inflammation, as already demonstrated in an *in vivo* model of retinal injury (excitotoxicity-induced neuroinflammation) (119).
As regards the safety profile of intravitreal anti-VEGF agents some adverse reactions such as endophthalmitis, intraocular inflammation, intraocular pressure elevation and ocular hemorrhage are sometimes associated with the treatment (120-122). Besides that, VEGF protein has a physiological role in the retina, so the prolonged period of treatment with these compounds could be deleterious (123). Moreover, these agents have a short half-life, and the widely treatment schedule is the treat-and-extend regimen with several injections for several months. For these reasons new agents and innovative delivery systems are under investigation.

NOVEL MOLECULAR TARGETS

Placental Growth Factor (PlGF)

PlGF has been implicated in pathological angiogenesis, especially in retinal disorders, although its function is less well understood (85), compared to VEGF-A. Oppositely to VEGF-A, PlGF is not required during physiological angiogenesis but plays a role only during pathological conditions (82, 83, 112, 124-128). Secreted PlGF specifically acts through VEGFR-1. Furthermore, it has been showed that VEGF-A and PlGF can form heterodimers (129) which can bind both VEGFR-1 and VEGFR-2, stimulating endothelial cells migration and vasorelaxation via the nitric oxide pathway (130, 131). Moreover, it has been found that VEGF/PlGF heterodimer can lead to activation of a positive feedback and overproduction of VEGF-A, which binds also the VEGFR-2. Therefore, PlGF may stimulate angiogenesis directly through VEGFR-1 but also indirectly through VEGFR-2 (83, 128). PlGF acts also through neuropilin receptor 1 (NRP1) (124, 127, 132), that is expressed in angiogenic vessels (133, 134). As well as VEGF-A, PlGF is expressed by endothelial cells in hypoxic environment (135-137). The PlGF overexpression is driven by HIF-1α, which is able to recognize a hypoxia responsive element (HRE) located in the second intron of PlGF gene (136). One interesting recent evidence demonstrated that aflibercept and a specific anti-PlGF antibody exert anti-inflammatory effects in the diabetic retina. Specifically, aflibercept and anti-PlGF antibody protected retinal endothelial cells (HRECs) and primary mouse retinal pigmented epithelial

cells (mRPEs) from cell damage induced by high glucose levels, blocking the activation of the ERK pathway with the subsequent suppression of TNF-α release (113).

miRNAs

There is an increasing interest on microRNAs as putative biomarkers for the progression of DR (138). Platania *et al.*, demonstrated that small set of miRNAs were dysregulated in serum and retina of diabetic mice. These miRNAs were also dysregulated in serum of patients with diabetic retinopathy. In the *in-vivo* study, these miRNAs modulated not only VEGF-A expression (up-regulation) but also the neurotrophic factor BDNF (down-regulated) (139). Moreover, Santovito *et al.*, reported that DR is associated with higher circulating levels of miR-25-3p and miR-320b and lower levels of miR-495-3p, in patients with type 2 diabetes and diabetic retinopathy (140). Interestingly, it has been demonstrated a specific association between miRNAs expression and hypoxic microenvironment; in fact, retinal hypoxia led to the upregulation of six miRNAs (miR-20a-5p, miR-20b-5p, miR-27a-3p, miR-27b-3p, miR-206-3p, miR-381-3p) in human retinal endothelial cells. These miRNAs, are capable to interfere with the expression of genes belonging to the TGF-β pathway at post-transcriptional level. In fact, the dysregulation of these miRNAs has driven and promoted angiogenesis and fibrosis, through the modulation of VEGF-A, TGF-β and HIF-1α, in retinal endothelial cells (135, 141-144). Moreover, Shao *et al.* identified miR-136 and miR-374 dysregulation as hallmark of proliferative DR (145). The putative involvement of miRNAs in the pathogenesis of DR is also linked to the direct activation of the inflammatory pathway through the TLR-4, as well demonstrated in several *in vitro* and *in vivo* models of DR; in fact, different miRNAs are associated with the regulation of TLR-4 expression during diabetic retinopathy (146-148). Currently, evidence about post-transcriptional regulation of PlGF expression by miRNAs has not been retrieved. A high-throughput screening of miR-

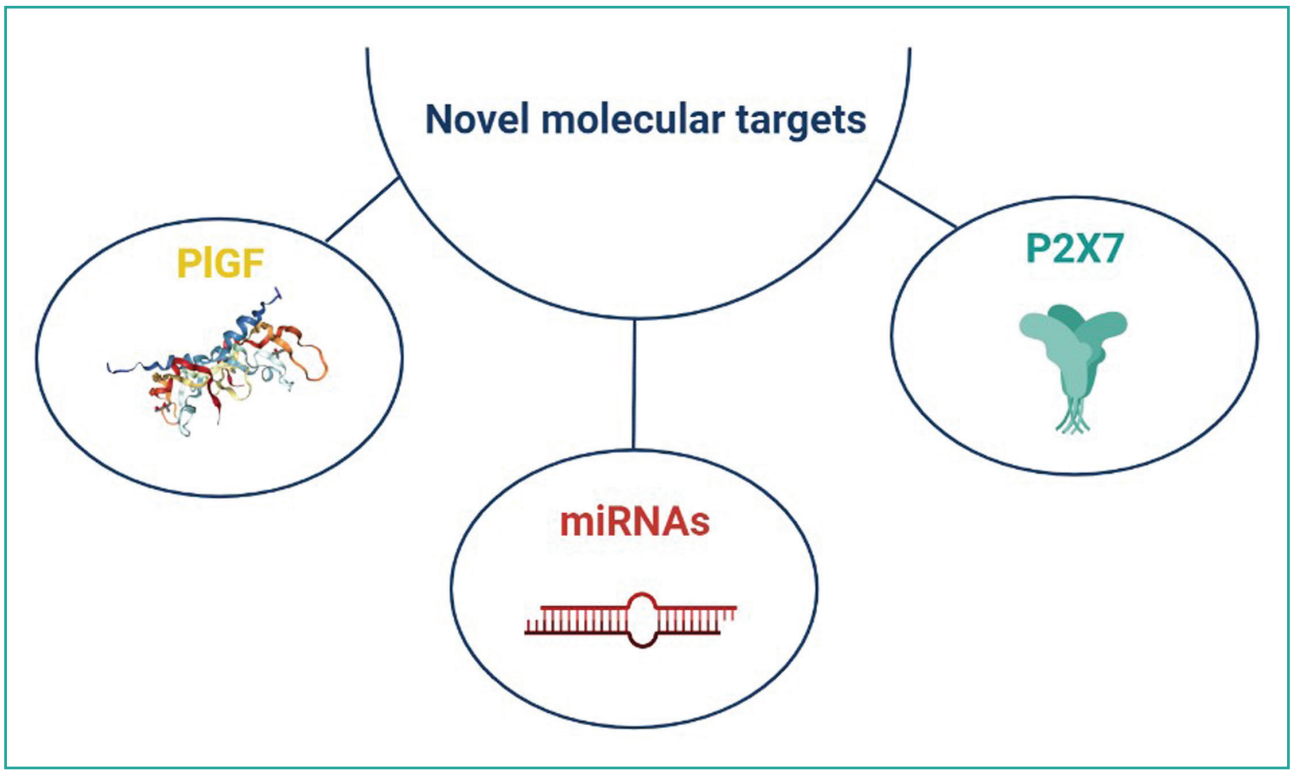

Figure 4. Novel molecular targets for the treatment of diabetic retinopathy.

NAs potentially related to PlGF would address this issue.

P2X7 receptor

In the last decade an important link between DR and purinergic receptor has been demonstrated, considering P2X7 receptor as a putative pharmacological target in this retinal disease (149-151). P2X7R is a member of the family of purinoceptors, ligand-gated membrane ion channels activated by extracellular ATP. This receptor is widely distributed in all retinal layers and also in retinal microvasculature. P2X7R stimulation promotes a wide range of cellular responses, ranging from proliferation to cell death, from cytokines release to reactive oxygen species (ROS) production. The early up-regulation and activation of P2X7R has been related to several types of retinal diseases, and its antagonism revealed benefits against inflammation, oxidative stress and angiogenesis, both *in vitro* and *in vivo* studies (152-156). In particular, the selective antagonist of this receptor (JNJ47965567) has shown anti-inflammatory effect, through the decreased activation of inflammasome and IL-1β production, in several pathological conditions. Moreover, P2X7R inhibition up-regulated the expression of junction proteins in the iBRB, which is compromised in early DR (156, 157). Furthermore, it was found a significant activation of P2X7R during retinal hypoxia, and the P2X7R blockade, by selective P2X7R antagonists A740003 and AZ10606120, inhibited the HIF-1α and VEGF-A retinal overexpression (151, 158).

CONCLUSIONS

Increasing evidence suggest that retinal neurodegeneration and inflammation are implicated in the pathogenesis of diabetic retinopathy. Several recent studies were carried out to explore new pharmacological targets, potentially able to counteract retinal neurodegeneration and inflammation. The present review summarizes new hints and puzzle pieces about the etiopathogenesis of DR, addressing several hypotheses and trying to identify and validate novel and promising pathways implicated in this pathology. Currently, the first-line pharmacological therapy for PDR and DME is represented by intravitreal injection of anti-VEGF agents and corticosteroids, respectively. However, the current proliferative diabetic retinopathy pharmacotherapy is characterized by frequent, invasive and expensive treatments, that have a significant impact on health system. There are several unmet medical needs in the management of DR that stimulate the pharmacological research to develop novel pharmacological targets and drugs to counteract the early phases of diabetic retinopathy.

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Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

All the authors contributed equally to conception, data collection, analysis and writing of this paper.

Availability of data and materials

N/A

Ethical approval

N/A

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ORIGINAL ARTICLE

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PRICING FOR MULTI-INDICATION MEDICINES: A DISCUSSION WITH ITALIAN EXPERTS

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SUMMARY

Negotiating the prices of drugs with multiple indications presents important challenges that impact a range of stakeholders and can result in delayed patient access to potential life-saving treatments. A broad range of stakeholders was recently assembled by the UK Office of Health Economics to work toward consensus on the challenges and solutions which promote better patient access and sustainable health care and innovation. The expert panel considered that differentiating payment based on value at the indication-level represents an important part of the solution for multi-indication therapies, for which implementation must recognise divergent country health systems and experience. Considering this conclusion the present paper represents a multi-step project developed by an Italian Board of experts with the primary aim of driving a deeper understanding of the critical issues and solutions relating to access to multi-indication therapies in Italy. By starting form evidence on the positive impact of value-based differential prices on innovation and access to innovative medicines, the experts discussed and defined the specific features of payment models that could be implemented in the Italian context.

Key words
Multi-indication medicines; value, pricing; innovative drugs.

Impact statement

- Negotiating the prices of drugs with multiple indications presents important challenges.
- In the case of extensions of indication throughout a drug lifecycle, it is necessary to perform a specific evaluation applying the principle of value-based pricing.

- Value-based pricing implies that a value framework is agreed, using a multi-inclusive approach, that value is measured, and that a decision-making path that converts a value into a price is defined.
- Value-based pricing managed per indication through specific entry agreements should be integrated and corrected with a price/volume approach every time the new indication extends the patient population from orphan/rare diseases to more prevalent ones.

INTRODUCTION

A cardinal feature of many innovative drugs approved in recent years is that they are effective in a plurality of therapeutic indications. This multi-indications pricing poses two critical challenges.

Firstly, while the regulatory processes proceed, it is usually questioned whether it would be preferable to have a single price for all indications or adopt differential prices for the same medicinal product in its different indications (1). In this respect, various strategies have been suggested for indication-based pricing:

- other brands or different list prices for each indication;
- a "blended" price, obtained as a weighted average (by volumes) of the prices appropriate for the other indications;
- a single list price with differential "adjustments" of the net prices, aligning them to a value- based payment model per individual indication.

Furthermore, value-based pricing for medicines, aimed at determining the price of a drug on the grounds of its added value, is generally well accepted (2, 3), but raises at least two issues: whether the added value should consider dimensions other than the primary endpoints employed in the drug registration trial (*e.g.*, convenience to patients or organizational impact on health care organizations) and which perspective should be used to calculate the net added cost generated by a new medicine. Secondly, a new indication expands the target population, thus challenging budget constraints: a price-cut is expected and often required by payers. This price cut depends on the new target population's dimension, but the health care system's budget impacts considerations.

A recent expert consensus report, published by the OHE (4), has investigated policies implication of indication-based pricing. Evidence on the positive impact of value-based differential prices on innovation and access is provided. More specifically, the international experts identified some examples of how to price flexibilities (or lack thereof) have:

- influenced the availability of therapies in individual countries, suggesting that better recognition of value at the indication-level may be associated with improved breadth and speed of access;
- incentivized innovation.

Three main elements characterize Indication-based pricing in Italy. First, when a new indication is approved, companies are asked to submit a full price and reimbursement dossier. Hence, a further indication is considered as a new medicine. Second, among the models mentioned above for indication-based pricing, a single list price with adjustment of net price aligning value-based payments per individual indication has prevailed so far. This choice has been supported by (i) the existence of web-based regulatory certified drug registries that allow tracking medicines use per indication and (ii) a diffused application of hidden discounts and managed entry agreements that permit different prices per indication to be charged to the National Health Service, keeping the public price the same across all indications (5, 6). Third, a price-cut is often requested due to price/volume considerations.

OBJECTIVE AND METHODS

A multi-step project was developed by an Italian Board of eight experts, from different pro-

fessional backgrounds, with the primary aim of discussing the critical issues posed by multi-indication medicines and payment models currently available for their reimbursement.
The experts were requested to express their opinions on what could best address critical issues and, ultimately, offer recommendations and solutions on recognizing differences in value across the different approved uses of a drug. To drive a deeper understanding of the critical issues and solutions relating to access to multi-indication therapies, each one of the eight experts participated in a personal interview followed by a written report approved individually. Finally, in an online Board meeting, the experts discussed the specific features of payment models that could be implemented in the Italian context.

FRAMING THE CRITICAL ISSUES

The first step was identifying the critical issues of the Italian P&R model for new indications.
A general concern was expressed on the presence of spending caps on drugs, which makes the price negotiation anchored only to the budget effects on the pharmaceutical market, disregarding the impact of a drug on other health services and expenditures, such as those linked to the reduction of adverse events, a better efficacy profile and the relevant avoided costs.
A second element is the actual role played by real-world data. It has been highlighted that, despite the emphasis put on their importance, there is no evidence on if and how they are used in the first negotiation (accurate world data on adverse events, available since the product has been already approved for other indications) and re-negotiations (data on effectiveness, cost-effectiveness, and budget impact).
In the past, Italy has successfully adopted a model that published a single public price, with different final net prices, through the use of hidden discounts, financial (*e.g.*, expenditure ceilings by product, price-volume agreements, coverage by companies of the costs of the first cycles of therapy, named cost-sharing in Italy) and outcome-based managed entry agreements (mainly performance-linked reimbursement, called payment by result in Italy) per different indications. However, outcome-based contracts have been increasingly challenged in recent years, and cross-indication discounts or price- cut have prevailed in the negotiation process. The proportion of pure payment-by-result agreements over total Managed Entry Agreements dropped from 55% in 2016 to 52% in 2018 and 44% in 2020 (7). In 2021 and 2002, only payment at results contracts for advance therapies has been signed. Furthermore, in a document signed by the Italian Ministry of Health and by nine other Countries (Draft Resolution aimed at "Improving the transparency of markets for medicines and other health technologies"), the issue of price transparency was raised (*i.e.*, confidential discounts and MEA agreements).
Applying a discount (or price-cut) on all indications when a new one is approved flattens everything, using a purely financial-impact approach, without recognizing the different possible value to each indication. Furthermore, the discount/price-cut is questionably presumptive (*i.e.*, based on estimates of future volumes).
Off-label use can also be seen as symptoms of the problem and evidence that change is needed.

DEFINING CONCEPTUAL ELEMENTS BEHIND THE SOLUTION

There was a consensus that health value must be the driver for defining the price of a drug. This requires to structure of the logic that converts such value into price models. Value-based pricing from an operational viewpoint implies that a value framework is agreed, that value is measured, and that a decision-making path that converts a value into a price is defined (2, 8).
Regardless of the final model to be adopted, indication-specific value-based price-set-

ting and reimbursement decisions should be agreed upon.

Current value frameworks need to be adapted to specific situations

It would be important starting for a broader definition of the value of a drug (9) considering the unmet medical and social need, the added value (and the quality of the evidence supporting its), including different dimensions such as the clinical impact and patient-reported outcome, implications for caregivers and the general impact on clinical pathway and health care organizations.

Value frameworks and economic impact

On the one hand, the actual value of a pharmaceutical innovation depends on its long-term incremental benefits and net incremental costs (*i.e.*, on value for money). On the other budgetary constraints cannot be denied, and the impact on a budget should be the second pillar of evaluating the economic impact.
Impact on health care costs, and, if possible, costs in the perspective of the society, should be considered in such an economic assessment. It would also be necessary to assess the value over the entire life cycle of the drug, systematically collecting real-world evidence data to re-assess the value as these data are generated, confirming the original assumptions or modifying them in any positive or negative direction (10). Actual world data must be collected using electronic health records following the regulatory authorities' specific requirements and standards. Optimally, and to minimize additional administrative effort, these would be facilitated by data extraction from routinely collected datasets (11). Mainly, it must be done compliant with data protection regulations.

Value-based pricing requires identifying an applicative model

Value-based pricing has been applied according to two models: (i) direct models driven by cost-effectiveness (mainly applied to the incremental cost-effectiveness ratio - ICER); and (ii) indirect, multi-attribute models characterized by greater discretion on the integration among the different value domains and the consistency evaluation between costs and value. In Italy, the second has prevailed so far, but the role of cost- effectiveness in the negotiation of prices should be better clarified.

Pricing and reimbursement decisions should recognize the uncertainty on evidence at market launch and the impact of medicines in real life

It is necessary to reconsider the benefit of pricing and reimbursement models that are outcome-based and adaptive. Conditional reimbursement mechanisms (*i.e.*, performance-linked reimbursement) of outcomes-based payment can improve the final allocation result, especially in the case of high uncertainty on the impact of medicines at market launch. Indeed, in the case of an outcomes-based mechanism, the reimbursement can be made conditional on actual therapeutic results as certified from real-world- evidence data registries (12).

IMPLEMENTING A PRICING AND PAYMENT MODEL FOR MULTI-INDICATION DRUGS

General recommendations (*i.e.*, applicable to any price negotiation)

Value-based pricing implies that a drug's value for money mainly drives prices and that the impact on budget (sustainability) is a second-order variable of price regulation. In general, and in the case of extensions of indication throughout a drug lifecycle, it is necessary to perform a specific evaluation applying the principle of value-based pricing. This assessment must respect a logical chain of drivers. First, there is the demonstration of value. For each patient under treatment, there should be an assessment of the consistency between value and cost (value for money) and, finally, consistency between the impact on bud-

get and the available funding for the National Health Service.

The added therapeutic value must be appropriately considered in the price negotiation process. It is necessary to define an actual evidence-based ranking for the additional therapeutic value that instructs the application of a premium price. This same approach should also be used when there is an extension of indication. A price negotiation process is much easier when a point scale of additional therapeutic value is established to obtain a premium price compared to existing comparators (or standard of care) and, therefore, avoiding an excessive price reduction due to problems of affordability by the health system.

Another relevant aspect is represented by the existing comparators and the reasonable cost. In addition to the value, it is also important to consider the characteristics of the comparators. This issue is also essential when there is an extension of indication as these comparators and having a differential value concerning the new indication of a drug already existing may have a different cost. This aspect must be governed by defining *ex-ante* the standard comparators and trying to understand how to balance the presence of many low-cost comparators and any added therapeutic advantage of the new indication.

Value-based pricing also implies that informative uncertainty on value be considered when prices are set. Outcome-based managed entry agreements should be re-implemented for this purpose, through either a population-based (*i.e.*, post-marketing study to verify the medicine's impact in real life) or a payment-by (or at-)-result contract in which the industry give back money for non- responders (or payers pay only for responders to therapy).

Specific recommendations (*i.e.*, applicable to pricing negotiation for new indications)

Price negotiation should be indication-specific. Generally, it is not always possible to envision a single contextual negotiation for the different indications of a drug that will be launched in the future, as very often the timing between one indication and another is different, spanning even several years, and there are a series of variables that are only partially predictable. Therefore, it is usually, although not always, more efficient to use individual negotiations for each indication unless the other new indications are expected to be discussed/approved very soon. Notwithstanding, separate negotiations for each indication should rely on the transparent sharing, between the HTA/payers' authorities and the pharmaceutical industry, of the horizon-scanning regarding the possible arrival of future indications.

When a new indication is approved, a choice must be made between two main approaches to convert the value into a pricing model. As mentioned before, pricing a further indication on value grounds can be mainly performed through two main courses (the third solution, *i.e.*, having different brands for different indications and prices per brand, is very rare). The first involves establishing a different price for each indication and, consequently, negotiating possible various managed entry agreements only when certain conditions are met or when there is significant uncertainty about the value of the drug (*i.e.,* single list price with adjustment of net worth to align with value-based payment per individual indication). The second approach involves redefining the price based on the weighted average value: when there is a new indication, the price for all indications of the drug about the weighted average value must be renegotiated (*i.e.,* "blended" price, obtained as a weighted average of the costs appropriate to the different indications and the volumes associated with each indication).

The first approach would be preferable as:

- reflects more the value per indication, while the blended price requires a complex assessment of the weighted average value (where the 'weights' could be based on the size of the target population, which is estimated for the new indication, whereas

for the indications already approved in the past there should evidence on the number of patients treated);
- makes negotiation more flexible: if, for example, for the new indication the added therapeutic value compared to comparators is low, there are no significant uncertainties about this value, and the impact on other costs is limited, a discount can be negotiated on the price valid for that indication, not affecting the other indications.

However, this approach is undoubtedly more complex to manage as it requires usage tracking by indication. Thanks to the drug registers, this is possible in Italy, but these registers have an administrative burden.

Price/volume should be a second-order driver of price negotiations. The price/volume logic (increased discounts due to increased volumes) should also be considered. This should occur when the extension of the indication provides for the transition from a minimal target to a more prevalent disease, considering, however, that if the effect of the launch on the new indication is the replacement of another therapy, the problem is more minor.

Finally, cross-coverage of multiple indications in the same patient should be considered. Sustainability assessment also requires considerations of specific situations. In the same patient, comorbid conditions are treated with a multi-indication drug; there is a cross-coverage of indications on the same individual. Such cross-coverage represents an economic advantage for the Payer System that must be considered when calculating the budget impact.

CONCLUSIONS

In recent years, due to advancements in biotechnology, agnostic targets definition, and the development of trans-nosography pharmacology, many pharmaceutical products are efficacious in multiple indications. However, the degree of such effects could differ substantially.

The price and reimbursement conditions should be re-negotiated every time a new indication is approved. These re-negotiations are challenging to prospectively manage, *i.e.*, capturing in one *ex- ante* model through a horizon scanning activity all new indications, expected to be approved in two/three years. The current tendency to enforce automatic mechanisms (*i.e.*, pre-defined price-cut based on expected volume increase) has proven to have serious flaws preventing the development of new indications. New models based on selective value considerations per further indication approved have now been discussed worldwide.

In general, value-based pricing implies that value frameworks are adopted, using as much as possible, a multi-inclusive approach, which computes pre-specified value expected metrics and, by looking at the total economic implications, weighs in value-for-money and budget impact as a first- and second-order criteria respectively.

There are two models for pricing new indications according to value: (i) establishing a different price for each indication on the grounds of outcome value, through discounts and managed entry agreements (mainly of the payment by result type) or (ii) redefining the price based on the weighted average value of the product for all indications. The first approach, despite it, requires maintaining regulatory and payer-certified drug registries. It is preferable to reflect the value per single indication, making subsequent negotiations more flexible. On the other hand, a single price reimbursement system per product would be rigid and unable to adapt to the evidence reflecting the effectiveness of various indications. In addition, patients could be delayed or denied access to drugs in a product- based single pricing and reimbursement model. At the same time, sponsors are discouraged from investing in developing other promising indications.

At the same time, value-based pricing managed per indication through specific entry agreements should be integrated and corrected with a price/volume approach (budget impact consideration) every time the new indication extends the patient population from orphan/rare diseases to more prevalent ones.

Regardless of the existence of outcome-based agreements, it's essential assessing the value over the entire life cycle of the drug per every single indication, systematically collecting real-world evidence data to re-negotiate the value as these data are generated, confirming that the original assumptions were correct or modifying them by increasing or decreasing the reimbursement in agreement with the data collected at least every other year or until the market access penetration and equilibrium have been completed.

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Authors' contribution

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The data underlying this manuscript are available in the article.

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N/A

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