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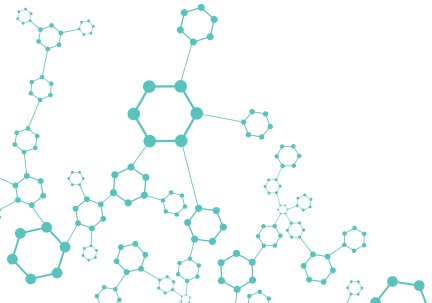

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INTERVIEW

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INTERVIEWS PhD AWARD

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As readers certainly remember, the **Italian Society of Pharmacology (SIF)** published a call asking recent PhD graduates to submit a review article based on their thesis. This initiative was well received and we congratulate the winners. The possibility exists that SIF will make this a tradition and we encourage future graduates to start thinking of a suitable paper and submit it to PharmAdvances. In addition to facilitating a cash prize, single-author papers are valued by funding bodies and provide some competitive advantages when it comes to obtain funds (read the interviews to see how this is a major hurdle in scientific research).
For this issue of PharmAdvances, we interviewed the winners of the PharmAdvances PhD awards, namely Drs. **Francesca Lazzara** (FL) (1), **Amer Ahmed** (AA) (2), **Paola Brivio** (PB) (3), and **Chiara Colarusso** (CC) (4). In particular, we wanted to learn about their experience in the lab and what obstacles they had to overcome.
Their answers are very instructive and should help us shape a better university and education system.

1 | What is your scientific background? What did you study?

FL: I graduated in biology and, thereafter, I've attended laboratories of pathology and immunology. In 2016 I started my experience in the Lab of ocular pharmacology in the department of Biomedical and Biotechnological Sciences (University of Catania). In 2020 I finished my PhD in Neuroscience.

AA: My background encompasses Biochemistry, Biotechnology, and Pharmacology. I finished my Bachelor and Master degrees in Biochemistry and PhD in life sciences (research area: Vascular Pharmacology).

PB: I studied Pharmacy at the University of Milan and I conducted the internship for my master thesis in the laboratory of "*Psychopharmacology and Molecular Psychiatry*" directed by Professor Marco Andrea Riva at the Department of Pharmacological and Biomolecular Sciences of the University of Milan under the supervision of Professor Francesca Calabrese. I graduated in July 2015 with a thesis entitled "*Exposure to the chronic mild stress, in rats, alters the molecular mechanisms activated in response to a cognitive test*". In October 2015 I started the PhD program in Experimental and Clinical Pharmacological Sciences at the University of Milan and I defended the Ph.D. thesis entitled "*Stress exposure as risk factor for psychiatric disorders: from functional characterization to pharmacological intervention*" in December 2018.
Since 2019 I'm a post-doctoral fellow in the laboratory of Experimental Pharmacology of Professor Fabio Fumagalli. Since I have started to work in science, the main purpose of my research has been to investigate the effect of stress exposure during adult life, by focusing on molecular mechanisms responsible for the dif-

ferent outcomes of stress response in the central nervous system.

CC: My scientific area of interest is to understand how lung inflammation fosters chronic lung diseases up to lung cancer. A particular interest concerns the role of the inflammasome, a multimeric complex that we proved to be involved in COPD-, pulmonary fibrosis- and lung cancer-related inflammation. During my PhD training I found that the inflammasome is at the crosstalk between chronic obstructive pulmonary disease (COPD) and lung cancer.

2 | What is the key message of your paper?

FL: Basically, the key message of this manuscript, and of my whole PhD thesis, is that pathological conditions, in my case diabetic retinopathy, can be characterized by novel and uninvestigated pathological mechanisms, that can be addressed after discovery and validation of druggable pharmacological targets. In particular, my thesis and this manuscript aimed at highlighting new putative pharmacological targets characteristic of the early stage of diabetic retinopathy.

AA: The key message of my paper, published in PharmAdvances, could be summarized as "Flavonoids consumption is associated with undoubtable beneficial effect in the cardiovascular context; this effect is the result of the pleiotropic mechanism of these valuable products of nature. These compounds exert anti-obesity effect, protect against hypertension development, ameliorate hyperlipidemia, and slow down the progression of diabetes and atherosclerosis".

PB: The key message of my paper "*The multifaceted aspects of stress*" is the fundamental need to study the consequences of stress exposure since it is one of the main environmental factors for developing psychiatric disorders. Moreover, it is necessary to pursue the research in this field to unravel the molecular mechanisms that may be at the basis of resilience for the study of novel pharmacological treatments to promote resilience.

CC: This paper highlights that the activation of a specific inflammasome receptor, AIM2, could be at the crossroad between COPD and lung cancer by acting as one of the orchestrators for the establishment of lung cancer in smokers. Therefore, we believe that this is a novel scientific approach for COPD patients that develop lung cancer, focusing on the biology of the AIM2 inflammasome as a potential pharmacological target which could on one side represent a diagnostic tool to early prevent COPD patients to develop lung cancer, and on the other side could open new therapeutic perspectives.

3 | If you had plenty of money, what would you study next?

FL: I would like to continue studies relative to several uninvestigated pathological mechanisms of retinal diseases. In particular, I would like to investigate further the role of specific angiogenic factors, which are the main protagonists of retinal degeneration (PlGF or the different isoforms of VEGFA). As everybody knows, researcher's activities and studies are costly, and my first purpose it would be to invest money in innovative lab equipment, in order to keep us at the forefront of the pharmacological research.

AA: If I had more money, I would continue my PhD work to investigate the long-term or chronic effect of flavonoids treatment on perivascular adipose tissue (PVAT) function. I would study the protective effects of flavonoids toward PVAT function in some diseases such as hypertension and obesity.

PB: In these years of academic research, I've increased my passion in science for the multifaceted effects of stress exposure, from the positive and negative consequences of stress at behavioral level to the molecular basis of stress. Hence, if I had plenty of money, I would employ novel and advanced techniques to better dissect the mechanisms altered in specific brain regions for the development of novel therapeutic strategies. Moreover, I would combine the results obtained at preclinical levels with collaborations with clinicians to identify innovative and specific targets for more effective interventions to treat stress-related disorders.

CC: I would like to further explore the molecular/cellular mechanisms involved in chronic lung inflammation at the basis of pulmonary diseases such as COPD, pulmonary idiopathic fibrosis, and lung cancer. In particular, I would focus on the inflammasome-dependent pathways puzzling from the process of lung cancer establishment up to progression that occurs after therapeutic treatment. In this regard, another goal I would like to reach is to understand cellular and molecular mechanism/s at the basis of immune checkpoint inhibitors' resistance in lung cancer patients and try to identify predictive and/or prognostic biomarkers able to define diagnosis of disease, treatment, efficacy assessment and disease progression. To achieve all these goals, I would like to take advantages of innovative experimental approaches, such as the spatial biology.

4 | In your opinion, what are the major obstacles in post-doctoral research?

FL: I think that all Italian PhD students and post-doc know well which is the main obstacles: access to research funds.

AA: In my opinion the major obstacles are funding and their scarcity.

PB: I believe that the major obstacles to post-doctoral research in Italy are the low number of grants to which post-doctoral fellows can apply to have money for conducting independent research and build their own group, the high level of competition in academy due to the few positions available with respect to the number of post-doctoral fellows and the precarious contracts and the low salaries in comparison to the other graduates who work in companies.

CC: Currently, I believe that the major obstacles in post-doctoral research are, on a hand, the poor financial support and resources and, on the other, the instability and uncertainty that characterize a post-doc researcher's life.

5 | What was the major challenge (technical, budgetary, *etc.*) you had to face in your own research?

FL: Maybe during PhD studies everything seems difficult. The main problem is the learning process: theory & practice. Students have to manage and balance time their own weakness and strength, in order to be productive and mentally healthy.

AA: The major challenge I faced in my PhD research was related to the approach I used to tackle my PhD research question. I have used a single classical pharmacological approach to investigate the modulation of flavonoids vascular reactivity by perivascular adipose tissue. I wanted to use in vivo experiments and or molecular approach to validate my result obtained by the classical pharmacological approach. This challenge also meant that I had less training during my PhD. This challenge was related to funding and bureaucracy re-

lated to the work with animal models as well as this challenge was in part caused by Covid-19 pandemic.

PB: In my opinion, the major challenge my colleagues and I had to face is the lack of personal budget to start conducting independent research. I believe that more calls for proposals directed to restricted groups or with specific thematic could increase the possibility to win a grant.

CC: The major challenge was to face a research project by using residual financial resources trying to obtain the more I could out of the planned experiments.

6 | What did your mentor teach you in addition to the scientific method?

FL: Scientific rigor and diplomacy.

AA: He taught me to be calm and cool when things are not going well in research.

PB: Since I started my journey in science, my mentor has passed to me the passion for this work, and the desire to continue research to reach my objectives in the field of neuroscience. Moreover, she has given me the possibility to join national and international congresses to share my data and to meet other young scientists to discuss about neuroscience, thus teaching me the importance of the attendance to these meetings for my personal growth thank to the face with other neuroscientists.

CC: My mentor taught me that in work, as well as in life, respect for the others and for the rules, honesty, humility, learning, listening to others and not being afraid to express your opinions are important. My mentor transmitted me the passion for scientific research and the importance of struggling to achieve a goal. This was for me a lesson of professional and personal growth, and of making mistakes to improve yourself.

7 | Should you become a mentor yourself, what would you tell your students upon joining your lab?

FL: Don't be too sure of yourself; always challenge yourself and be humble. Work and make sacrifices, that's the only way to get results.

AA: Yes, I wish to become a mentor and I will tell my student upon joining my lab "do not delay what you can do today until tomorrow, manage your time effectively, work as hard but wise as possible, do not try to do all things together, have enough time to work, read, relax and sleep".

PB: During these years in academy, I have had the opportunity to be the tutor of several students during their master thesis internship. I hope to have passed onto them (and as mentor I will do the same) not only my passion for this work but also the importance to work with passion and respect.

CC: My mentor taught me that in work, as well as in life, respect for the others and for the rules, honesty, humility, learning, listening to others and not being afraid to express your opinions are important. My mentor transmitted me the passion for scientific research and the importance of struggling to achieve a goal. This was for me lesson of professional and personal growth, and of making mistakes to improve yourself.

8 | If you were the Ministry of Research, how would you distribute funds?

FL: An equal distribution between Italian universities. Grades and scores assigned

to universities can be influenced by factors (*e.g.* environmental, economic) that create disparities between universities located in the northern and southern parts of Italy.
AA: I would focus the funds distribution towards more basic scientific research in medical and life sciences and toward innovative technologies in engineering and industrial sectors.

PB: If I were the Ministry of Research, I would distribute funds by opening several applications for the young scientists divided for specific themes and directed to more closed groups (for example, 1-2/3-4 (and so on) years after the PhD) to balance the curriculum vitae of the candidates and to increase the possibility of younger to win the grants.

CC: I would try to distribute funding in order to reduce the gap between the northern and southern universities, and between small and large universities. I would give more funding to applied research, and in the field of pharmacological research, to the basic one, and I would provide an increased number of grants pointing at increase investment in junior scientists.

9| What are you planning to do in the future?

FL: I hope to continue my post-doc in the field of ocular pharmacology, specifically on retinal function in *in-vivo* model of retinal degenerative disease. After that, "what's meant to be will be".

AA: In future, my plan is to pursue some postdoc research training (3-4 years) in order to enhance my skills and technical expertise, after which I would like to apply for some fixed-term or permanent positions and to establish my own research lines.

PB: I hope to continue my career in neuroscience and to conduct my independent research in the field of studying the effects of stress during the different phases of life.
CC: I'm planning to continue my research work by further enriching my cultural background, by expanding my knowledge in the pharmacological research field, by discovering innovative experimental approaches. I would like to learn about novel scientific discoveries and integrate them with my ideas, confront myself with leading researchers/scientists across the world in order to boost my career.

10| Is there anything you would say to undergraduates?

FL: Study, be patient and try to understand exactly what you want to do after graduation.

AA: Yes I would say them "find your interest intellectually, try to be creative with it, seek help whenever you need, do not plan to go for higher studies because it is not the best choice if you think about making money and enjoying life with family and friend, try to establish your own enterprise, higher studies do not suite everyone, academic life may compromise your happiness and enjoyment at several stages of life, and if mandatory that if you opt for higher studies you are mentally prepared for it".

PB: Even if is not an obligatory step of your studies, join the laboratory of research of your faculties to know this wonderful world!

CC: As stated by Steve Jobs '*The only way to do great work is to love what you do… Have the courage to follow your heart and intuition. They somehow already know what you truly want to become'*

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RESEARCH ARTICLE

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FASTER ABSORPTION OF IBUPROFEN LYSINATE THAN STANDARD IBUPROFEN ACID IN PEDIATRIC POST-SURGICAL PAIN

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SUMMARY

Surgery represents one of the most painful events that a child may experience. Advanced pharmaceutical formulations, including salts of ibuprofen, were developed to provide faster drug absorption and rapid onset of analgesic effects. The aim of this pilot study was to evaluate the rate of early drug absorption of ibuprofen lysinate (Algidrin® Pediatrico, FARDI S.A., Barcelona, Spain) compared to standard ibuprofen (MomentKid®, Aziende Chimiche Riunite Angelini Francesco, Rome, Italy) in children receiving the drug for the treatment of post-surgical pain.

Twenty-one children (4-16 years) were enrolled in a randomized, open-label, controlled, pilot study. Patients were randomly assigned to the experimental-group (LYS-group, n = 10, treated with the lysinate formulation after surgery) or the standard-group (STAND-group, n = 11 treated with standard ibuprofen formulation). Four blood samples (immediately before and 5, 15 and 20 minutes after the oral administration) were collected 24-hours after starting ibuprofen; pain (faces pain scale) and vital parameters (heart rate, blood pressure, oxygen saturation) were also considered.

Patients from the LYS-group had significantly higher ibuprofen concentrations at 5 minutes after drug intake compared with those from the STAND-group (11.9 ± 8.6 *versus* 3.6 ± 3.6 mg/L, p = 0.010), with the same trend for all other pharmacokinetic parameters. Remarkably, ibuprofen basal concentrations, were more than doubled in the LYS- *versus* STAND-group (5.7 ± 7.8 *versus* 2.1 ± 1.0 mg/L, p = 0.141). The LYS-group was also associated with a trend for reduced inter-individual variability in the drug exposure compared with the STAND-group (coefficient of variation of the AUC0-20 min: 52% *versus* 84%. Pain control was also obtained.

The use of ibuprofen lysinate was associated with an early fast absorption and reduced pharmacokinetic variability compared to the traditional ibuprofen acid formulation, supporting fast action and an improved clinical response to mild-moderate post-surgical pain in children.

Key words
Ibuprofen lysinate; Ibuprofen; children; post-surgical pain; pharmacokinetic.

Impact statement
Ibuprofen lysinate presents faster absorption and reduced pharmacokinetic variability compared to standard ibuprofen in children.

INTRODUCTION

According to the revised definition provided by Williams and Craig in 2016, pain is a "distressing experience associated with actual or potential tissue damage with sensory, emotional, cognitive and social components" (1). Pain, among all the symptoms, undermines physical and psychological integrity of the subject who is experiencing it, affecting, at the same time, also family and caregivers. This applies especially to pediatric population, in which pain is a frequent finding. In fact, up to 80% of hospital admissions into pediatric departments are due to pathologies which involve pain as a symptom.

Despite the tremendous advantages in knowledge about pain reached in the last decades, this condition is still frequently under-recognized and thus inadequately treated in children. It is now widely known that there is no age limit to perception of pain, as the development of anatomic substrates required for pain transmission occurs mainly during fetal life (2, 3).

The Italian Health Ministry in 1995 issued a document about correct pediatric pain management, in which is stated that "pain should always be assessed and treated whenever there are signs and symptoms of its presence, even if the child does not verbally express his discomfort, and when possible, with a prophylactic approach" (4).

In particular, in case of predictable onset of pain, such as in the postoperative period, it must be prevented with adequate prophylaxis. Children who underwent surgery may not feel pain immediately after awakening from anesthesia, but this should not discourage clinicians from starting a pharmacological pain prophylaxis. In fact, analgesic therapy should be administered "by the clock" and not on demand (5).

In pediatric age, paracetamol and ibuprofen are the first-choice drugs to treat acute mild to moderate pain.

Ibuprofen, (±)-(R,S)-2-(4-isobutylphenyl)-propionic acid, is a chiral 2-arylpropionic acid derivative nonsteroidal anti-inflammatory drug (NSAID) widely used in the management of mild to moderate pain, fever and inflammation since early seventies acting as is a non-selective inhibitor of cycloxygenase-1 and -2 derived prostaglandin biosynthesis (6).

Ibuprofen free acid is a lipophilic compound with limited aqueous solubility. As dissolution is a key factor in the process of drug permeation through the cellular membranes, poor water solubility may limit drug absorption, ultimately delaying systemic bioavailability and in some cases restraining it. Therefore, one common way pursued to improve aqueous solubility and dissolution rate of a drug without changing its chemical structure and biological properties is by the formation of salts with the conjugate acids. This concept well applies to ibuprofen, due to its carboxylic acid moiety. Indeed, although the free acid formulation is still largely used and prescribed worldwide, different salts of ibuprofen have been introduced on the market in the past few years as gastrointestinal absorption enhancers, with the goal to improve ibuprofen absorption in terms either of higher peak of drug levels (C_{max}) and faster time to reach maximum concentration (T_{max}) compared with the conventional formulation (7). Testing alternative ibuprofen formulations, with rapid absorption and improved oral bioavailability may, therefore, be useful to obtain a more efficient acute pain control.

Limited data is available on the lysinate formulation of ibuprofen. In 2015 Ferrero-Cafiero et al evaluated the bioavailability of pediatric suspension of lysinate ibuprofen compared to pediatric suspension of standard ibuprofen, in healthy, adult volunteers and found that the rate of absorption of the ibuprofen lysinate suspension is quicker and less variable than that of the ibuprofen base reference suspension and it exhibits a shorter T_{max}, which is of particular interest for achieving a rapid and homogeneous analgesic and antipyretic effect (8). However, to our knowledge, no comparative pharmacokinetic study comparing the early absorption of lysinate ibuprofen and stan-

dard ibuprofen in the pediatric population has been carried out so far.
The aim of the present pilot study was to evaluate the rate of early drug absorption of ibuprofen lysinate compared to standard ibuprofen in children receiving the drug for the treatment of post-surgery pain. The secondary outcome was to evaluate the analgesic efficacy to control post-operative pain of the two ibuprofen formulations.

MATERIALS AND METHODS

Patients

Twenty-one pediatric patients (both genders), aged 4 to 16 years, undergoing surgical procedures at the Pediatric Surgery Unit, Vittore Buzzi Children's Hospital, Milan, were sequentially enrolled in this study. Inclusion criteria included: abdominal or thoracic surgery, operative time < 3 hours, mild (FPS-R: 2-4) or moderate (FPS-R: 4-6) self-reported postoperative pain according to Faces pain scale–revised (FPS-R) (9), no complications of surgery. Exclusion criteria included: chronic illness (including heart failure, kidney disease, inflammatory bowel diseases), history of NSAIDs' related gastrointestinal bleeding, use of any analgesic medication before surgery, hypersensitivity or allergy to ibuprofen or excipients, language barrier.
Participants were recruited between June 1, 2021 and November 30, 2021.
This pilot study was performed in accordance with the Helsinki Declaration of 1975, as revised in 2008. The institutional Ethics Committee approved the study (2020/EM/210). Written informed consent was obtained from subjects' parents or guardians; the assent was also recorded in children and adolescents from 8 to 16 years of age.

Study protocol

This was a randomized, open-label, controlled, pilot study. The two arms consisted of an experimental group, treated with an Ibuprofen Lysinate (Algidrin® Pediatrico, FARDI S.A., Barcelona, Spain, 20 mg/ml oral suspension) formulation (LYS-group) after a surgical procedure and a control group, treated with a standard ibuprofen (MomentKid®, Aziende Chimiche Riunite Angelini Francesco, Rome, Italy, 100 mg/5ml oral suspension) formulation (STAND-group) after surgery; doses administered according to weight as reported in the summary of product characteristics (or in the patient leaflet) of the medicinal product. The flow chart of the progress through the phases of our pilot study is showed in **figure 1**.
The study variables were determined in each patient independently of the assigned group, before and after the experimental intervention.

Procedures and data collection

At admission, a complete clinical examination including weight measurement and detections of the vital signs, including heart rate (HR), blood pressure (BP), oxygen saturation (SpO2) was performed in all enrolled children. All subjects were in good physical condition. Pre-surgery, enrolled children were randomly assigned to the experimental-group, where the lysinate formulation was adopted (LYS-group), or the standard-group (STAND-group) where they received standard ibuprofen formulation as analgesic medication for pain control after surgery. A simple randomization based on a single sequence of random assignments was carried out. In all children, surgery was performed between 8.30 am and 12 am under general anesthesia.
After surgery, all patients were transferred from the operating theater to the recovery room. After a complete awakening from anesthesia, the children received the first weight-calculated dose of one of the two ibuprofen formulations, as an analgesic, according to the randomization list. Administration of the assigned ibuprofen formulation was then continued on an 8-hours interval until 48 ± 6 hours after surgery.
Vital signs, including HR, BP, SpO2 and measure the child's self-reported pain, according

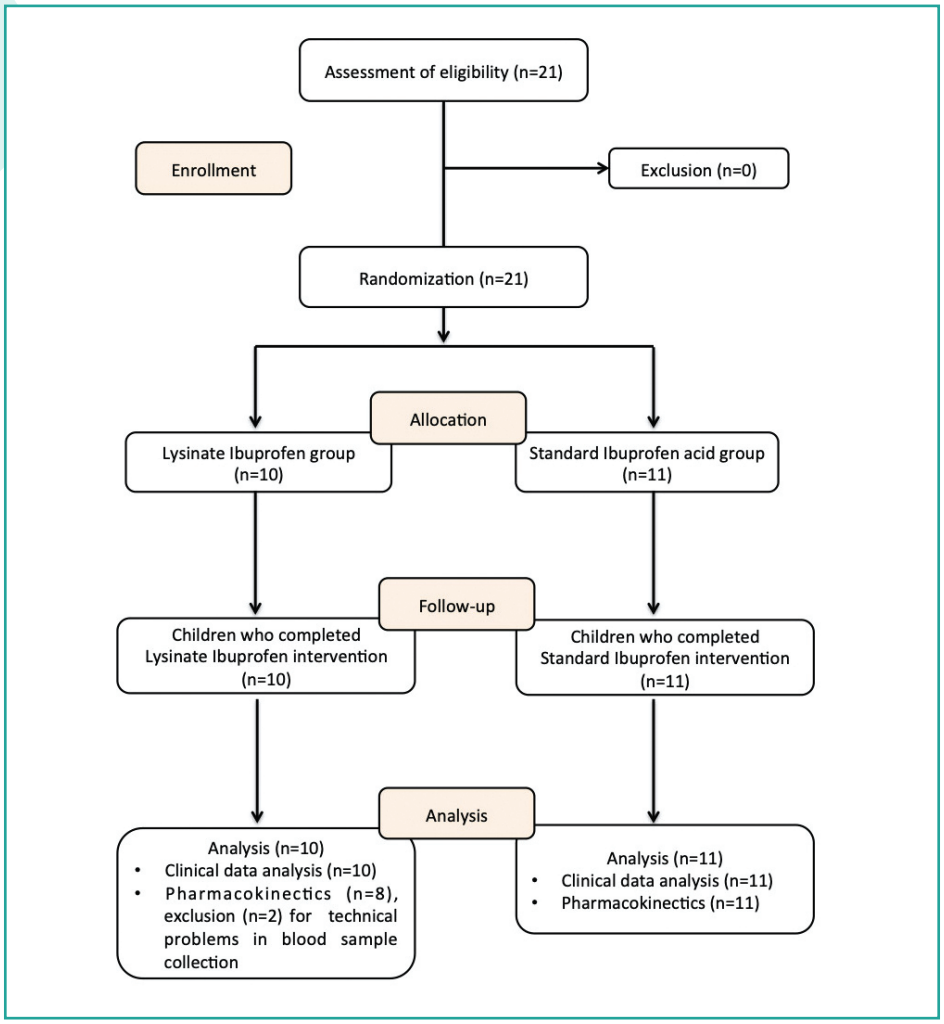

Figure 1. Flow chart of the phases of the randomized pilot study of two groups (lysinate ibuprofen and standard ibuprofen after surgery).

to Faces pain scale – revised (FPS-R) (9) (**figure 2**), were monitored before the first administration of one of the two ibuprofen formulations on day 1 post-surgery, 20 minutes after, and 48 hours after surgery.

Estimation of early ibuprofen absorption

Based on early pharmacokinetic assessments in adult healthy volunteers (8) and with the goal to limit discomforts for the pediatric patients, we decided to focus on the rate of ibuprofen absorption in the first 20 minutes after oral drug intake. In particular, after 24 hours (to ensure steady state conditions) of the three times a day oral administration of one of the 2 ibuprofen formulations, we collected 4 blood samples at 0 (immediately before the administration of the fourth dose) and 5, 15 and 20 minutes after the oral administration for a maximum total volume collected for each individual equal to 8 mL (2 mL for pharmacokinetic evaluation).

Ibuprofen concentrations were quantified by a validated liquid-chromatography tandem mass spectrometry method. The lower limit of quantification was set at 0,5 mg/L. Inaccuracy and imprecisions, tested during each analytical run by internal quality control samples, were in every instance less than 15%.

As the main ibuprofen pharmacokinetic parameters, we considered the basal (trough) drug concentrations, drug concentrations at 5, 10

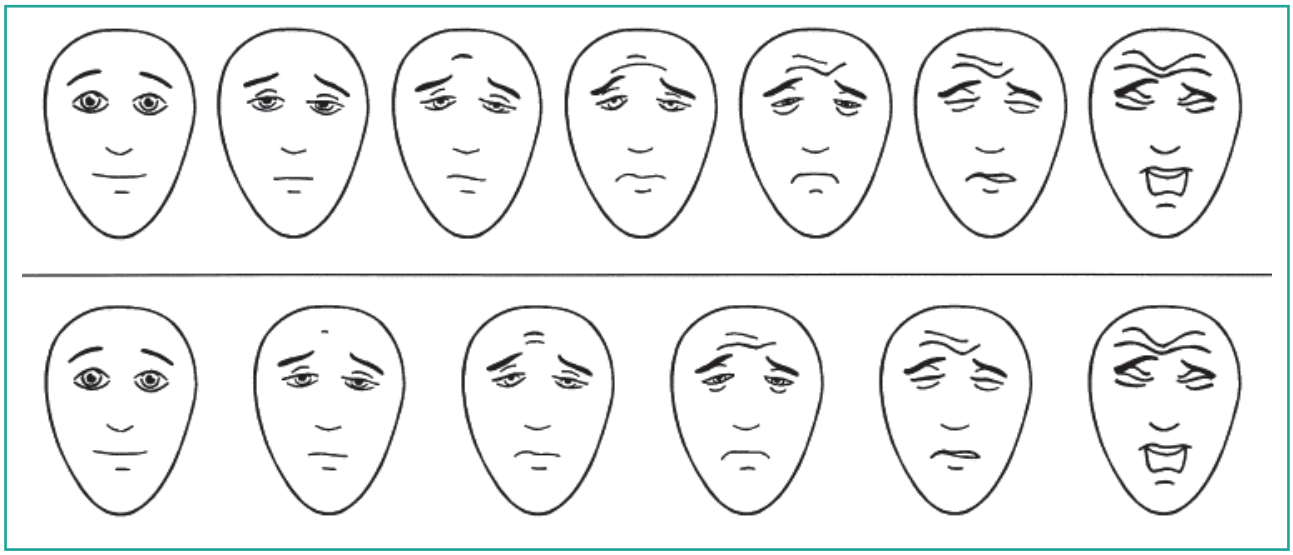

Figure 2. Faces pain scale-revised. Adapted from: Young KD. Assessment of Acute Pain in Children. Clin Pediatr Emerg Med. 2017;18:235-41.

and 20 min after drug intake and the $AUC_{0\text{-}20min}$ estimated using the trapezoidal rule.

Statistical analysis

Continuous variables were described as the mean and standard deviation (SD) or median and quartiles, and categorical variables as counts and percentages. The statistical significance of the continuous variable comparisons was assessed using the unpaired Student's t-test; the comparison of categorical variables was conducted using the chi square test or Fisher's Exact test if there was a small (< 5) expected cell size. A p-value below 0.05 was considered statistically significant. No multiple test correction was applied given the exploratory nature of the pilot study. The data analysis was performed with the STATA statistical package (release 15.1, 2017, Stata Corporation, College Station, Texas, USA).

RESULTS

Clinical data at the enrollment and follow-up

21 children (12 M and 9 F; mean age 10.46 ± 3.51 years; range 6.9-16.7 years), were randomly assigned to one of the two groups: the LYS-group (n = 10) and STAND-group (n = 11). In LYS-group, 9 patients were submitted to abdominal surgery (laparoscopic appendectomy) and one to thoracic surgery (breast abscess surgery); all STAND-patients were submitted to abdominal surgery (laparoscopic appendectomy).
Pre-experimental intervention clinical, demographics and vital signs in the two groups are reported in **table I**; no significant differences for age, gender and vital signs were noted ($p > 0.05$ for all parameters).
Twenty minutes after ibuprofen administration, pain control (FPS 0-2) was obtained in 19/21 (90.4%). At 48 ± 6 hours after surgery, pain control was reached in all but one patient in the lysinate group (with FPS = 4).
As reported in **figure 3**, vital signs remained stable during monitoring, without significant differences between groups ($p > 0.05$). No adverse effects were recorded.

Ibuprofen absorption

Data on ibuprofen pharmacokinetics during the absorption phase were available in 11 patients in the STAND-group and 8 patients in the LYS-group (blood samples collection was not performed in 2 LYS-children due to technical problems).

Features	LYS-group (n = 10)	STAND-group (n = 11)	p-value
Age (years)	11.61 ± 3.3	10.18 ± 1.7	0.223
Gender (M/F)	5/5	7/4	0.397
Weight (kg)	43.6 ± 16.5	38.9 ± 17.6	0.545
Heart rate (bpm)	67.8 ± 7.1	66.7 ± 12.7	0.828
Systolic blood pressure (mmHg)	107.7 ± 12.0	107.0 ± 11.1	0.882
Diastolic blood pressure (mmHg)	60.5 ± 16.6	59.8 ± 12.2	0.910
Oxygen saturation (%)	99.8 ± 0.4	99.5 ± 0.5	0.304
Face pain scale - < 4 - ≥ 4	 7 3	 9 2	0.403

Table I. Clinical and demographic features pre-experimental intervention in Lysinate Ibuprofen group (LYS-group) and Standard Ibuprofen acid (STAND-group).

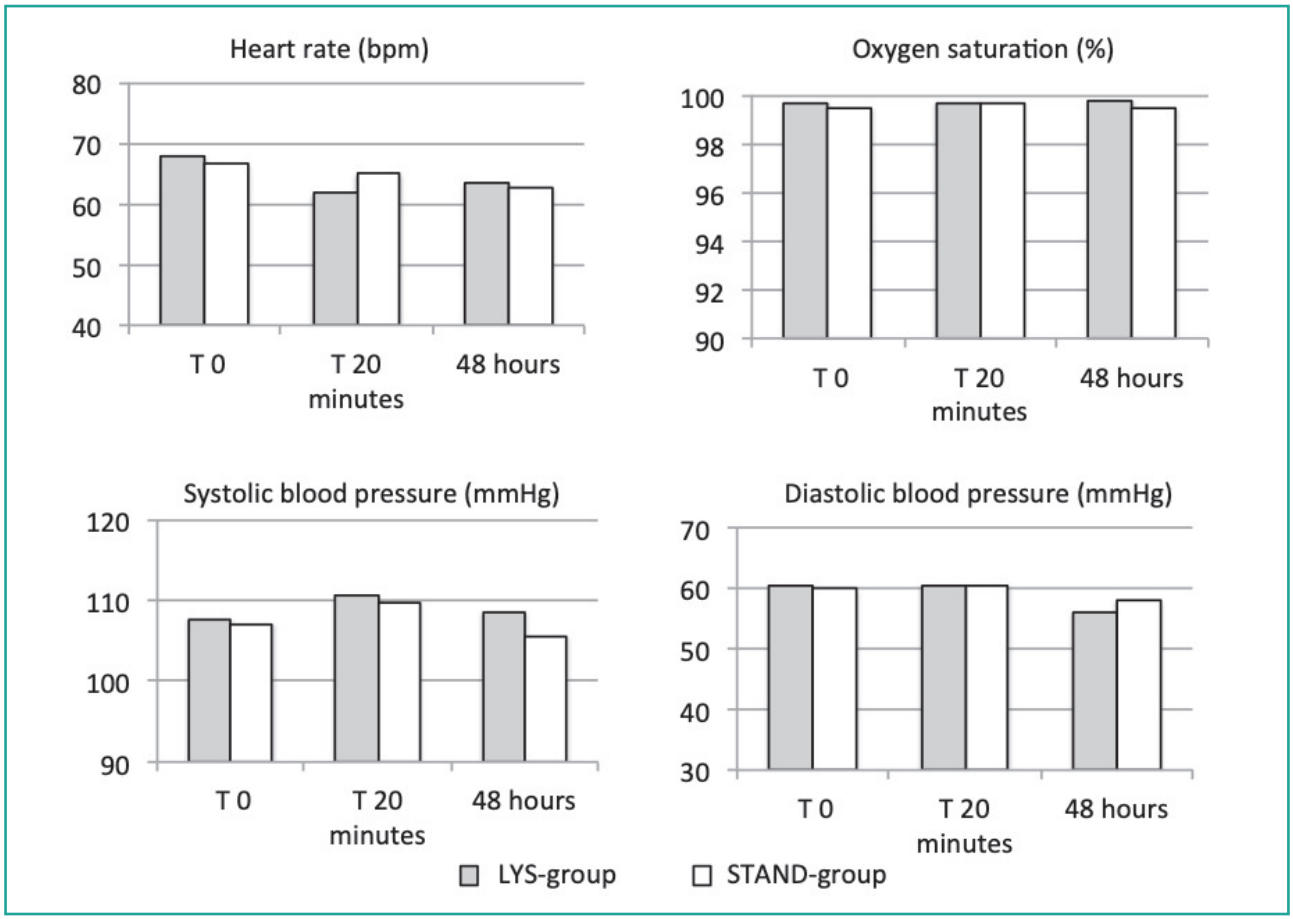

Figure 3. Average values of the vital signs during monitoring in Lysinate Ibuprofen group (LYS-group) and Standard Ibuprofen acid (STAND-group).

As shown in **table II**, pediatric patients from the LYS-group had significantly higher ibuprofen concentrations at 5 minutes after drug intake compared with those from the STAND-group (11.9 ± 8.6 *versus* 3.6 ± 3.6 mg/L, p = 0.010). Although not reaching statistical significance,

Main pharmacokinetic parameters	Ibuprofen lysinate	Ibuprofen acid	P-value
Patients, n	8	11	------
Ibuprofen basal, mg/L	5.7 ± 7.8	2.1 ± 1.0	0.141
Ibuprofen 5 min after intake, mg/L	11.9 ± 8.6	3.6 ± 3.6	0.010
Ibuprofen 10 min after intake, mg/L	17.3 ± 9.0	10.6 ± 10.2	0.153
Ibuprofen 20 min after intake, mg/L	23.0 ± 12.4	20.7 ± 18.0	0.762
Ibuprofen $AUC_{0\text{-}20\ min}$, mg/L*min	318 ± 168	206 ± 173	0.176
Min: minutes; AUC: area under the curve			

Table II. Main ibuprofen pharmacokinetic parameters in pediatric patients treated with ibuprofen lysine *versus* standard ibuprofen base.

the same trend was confirmed also for all the other pharmacokinetic parameters. Remarkably, ibuprofen basal concentrations, that represent the minimum measurable drug concentrations between two consecutive drug doses, were more than doubled in the LYS- versus STAND-group (5.7 ± 7.8 *versus* 2.1 ± 1.0 mg/L, p = 0.141). The LYS-group was also associated with a trend for reduced inter-individual variability in the drug exposure compared with the STAND-group (coefficient of variation of the $AUC_{0\text{-}20min}$: 52% *versus* 84%).

DISCUSSION

Surgery represents one of the most painful events that a child may experience (10). Inadequate pain treatment may lead to short-term consequences, such as prolongation of hospitalization and clinical worsening, but also to long term-consequences, as impairment of pain threshold and increased risk of chronic pain (11). Post-surgical pain is usually managed with multiple analgesics. The appropriate type, delivery and dose of medications depend on the type of surgery and the age of patients. In pediatrics, paracetamol and ibuprofen have been thoroughly described and are widely used in children to treat mild-moderate pain (12, 13). To obtain a correct management of pain, advanced pharmaceutical formulations, including salts of ibuprofen, were developed to provide faster-acting analgesics (14).

In the present observational, pilot study we have evaluated the rate of early drug absorption of two formulations of ibuprofen in pediatric patients requiring analgesic treatment for post-surgery pain and we have documented that the use of the lysinate salt of ibuprofen was associated with a fast absorption in the first 20 minutes after drug intake compared with the traditional ibuprofen acid formulation, confirming previous results in adult healthy volunteers (8). Moreover, our study extends previous findings by showing that the rate of absorption of the ibuprofen lysinate suspension is quicker and less variable than that of the ibuprofen acid reference in a population of pediatric patients at steady state conditions (that is 24-hours after starting ibuprofen treatment). More specifically, we have documented that, once given at the same molar drug doses, patients in the LYS-group at 5 minutes post-dosing had ibuprofen concentrations nearly 3-fold higher that those measured in the STAND-group. Remarkably, this trend was confirmed up to 20 minutes after drug intake.

The main issue with pain management is often the difficulty in evaluating it, especially in younger children. Many different tools and pain scales have been developed to help clinicians to identify pain in pediatric population, either based on observation of the child or on self-assessment, depending on the child's age. Faces pain scale has firstly been developed in

1990 and then revised in 2001 and it is widely used. As used in our study, FPS-R is made up of 6 faces resembling different severity of pain, starting from the first face on the left, corresponding to "absence of pain" and finishing with the last face on the right, corresponding to "the worst pain ever". The scale has been validated in children and has some advantages respect to other self-reported pain scales, as the absence of smiles and tears, that may be associated with misinterpretation of severity of pain with the related emotions. Moreover, the use of this scale has been recommended in clinical trials involving pediatric patients reporting their pain (15). With the aid of these tools, together with clinical information such as vitals and physical examination, pain can be classified as mild, moderate, or severe, and, for each category, appropriate treatment can be started (5).

Even though in our patients a pain control and vital signs stability were obtained, the study was not powered to test potential differences between the two formulations in terms of clinical efficacy. However, consistent evidence is available showing that a clear relationship exists between the maximum ibuprofen concentrations and the peak of analgesic effect or duration of analgesia (16, 17). Consequently, it can be assumed that the use of ibuprofen lysinate – due to the improved absorption – may result in a fast drug action and improved clinical response, especially when ibuprofen is given with the goal to treat acute pain, such as migraine attacks, dental or ears pain. In the only clinical trial published so far, Kyselovic *et al*. have documented that a single dose of ibuprofen lysinate was non-inferior to ibuprofen acid in terms of analgesic efficacy, onset of action, and tolerability in patients who have recently undergone dental surgery (18). However, as indirect support of this hypothesis, several investigations are available in literature showing that ibuprofen associated with arginine, which also provides fast absorption was significantly more effective than standard ibuprofen at the same dose in patients with osteoarticular pain, postoperative dental pain, periodontitis and primary dysmenorrhea (7).

We acknowledge some study limitations, including the small sample size; thus, further studies with a larger number of patients are mandatory to confirm the results. Secondly, to limit discomforts for the pediatric patients, we decided to focus on the rate of early drug absorption; additional data are useful to define a detailed pharmacokinetic profile of the drug. Finally, in addition to pain scale, endocrinological response on the hypothalamic–pituitary–adrenal axis could be useful to a better evaluation of the adaptive behavioral response to pain and to define the clinical efficacy of the formulation. The safety profile and the tolerability of ibuprofen lysinate have been thoroughly described by previous studies (19, 20). As of today, there is no evidence of increased rate of ADRs in lysinate ibuprofen compared to standard formulations, as both pharmacokinetic studies and efficacy ones, have shown a comparable incidence of ADRs between the two formulations (18, 21, 22). Although we did not include safety profile evaluation as an aim of our study, we did not record any ADRs in both LYS and STAND-groups.

CONCLUSIONS

Despite the acknowledged limitations, this study may be considered a first step suggesting the necessity to test new drug formulations for treating pain in pediatrics.

In conclusion, the use of ibuprofen lysinate in the form of oral suspension was associated with an early fast absorption after drug intake, compared to the traditional ibuprofen acid formulation, supporting fast action and an improved clinical response to mild-moderate postsurgical pain in children.

ETHICS

Fundings

Study design was funded by Dicofarm.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contributions

VF, DC, EC, GVZ conceptualized the research; AM, VC, FD, GP collected research data; VF, VC, DC analyzed data; VF, AM, VC, DC, drafted the manuscript, EC, GVZ revised the manuscript, VF, VC, AM, FD, GP, EC, GVZ and DC approved the final version of the manuscript.

Availability of data and materials

The data underlying this article cannot be shared publicly due to privacy of research participants. The data can be shared just before a reasonable request to the corresponding author.

Ethical approval

Authors intend to respect the autonomy of individuals involved in the research by supplying the research's participants with sufficient and clear information to make an informed decision as to participate (informed consent to participate); ensuring that participants are not subject to coercion to participate or penalty for not participating; assuring that all the participants are free to withdraw from the research at any time without giving a reason and without any form of prejudice; respecting and defending the personal data made available by the participants following rigorous and recommended procedures to take in account both the confidentiality and anonymisation.

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RESEARCH ARTICLE

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EVALUATION OF SINGLE AND ASSOCIATED BOTANICALS ON GLUCOSE TOLERANCE IN GLUCOSE-INDUCED HYPERGLYCEMIC MICE

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SUMMARY

Nutraceuticals can be used in addition to conventional treatments to improve glycemic control. The aim of this study was to evaluate the efficacy of *Morus alba*, *Ilex paraguariensis*, and *Chromium picolinate*, alone and in association, on glucose tolerance in glucose-induced hyperglycemic mice. Male CD1 mice were treated for 6 weeks with *Chromium picolinate* (0.8 mg/kg) (A), *Ilex paraguariensis* (1000 mg/kg) (B) and Morus alba (50 mg/kg) (C), following these combinations: A; B; C; A + B + C; A + B; A + C; B + C. The control animals were administered with the vehicle only. The oral glucose tolerance test (OGTT) was carried out in mice 4 and 6 weeks after the start of treatment with *Chromium picolinate, Ilex paraguariensis* and *Morus alba*, alone or in combination, or by the administration of vehicle (CMC 1%) in the control group, 24 h after the last daily administration.

The complete mixture A + B + C reduced the glycemic values recorded in animals 60 min after glucose administration compared to the control group values and the B + C mixture showed a significant prevention of the glycemic peak at 30 min after 4 weeks of treatment. The combination of A + B + C induced the best effect, preventing the glycemia increase at 60 min after 6 weeks of treatment. In conclusion, the nutraceutical resulted effective for use in the prevention of diabetes mellitus.

Key words
Ilex paraguariensis; Morus alba; botanicals; glucose; mice.

Impact statement
The synergic effect of a nutraceutical containing all three components *Morus alba*, *Ilex paraguariensis*, and *Chromium picolinate*, at 1000 mg of dosage, could be effective for use in the prevention of diabetes mellitus.

List of abbreviations:
OGTT: Oral Glucose Tolerance Test; AUC: area under the curve.

INTRODUCTION

The World Health Organization (WHO) estimates that more than 190 million people worldwide are affected by type 2 diabetes mellitus and this number is constantly on the rise. The pathogenesis of type 2 diabetes mel-

litus is very complex and see different actors playing a role: the main mechanisms involved are an increased peripheral insulin resistance and a decrease of beta cell function (1). Due to these mechanisms, blood glucose levels gradually rise, causing endothelial damage and increasing cardiovascular risk. In the latest years, several new drugs have been marketed for diabetes, but also phytotherapy may play a role in improving glucose metabolism and insulin resistance (2-4).

Nutraceuticals can be used in addition to conventional treatments to improve glycemic control (5, 6), or can be used to prevent type 2 diabetes mellitus, in addition to diet and physical activity, in subjects affected by dysglycemia (7).

Recently, numerous herbs, such as various Mulberry species (*Moraceae* family), showed anti-diabetic action by acting on various aspects of the pathology. White Mulberry (Morus Alba), for example, decreases body weight and adiposity (8), improves insulin resistance (9), increases glucose uptake, GLUT4 translocation and adiponectin (10), inhibits α-glucosidase activity (11), and improves endothelial function (12). On the other hand, *Ilex paraguariensis* (Yerba Maté) has some beneficial effects on glucose absorption (13), it also has hypocholesterolemic, anti-inflammatory (14), and antioxidant effects (15). Xanthines and Polyphenols, Caffeoyl derivatives, and Saponins have a role in many of the pharmacological activities of Yerba Maté (16, 17). Finally, chromium apparently has a role in maintaining proper carbohydrate and lipid metabolism in mammals. This role probably involves empowerment of insulin signaling, reduction of fat mass and increasing of lean body mass. Chromium picolinate has a greater bioavailability compared to Chromium and this formulation may explain the superior efficacy in glucose and lipid control (3). Human studies suggest that Chromium picolinate decreases insulin levels and improves glucose disposal in obese and type 2 diabetic population (18, 19).

On this basis, we evaluated the effects and synergy of a nutraceutical supplementation (Glicoset® 1000, produced by Nutrilinea S,r.l., Gallarate (VA), Italy), containing a mineral (Chromium picolinate, A) and two botanicals (*Ilex paraguariensis*, B, and *Morus alba*, C) (**table I**), in normal mice on glucose tolerance after an Oral Glucose Tolerance Test (OGTT). In particular, the aim of this study was to evaluate the anti-hyperglycemic effect of three substances: A, B, and C, alone and in combination, on glycemic profile in a mice model.

MATERIALS AND METHODS

Study design

In this study we valuated the anti-hyperglycemic effect of A, B and C, alone and in association, in a sample of mice subjected to a

Table I. Composition of the nutraceutical supplement (Glicoset® 1000).

Ingredients	Daily intake:
Chromium picolinate	100 mcg (250% RDD)
Ilex paraguariensis	1000 mg
Morus alba 2% I-deoxinojirimcina	Of which 1 mg DNJ
Silicon dioxide	q.s.
Magnesium stearate	q.s.
Dicalcium Posphate	q.s.
Microcrystalline cellulose	q.s.
E172	q.s.

RDD: Recommended Daily Dose; DNJ: I-deoxinojirimcina; q.s.: quantum sufficit.

load of glucose. The following combinations were evaluated: A; B; C; A + B + C; A + B; A + C; B + C.

Animals

Male CD1 mice (Envigo, Varese) that weighed about 20-25 g at the start of the experiment were used, housed in the Laboratory Animal Stable Center of the University of Florence (Ce.S.A.L.). The animals were placed in cages of 26 cm x 41cm, in environments with a temperature of 23 ± 1 °C with a 12-hour circadian cycle and fed according to the standard diet and ad libitum water.

All treatments were carried out following the Directives 2010/63/EU of the European Parliament and of the Council of the European Union (September 22, 2010) regarding the protection of animals used for scientific purposes. The ethical policy of the University of Florence conforms to the National Institutes of Health Guide for the care and use of laboratory animals (NIH Publication n. 85-23, revised 1996; University of Florence Assurance n. A5278-01). Formal approval for conducting the experiments was given by the university council. The experiments were carried out according to the ARRIVE guidelines (20) trying as much as possible to minimize the suffering of the animals and their number.

Administration of the mixture

The mixture, consisting of Chromium picolinate (0.8 mg/kg), *Ilex paraguariensis* (1000 mg/kg) and *Morus alba* (50 mg/kg), was suspended in a 1% carboxymethylcellulose (CMC) solution and administered orally daily for 6 consecutive weeks. The control animals were administered with the vehicle only.

Oral glucose tolerance test

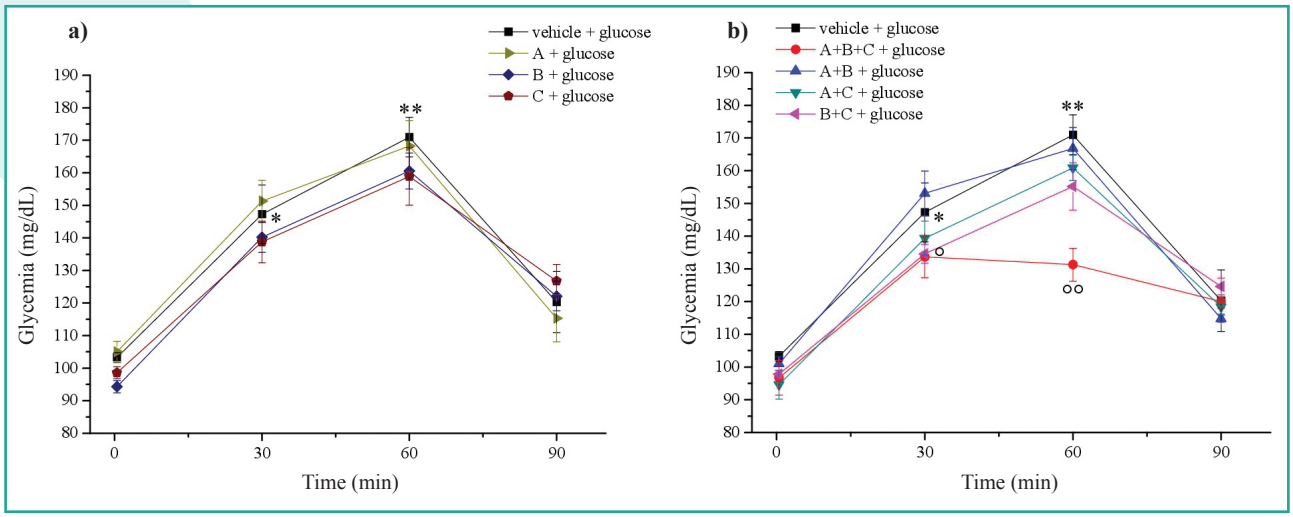
The OGTT was carried out in mice 4 and 6 weeks after the start of treatment with the mixture consisting of Chromium picolinate, *Ilex paraguariensis* and *Morus alba* or by the administration of vehicle (CMC 1%) in the control group, 24 h after the last daily administration. Glucose (3 g/kg) was solubilized in water and administered orally after fasting the animals for 4 h; blood glucose values were measured at minute 0, 30, 60 and 90 by blood sampling from the caudal vein and analysis with the Accu-Check Aviva planar sensor based on the glucose oxidase method.

Statistical analysis

All experimental results were expressed as mean ± standard error (M ± SE). Each group of treatment was represented by 10 mice. A one-way analysis of variance (one-way ANOVA) was conducted, followed by the Bonferroni test to verify the significance between two averages. Residual analysis showed that ANOVA residuals followed a normal distribution. The analysis of variance and the Bonferroni test were performed with the statistical program Origin 9.1. Differences with p value < 0.05 were considered significant.

RESULTS

The animals were treated daily for 6 consecutive weeks orally using, alone or in combination, 0.8 mg/kg of A, 1000 mg/kg of B and 50 mg/kg of C. On weeks 4 and 6, we evaluated the effectiveness of these substances in protecting animals from the glycemic peak induced by a glucose load (3 g/kg per os; performed after a 4 h fast). The results were compared with those of a group of vehicle-treated animals. The results obtained were reported in **figures 1 and 2**. On week 4 of treatment, the acute administration of glucose 3 g/kg significantly increased the glycemic values in the control animals after 30 min (147.3 ± 9.0 mg/dL *vs.* 103.3 ± 1.5 mg/dL), this increase peaked 60 min (171.0 ± 6.1 mg/dL) after the administration of sugar (**figure 1 a**). Daily treatment with the complete mixture A + B + C significantly reduced the glycemic values recorded in animals 60 min after glucose administration (131.3 ± 5.0 mg/dL) compared to the control group values (**figure 1 b**). The B + C



Figures 1 a, b. Evaluation of glycemia levels after 4 weeks of treatment. Each value was expressed as mean ± S.E.M. of 10 mice. * P < 0.05 and ** P < 0.01 *vs* values recorded at time 0 (0 min) in the same group; ° P < 0.05 and °° P < 0.01 *vs.* vehicle-treated group.

mixture showed a significant prevention of the glycemic peak at 30 min (134.6 ± 2.9 mg/dL) (**figure 1 b**).

The experiments were repeated after 6 weeks of treatment with the mixture. The glucose load induced a significant increase after 30 and 60 min after administration (144.8 ± 7.7 mg/dL and 180.5 ± 4.6 mg/dL, respectively *vs* the control group -93.5 ± 6.1 mg/dL) (**figure 2**). The repeated treatment with the complete mixture A + B + C induced the best effect, significantly preventing the glycemia increase at 60 min (138.5 ± 4.3 mg/dL) (**figure 2 b**). At 60 min also the mixtures B + C (154.6 ± 4.7 mg/dL), A + B (158.4 ± 6.1 mg/dL) (**figure 2 b**) and the product A (166.4 ± 3.3 mg/dL; **figure 2 a**) gave significant lower results. To note, neither after 4 weeks nor after 6 weeks none the tested formulations modified the basal glycemic threshold measured at time 0 before the glucose intake.

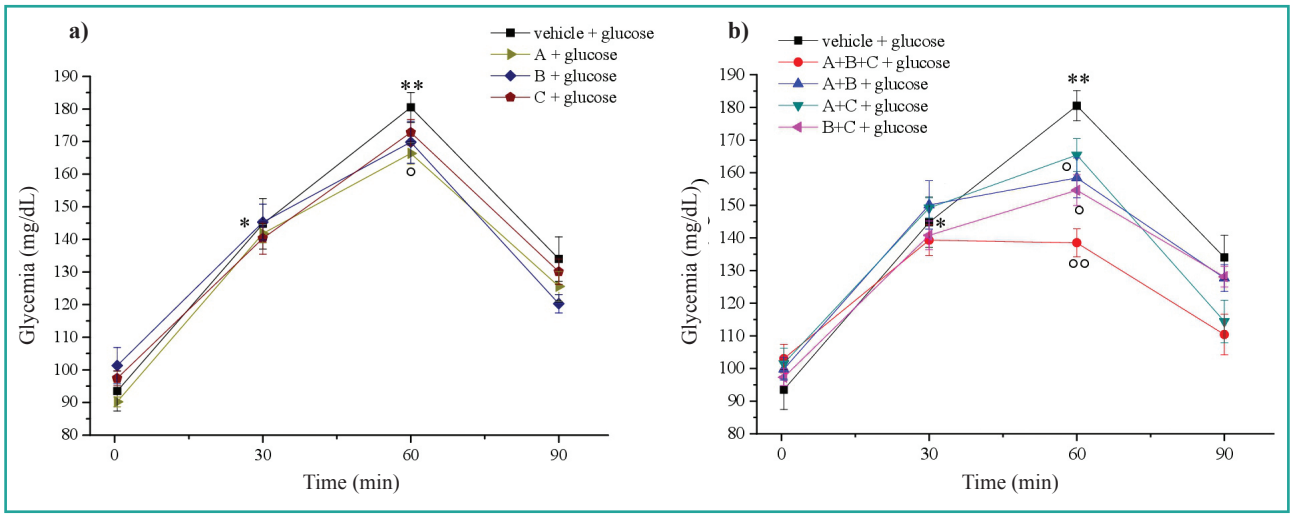
DISCUSSION

In this study, it has been demonstrated that Chromium picolinate (one of the three components) reduced glycemia values (-14.1 mg/dL, -7.8%) 60 min after the oral glucose load at 6 week of treatment.

Mita et al showed that everyday consumption of Chromium picolinate, at the dose of 2 mg/kg or 10 mg/kg for 12 weeks and of 10 mg/kg for 4 weeks in obese diabetic mice, significantly decreased blood glucose levels at 120 and 180 min after OGTT compared to control when the higher dosage was utilized for a 12-week period (21). It has been also reported that, in diabetic rats subjected to glucose tolerance test, Chromium picolinate daily intake at the dose of 1 and 10 mg/kg for 32 and 16 weeks, respectively, ameliorated glucose tolerance similarly for both amounts used. However, the lowest dose of Chromium picolinate after 6 weeks produced reductions in the relative changes in glucose area under the curve (AUC) that were resulted significant only at the end of the treatment period for both dosages (22).

The consumption, separately, of *Ilex paraguariensis* and *Morus alba*, the two other compounds contained in nutraceutical used in our study, slightly reduced glycemic values at 60 min following OGTT after 4 and 6 weeks of treatment. However, though the lowering did not attain statistical significance, it represents a trend towards reduction.

Mate tea is an infusion derived from *Ilex paraguariensis* leaves. Hussein *et al.* reported that, in obese diabetic mice subjected to intraperitone-



Figures 2 a, b. Evaluation of glycemia levels after 6 weeks of treatment. Each value was expressed as mean ± S.E.M. of 10 mice. * P < 0.05 and ** P < 0.01 *vs.* values recorded at time 0 (0 min) in the same group; ° P < 0.05 and °° P < 0.01 *vs.* vehicle-treated group.

al glucose tolerance test, daily consumption of mate aqueous extract at the dose of 100 mg/kg for 7 weeks significantly decreased blood glucose levels at 60 (-69.2 mg/dL, -13.0%) and 120 min (-93.0 mg/dL, -22.1%) after sugar load compared to untreated obese diabetic mice utilized as controls (16). In addition, Pereira *et al*. investigated the acute effect of two fractions (ethyl acetate (EtOAc) and n-butanol (n-BuOH)) of native *Ilex paraguariensis* and two infusions (green and roasted mate) of commercial Ilex paraguariensis in rats after OGTT. The authors observed that 200 mg/kg of EtOAc fraction significantly reduced glycemia at 15 (-48.6 mg/dL, -28.5%), 30 (-54.0 mg/dL, -28.2%) and 60 min (-36.0 mg/dL, -21.2%) after oral sugar administration, respect to hyperglycemic rats adopted as controls. The n-BuOH fraction (200 mg/kg) showed a significant blood glucose lowering 15 (-28.0 mg/dL, -16.4%), 30 (-46.5 mg/dL, -24.3%) and 60 min (-28.8 mg/dL, -17.0%) following OGTT compared to hyperglycemic controls, respectively. The n-BuOH and EtOAc fractions at the dose of 100 mg/kg reduced likewise glycemia (-19.5 mg/dL, -11.4% for n-BuOH and -19.3 mg/dL, -11.4% for EtOAc) at 15 and 60 min respectively compared to hyperglycemic control group. The green mate infusion (200 mg/mL) decreased blood glucose levels at 15 (-41.4 mg/dL, -24.3%), 30 (-35.6 mg/dL, -18.6%) and 60 min (-30.1 mg/dL, -17.7%) after OGTT, respect to hyperglycemic controls, and this dose has resulted more effective than 50 and 100 mg/mL in improving glucose tolerance. As regard roasted mate infusion, the best sugar-lowering effect was obtained with the dose of 100 mg/mL, compared to 50 and 200 mg/mL, and this dosage led to a glycemia reduction of -27.9 mg/dL (-16.3%) at 15 min and of -26.4 mg/dL (-13.8%) at 30 min after oral glucose administration respect to hyperglycemic control group (23).

The consumption of two polysaccharides extracted from *Morus alba* fruit, generally known as White Mulberry, in murine model of type 2 diabetes mellitus has been also evaluated. The two fractions have significantly decreased blood glucose levels at 180 min after oral sugar administration and the OGTT-AUC values (158.71 and 157.53, respectively) compared to that of untreated diabetic rats (176.83) following 7 weeks of supplementation (24). Another study reported that, in type 2 diabetes mellitus rats subjected to oral glucose tolerance test, the extract of Mulberry leaf (Folium Mori) at the daily dose of 2 g/kg b.w. determined a significant lowering in the AUC of OGTT after 4 weeks of treatment compared to normal controls and untreated diabetic rats (25).

In agreement to the literature data, our results highlight that mostly Chromium picolinate, and to a lesser extent Ilex paraguariensis and *Morus alba*, individually, are able to ameliorate glucose tolerance thanks to their glucose-lowering activity. As regard the associations of different nutraceutical compounds, we observed that the combination of Chromium picolinate, *Ilex paraguariensis* and *Morus alba* (A + B + C) caused, at 60 min afterwards OGTT, a significant blood glucose lowering of -39.7 mg/dL (-23.2%) and -42.0 mg/dL (-23.3%) after 4 and 6 weeks of treatment respectively. Also the association of *Ilex paraguariensis* and *Morus alba* (B + C) has decreased glycemia levels at 30 (-12.7 mg/dL, -8.6%) and 60 min (-25.9 mg/dL, -14.3%) following oral glucose administration after 4 and 6 weeks of supplementation, respectively. Moreover, Chromium picolinate plus Ilex paraguariensis (A + B) 60 min after oral sugar load has reduced blood glucose values (-22.1 mg/dL, -12.2%) at 6 weeks of treatment.

Kan et al reported that, in a murine model of insulin resistance and type 2 diabetes mellitus, the administration of a mixture containing Mulberry leaf (120 mg), Fenugreek seed (88 mg) and American ginseng (300 mg) extracts at 3 different doses (42.33, 84.66 and 169.33 mg/kg b.w.) has significantly decreased glycemia levels at 30 and 120 min after OGTT with a significant AUC reduction respect to control. Moreover, the lowering induced by the three dosages was similar (26).

In our study, the combination of Chromium picolinate, Ilex paraguariensis and *Morus alba* (A + B + C) was found to be the most effective in reducing glycemia following OGTT at the end of treatment period. Since Chromium picolinate, alone, exhibited the best anti-hyperglycemic effect after OGTT at 6 week and each compound of our supplement has its own mechanism of anti-hyperglycemic action, although still unclear, the higher effect of Chromium picolinate, *Ilex paraguariensis* and *Morus alba* mixture on postprandial glycemia than that of the single compounds as well as the other associations has proved that the improvement of glucose tolerance is due to the ability of the different hypoglycemic agents to synergize among them.

CONCLUSIONS

Our study showed the synergic effect of the three components (A + B + C) of the proposed nutraceutical, suggesting that a nutraceutical containing all three components, at 1000 mg of dosage, could be effective for use in the prevention of diabetes mellitus.

ETHICS

Fundings

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Stefania Murzilli and Arianna Vanelli are employed by Nutrilinea srl, Varese, Italy. The other authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

Authors' contributions

Design and conduction of the study: GD and CG; data collection: LDCM; data interpretation and manuscript writing: GD, PM and ADA. All authors read and approved the final version of the manuscript.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article.

Ethical approval

All treatments were carried out following the Directives 2010/63 / EU of the European Par-

liament and of the Council of the European Union (September 22, 2010) regarding the protection of animals used for scientific purposes. The ethical policy of the University of Florence conforms to the National Institutes of Health Guide for the care and use of laboratory animals (NIH Publication n. 85-23, revised 1996; University of Florence Assurance n. A5278-01).

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REVIEW

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PHARMACOLOGICAL APPROACHES TO SARS-CoV-2 INFECTION: FROM DRUG REPOSITIONING FOR COVID-19 TREATMENT TO DISEASE ARREST/ PREVENTION WITH MoAbs AND NOVEL ANTIVIRALS

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SUMMARY

COVID-19 disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is one of the major emergencies that have affected health care systems and society in recent decades. At the end of winter 2021-2022, the number of patients infected with SARS-CoV-2 and especially those suffering from severe COVID-19 is decreasing in Europe. This is due to the protective effect of anti-SARS-CoV-2 vaccines and the increasing number of people who had COVID-19, thus developing a certain immunity. However, vaccines to prevent the disease did not appear until more than one year after the emergence of SARS-CoV-2, so the initial medical approaches to control the disease focused on the existing drugs that were considered suitable for controlling the pathological events caused by the virus as far as was known at the time. Unfortunately, due in part to the limited initial knowledge of the molecular details of the pathology of COVID-19, many of the proposed drugs fell short of expectations and were abandoned. Over time, the challenge of understanding the mechanisms behind COVID-19 has generated a large body of knowledge about how this beta-coronavirus gains control of the host during infection, a knowledge that has been used to redefine treatment strategies by repurposing existing drugs and to explore new drugs. Here, we draw a picture of the major strategies and groups of drugs studied and provide a critical overview of their efficacy and safety based on the available literature data. The main topics covered are repurposed drugs, anticoagulants, anti-cytokine agents, monoclonal antibodies against SARS-CoV-2, and small antiviral molecules.

Key words
COVID-19; drug repositioning; anti-inflammatory and anticoagulants; antiviral MoAbs; antiviral small molecules.

Impact statement
The impact of the review is to collect together successes and failures in the use of drugs to treat COVID-19, the reasons for the repositioned drugs and the corresponding responses of the relevant clinical trials as well as the responses to new monoclonal antibodies and antiviral drugs.

INTRODUCTION

Severe Acute Respiratory Syndrome due to CoronaVirus-2 (SARS-CoV-2) appeared as a novel, highly dangerous, virus that caused the coronavirus disease-2019 (COVID-19) in humans at the end of 2019. First identified in Wuhan, China, SARS-CoV-2 rapidly spread throughout the world, leading to a public health emergency. SARS-CoV-2 infection caused patients to develop severe disease with an acute respiratory distress syndrome (ARDS), associated with coagulation disorders, an exuberant cytokine storm leading to multiple organ failure, and resulting in fatal events in about 3% of the infected people (1). The risk for the severity of COVID-19 disease depends on several comorbidities (diabetes, hypertension, lung-related diseases, cardiovascular diseases and obesity), older age, ethnicity, genetic factors, vaccination status and other conditions (2). The morbidity and mortality associated with the COVID-19 have pushed the development of SARS-CoV-2 vaccines as a priority for human health. As a result of that emergency, several effective vaccines, targeting the SARS-CoV-2 spike protein, rapidly emerged and gained conditioned approvals by the regulatory agencies (3).

However, vaccines dedicated to the prevention of the disease appeared after more than one year after SARS-CoV-2 appearance. Therefore, the initial medical approaches to this virus and the COVID-19 were focused on the existing drugs suitable for the control of the pathological events caused by the virus. Unfortunately, also because of the limited initial knowledge of the molecular pathology details of COVID-19, many of the proposed drugs have often missed expectations and were abandoned. Indeed, it was crucial to understand how this beta coronavirus gained control of the host during infection, a knowledge that was applied to the development of treatment strategies by the repurposing of existing drugs but also to the study of new ones. The emergency of the pandemic made the marketed drug repurposing the best approach to identify therapeutic options for COVID-19 in a limited time (4). In the absence of clear clinical evidence, many treatment regimens have been explored in the treatment of COVID-19. Some of these treatments could refer to the experience gained with the Middle East Respiratory Syndrome (MERS) and with the Severe Acute Respiratory Syndrome (SARS); some of them showed effects on COVID-19 patients (5). However, the published data often suffered from limited rigorousness of the clinical trials, particularly regarding randomization, genetic causes and differences in study design and treatment regimens, leading to contrasting results (6). In other cases, side effects precluded the use of the drug itself (4).

Although vaccines have made a difference in significantly reducing SARS-CoV-2 diffusion and COVID-19 frequency, with the acquired and increased knowledge on the modalities of virus infection, it became possible for researchers and pharmaceutical companies worldwide to work and develop new drug candidates. There is still a need for effective therapies for COVID-19 for many reasons: 1) some people do not properly respond to vaccines, 2) the appearance of virus variants that escape or reduce the vaccine effectiveness and 3) some patients develop severe forms of the pathology (7). Also, drugs can be useful in patients on chemotherapy, patients with hematologic malignancies, immunocompromised people or in other pathologic conditions.

Drug development is mainly focused on different strategies: i) to avoid the virus entry into the cells, ii) to inhibit viral replication and vitality, and iii) to regulate the human immune system. These drug categories include anticoagulants, immunosuppressors, anti-inflammatory, corticosteroids, janus kinase inhibitors, immunoglobulins, monoclonal antibodies, antivirals and cell therapy (8).

The aim of this review is to examine all the strategies adopted for the control and treatment of COVID-19, with particular emphasis of the role and effectiveness of the different categories of drugs on the stages of the disease.

REPURPOSED DRUGS FOR THE CONTROL OF COVID-19

In the search for an effective treatment for SARS-CoV-2 infection, many attempts have been made using existing drugs which, on the basis of their mechanisms of action, or given some preliminary clinical evidence, seemed to be effective in managing the disease (9). If, before vaccination and up to the spread of the omicron variant, the medical need was urgent, nowadays clinicians are more cautious in prescribing unapproved drugs for COVID-19. A database has also been developed (10) containing all the available in in vitro anti-SARS-CoV-2 activity and in vivo pharmacokinetic data to facilitate the extrapolation from in vitro antiviral activity to potential in vivo antiviral activity for choosing drugs that could be useful in saving lives.

The main problem concerning repurposed, or any other drug treatment for COVID-19 is the need for mechanical ventilation or high flux, as the availability of resources and the real severity of the patient respiratory function greatly influence the efficacy of the therapy. As an example, tocilizumab, an interleukin 6 antagonist, in randomized clinical trials has shown mixed results compared with control or usual care in hospitalized patients with COVID-19 (11). However, the real benefit was evident only for those patients who did not require invasive mechanical ventilation (IMV) at randomization and no further details were provided regarding the respiratory status. Despite the absence of these data, guidelines have suggested the use of tocilizumab in patients with either severe or critical COVID-19 independent of their respiratory condition. From a re-analysis of the published evidence, it appears that the real benefit of using tocilizumab might have been overestimated also in subjects without IMV, as it was used in association with high doses of corticosteroids that by themselves blunt the inflammatory reaction (12).

Another drug that has been used for treating COVID-19 was Ivermectin, an inexpensive, easy-to-administer, and widely available antiparasitic drug, because an in vitro study showed inhibitory effects against SARS-CoV-2. Despite the initial reports on its supposed efficacy in reducing viral load in 45 patients (13), a 5-day course of ivermectin, compared with placebo, did not significantly improve the time to resolution of symptoms in 400 patients (14). Further evidence on 490 high-risk hospitalized patients with mild to moderate COVID-19, demonstrated no benefit from this treatment regarding the need for mechanical ventilation, intensive care unit admission, or death (15).

Among the drugs used to treat COVID-19, the anti-malarial drugs chloroquine (CQ) and hydroxychloroquine (HCQ) deserve special attention. These have been suggested as promising agents, from early trials in China, for shortening the duration of the viral disease, reducing the fever duration, and improving lung health (16) also in combination with azithromycin, a commonly prescribed antibiotic for lung infections (17). The antiviral effects of CQ and HCQ have been demonstrated in vitro due to their ability to block viruses like coronavirus SARS in cell culture (18, 19). Given this preliminary evidence and considering the low cost of HCQ, most pharmacies, as a detrimental consequence of the rapid dissemination of over-interpreted data, in Europe and Italy have been struck by people asking for this drug, hoping for a miraculous cure for the deadly virus (20). However, a few months later, emerging clinical evidence demonstrated the ineffectiveness of HCQ, together with the severe adverse events, including death, when used at high dose. Currently, no direct supporting data on the effective role of CQ and HCQ in the treatment for COVID-19 exist, and the international RCTs for COVID-19 treatments launched by WHO (21) concluded that HCQ had little or no effect on overall mortality, initiation of ventilation, and duration of hospital stay in hospitalized patients (22, 23).

Finally, also azithromycin, a macrolide antibiotic with alleged antiviral efficacy against COVID-19, was widely prescribed up to the second quarter of 2021 because several

guidelines in 2020 recommended the use of empirical antimicrobial treatment (24). However, its alleged efficacy was eventually unsupported by the results of the Recovery trial that enrolled over 7000 patients that did not benefit from azithromycin treatment, in terms of the need for IMV or death (25). The same trial indeed revealed that corticosteroids are indeed useful in the treatment of COVID-19. In fact, before Recovery trial results were available, there was a wide debate regarding the role of corticosteroids in mitigating inflammatory organ injury (26, 27) and these were generally not included in most guidelines. However, the first results obtained in 2104 hospitalized patients showed that dexamethasone lowered mortality among those who were receiving either invasive mechanical ventilation or oxygen alone at randomization (28).

Considering the great medical need, a special effort has been made in these past 2 years to assess the safety and efficacy of drugs proposed or used to treat COVID-19, but little evidence exists to date on the prescribing patterns for repurposed and adjuvant drugs in routine clinical practice.

ANTICOAGULATION TREATMENTS IN COVID-19

Since the beginning of the COVID-19 pandemic, altered coagulation has been reported in hospitalized patients, with both thrombotic as well as hemorrhagic events. In some patients, a pro-thrombotic status was the alleged cause, but for many others, the problems seem related to the cytokine storm, leading to hyperinflammation, endothelial disruption, platelet activation, and thrombotic complications (29). Arterial and venous thrombotic complications are common in hospitalized patients with COVID-19 and are an independent predictor of poor outcome. Microvascular thrombi also determine multi-organ dysfunction, starting with acute respiratory distress and then involving other tissues (30). Early studies also indicated that standard prophylactic doses of anticoagulant therapy appeared to be inadequate for preventing thrombotic events in hospitalized patients (31).

More recently, larger trials have been published, providing more insight into treatment strategies for hospitalized patients with COVID-19. The ACTION trial (32) showed that clinically stable hospitalized patients with COVID-19 receiving rivaroxaban, compared to unstable patients receiving enoxaparin, did not improve the primary efficacy outcome on the death rate, duration of hospitalization, or duration of supplemental oxygen. Therapeutic anticoagulation was associated with increased bleeding in both clinically stable and clinically unstable patients.

The ATTACC, ACTIV-4a, and REMAP-CAP Investigators in 2 trials (33, 34) using therapeutic-doses anticoagulation compared with "usual-care" thromboprophylaxis in noncritically ill patients, defined as not needing respiratory or cardiovascular support, showed that therapeutic dosing improved survival and reduced the use of cardiovascular or respiratory organ support as compared with usual-care thromboprophylaxis.

In the HEP-COVID study (35), adult patients with evidence of coagulopathy (by laboratory means) affected by COVID-19 and randomized to receive standard prophylactic or intermediate-dose low-molecular-weight heparin (LMWH) or unfractionated heparin (UFH) or therapeutic-dose LMWH throughout hospitalization, demonstrated interesting results. The primary efficacy outcome of thromboembolic occurrence, or all-cause mortality, was reached only in non-severe patients with therapeutic-dose anticoagulation, but ICU patients did not improve with this therapeutic regimen.

Anticoagulation with LMWH or UFH at a therapeutic dose in COVID-19 hospitalized patients with an elevated D-dimer level did not significantly reduce the rate of death or severe consequences such as ICU admission, noninvasive or invasive mechanical ventilation, as demonstrated in the RAPID Trial (36). Also, considering the results of the INSPIRATION trial in ICU

patients, no benefit was obtained from using an intermediate dose of LMWH over standard prophylactic-dose anticoagulation in preventing thromboembolic events or death (37).
Despite the methodological differences in defining the criteria for considering critically or non-critically ill COVID-19 patients in these studies, the take-home message regarding the efficacy and safety of anticoagulant therapy in hospitalized patients can be summarized in 3 crucial points. First, patients that are non-critical and have elevated D-dimer levels benefit of therapeutic anticoagulation with LMWH or UFH; second, critically ill and/or ICU patients do not benefit from therapeutic anticoagulation and have a higher risk of hemorrhage; finally, a dose between prophylactic and therapeutic is not recommended in either ICU or non-ICU patients.
The use of LMWH in the prophylaxis of thromboembolic events or in patients with an acute respiratory infection is recommended by the main guidelines in the absence of contraindications. LMWH or UFH are necessary in case of thromboembolic manifestations; it is indeed reasonable to recommend enoxaparin prophylaxis or an intermediate dose when pneumonia is present and hypomobility occurs in the bed rest patient (38).
Although many limitations and a small number of high-quality, well-designed studies, heparin treatment should be preferred to anticoagulants in the treatment of COVID-19 patients at high risk or with thromboembolism.

ANTI-CYTOKINE AGENTS FOR COVID-19 TREATMENT

SARS-CoV-2 virus infection triggers an inflammatory response and subsequent production of immune mediators such as cytokines, chemokines, and complement, initially locally and in moderate amounts: this response is essential to fight the infection. However, in severe COVID-19 infection, cytokines and chemokines are released in increased amounts, leading to massive recruitment of immune cells and consequent hyperinflammation, which eventually causes the cytokine storm (CS) (39). This increased inflammatory response leads to severe complications such as acute respiratory distress syndrome (ARDS) in the lungs, intravascular coagulation, multiorgan failure, and ultimately death. Higher concentrations of cytokines in the plasma of patients have been associated with disease severity (40, 41). These pro-inflammatory cytokines and chemokines include tumour necrosis factor alpha (TNF-α), interleukin 1beta (IL-1β), IL-6, IL-10, IL-17, Granulocyte/macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1-alpha (MIP-1α) (42-45). The IL-1/IL-6 axis is probably one of the most biologically relevant signalling pathways in the SARS-CoV-2-induced hyperinflammatory response (44, 46, 47). Consequently, monoclonal antibodies or drugs targeting specific cytokines among the host defence immune mediators triggered by the virus were considered early on as a potential class of adjunctive therapies for COVID-19 (48).

IL-1 blockers

IL-1 induces local effects such as macrophage activation, endothelial leakage, and fluid extravasation, as well as systemic effects such as fever, drowsiness, and synthesis of acute-phase proteins. Blocking IL-1 signals reduces inflammation, which in turn may reduce the need for respiratory support and deaths from COVID-19. Three IL-1 blockers are available: anakinra, canakinumab, and rilonacept.
Anakinra is a recombinant soluble IL-1 receptor antagonist (IL -1Ra) that competitively inhibits the binding of both IL-1α and IL-1β to their receptor (IL-1 type I) (49-51) and is currently approved for rheumatoid arthritis and other autoinflammatory diseases. Randomized trials with anakinra, compared to placebo, in patients with mild to moderate COVID-19 pneumonia reported no significant effect on the proportion of patients who died or re-

quired non-invasive or mechanical ventilation, or on survival without the need for mechanical or non-invasive ventilation, or on discharge from organ support in the intensive care unit (ICU) (52, 53). These findings are consistent with a Cochrane systematic review that examined the effects of IL-1 blockers compared with standard of care (SoC) alone or placebo on efficacy and safety in patients with moderate to severe COVID-19 (54). Overall, there was no evidence of a significant beneficial effect of IL-1 blockers or of adverse effects. Similarly, a study on canakinumab, a monoclonal antibody that blocks only IL -1β, did not reach significance for its primary outcome, survival without invasive mechanical ventilation at day 29 (55). Again, the results are supported by the findings of the Cochrane systematic review, which states that canakinumab is likely to result in little or no improvement in COVID -19 symptoms, defined as improvement on a clinical scale or discharge from hospital at day 28 after treatment. No studies of rilonacept in COVID-19 were found in either the EU Clinical Trials Register or on ClinicalTrials.gov (accessed February 17, 2022).

In contrast to these disappointing results, a different approach based on stratifying patients by immunologic profiles identified patients who would likely benefit from IL-1 blockade. In the SAVE-MORE trial, treatment with anakinra was guided by plasma levels of soluble urokinase plasminogen receptor (suPAR) as a biomarker of risk of progression to severe respiratory failure (56-58). Treatment with anakinra resulted in significant clinical improvement on the 11-point WHO clinical outcome scale, both toward complete resolution and toward critical illness or death at 28 days (59). In this study, anakinra also improved outcomes in patients treated concomitantly with dexamethasone, suggesting that suPAR-based treatment with anakinra is a therapeutic strategy before critical illness occurs. Other useful information on the use of anakinra comes from a retrospective observational study suggesting that a shorter time between hospitalization and treatment with anakinra in patients with moderate/severe COVID-19 is associated with a significantly lower number of intensive care admissions and lower mortality (60).

IL-6 blockers

In severe COVID -19 patients, a significant increase in the levels of IL-6 is observed (47, 61). IL-6 is a strong predictive marker of acute severe systemic inflammatory response requiring support by mechanical ventilation. Moreover, elevated levels of IL-6 activate the coagulation cascade and increase the risk of death (62-64). Accordingly, blockade of IL-6 has emerged as a potentially promising approach to control SARS-CoV-2-associated cytokine release syndrome (CRS). IL-6 promotes monocyte differentiation into macrophages, recruits immune cells to the site of injury, and increases cytokine production. Interaction of IL-6 with its transmembrane IL-6 receptor (IL-6R) leads to dimerization of glycoprotein 130 and the "classical" signalling process via JAK /STAT, MAPK and RAS /RAF. However, cells that do not express IL-6R also respond to IL-6 through circulating soluble IL-6Rα (sIL-R), known as "trans-signaling". Recently, three drugs have been used to treat COVID-19 infections and are in clinical trials: tocilizumab, sarilumab, siltuximab.

Tocilizumab is a humanized IgG1-type mAb that targets both the membrane-bound and soluble forms of IL-6R (63), inhibiting both classical and trans-signalling. It is used to treat rheumatoid arthritis (RA) and CRS concomitant with CAR-T therapy in cancer, a syndrome similar to the hyperinflammatory phase of COVID-19 (64, 65). A prospective meta-analysis of clinical trials of patients hospitalized for COVID -19 showed an association with lower 28-day all-cause mortality in patients treated with IL-6 antagonists compared with patients receiving usual care or placebo (65). Tocilizumab resolved respiratory symptoms and improved overall health (11). In addition, patients with hypoxemia requiring oxygen therapy have benefited from anti-IL-6 strategies,

as shown by the results of two large-scale randomized clinical trials (65, 66). In the open-label trial RECOVERY, which enrolled predominantly non-critically ill patients, a significant reduction in mortality was observed in the tocilizumab arm compared with the usual care arm (66). In the REMAP-CAP trial, both tocilizumab and sarilumab were effective compared with the control group and likely equivalent in improving survival and discharge from organ support (67). In the same study, treatment with anakinra was not effective, as previously reported. Overall, these data support the use of blockade of IL-6 in patients with COVID-19 who are hospitalized and require oxygenation. Unlike tocilizumab and sarilumab, which target the IL-6 receptor, siltuximab modulates IL-6 signalling by directly binding the cytokine (68). The COV-AID study examined the effects of tocilizumab and siltuximab within the anti-IL-6 therapy group and found no significant difference between the two different anti-IL-6 strategies (69).

Inhibitors of JAK /STAT

Several studies suggest that activation of host NF-kB and IL-6/JAK/STAT signalling pathways by SARS-CoV-2 viral proteins is likely a critical factor in virulence, promoting overexpression of proinflammatory cytokines, viral replication, and pathogenicity. The JAK/STAT pathway transmits extracellular signals conveyed by a large number of cytokines, lymphokines, and growth factors, with IL-6 being one of the most important activators (70). Binding of IL-6 to its receptor activates STAT3, which contributes to the cytokine storm, then the ability of STAT3 to promote IL-6 gene expression leads to an autocrine loop that enhances cytokine expression (71). JAK/STAT signalling pathway in COVID-19 has also been implicated in the inflammatory response of IFN-γ, the signalling of which involves JAK1 and JAK2 as well as STAT1 (72). Last but not least, detachment of ACE2 from the cell surface after endocytosis increases angiotensin II levels (Ang II), whose effects are also mediated by the JAK/STAT pathway and contribute to the development of ARDS (73). Therefore, it is not surprising that one of the therapeutic strategies being investigated for COVID-19 is targeting the JAK/STAT pathway, whose inhibition may have pleiotropic effects on the actions of multiple cytokines, including IL-6 and GM-CSF, while overcoming the limitations of mAbs that normally target only one cytokine. There are several JAK/STAT inhibitors that differ in their selectivity toward members of the family, namely JAK1, JAK2, JAK3, and Tyk2 (74).

The efficacy and safety of the pan-JAK inhibitor tofacitinib were evaluated in a clinical trial of 289 patients hospitalized with COVID-19 pneumonia (75). Tofacitinib resulted in a lower risk of death or respiratory failure than placebo by day 28, with serious adverse events occurring in 14.1% in the tofacitinib group and 12.0% in the placebo group. Further promising results were also obtained in combination with hydroxychloroquine (76). Further evidence is available on the use of ruxolitinib and baricitinib, both inhibitors of JAK1 and JAK2. Ruxolitinib is a potent JAK1/2 inhibitor and significantly suppresses the increase in IL-6 and TNF-α levels in COVID-19 patients. Compared with placebo, treatment with ruxolitinib resulted in significantly improved chest computed tomography and faster recovery from lymphopenia (77). In addition, ruxolitinib in combination with steroids reduced mortality and resulted in a 75% recovery rate in COVID-19 patients enrolled in the MAP program (78). Nevertheless, ruxolitinib failed to significantly reduce inflammation in patients who experienced respiratory failure or ICU admission. Baricitinb is not only a JAK inhibitor but also impedes the entry of SARS-CoV-2 into target cells (73). Although the virus enters the host cell mainly through ACE2 receptors, JAK and AP-2 (Adaptor Protein Complex 2) associated protein kinase-1 (AAK1) are also involved in viral attack and endocytosis (79, 80). Baricitinb inhibits viral endocytosis and assembly by inhibiting AAK1 and cyclin G-associated kinase (GAK). Baricitinib treatment attenuates the cy-

tokine storm by decreasing expression levels of IL-6, IL-1β, and TNF-α, resulting in improvement in lymphocyte counts in patients with COVID-19 (81). A double-blind, randomized, placebo-controlled trial of 1,033 adults hospitalized with COVID-19 who were randomized to receive either baricitinib or placebo showed that patients receiving this JAK inhibitor had a shorter time to recovery than patients in the placebo group (82). Importantly, the effect was more pronounced in the subgroup that required high-flow oxygen or non-invasive ventilation compared with placebo. Encouraging results came from a double-blind phase 3 trial of 1,525 participants randomized to baricitinib or placebo (83). The relative reduction in mortality was 38.2% for baricitinib versus placebo when considering 28-day all-cause mortality; this effect is in addition to standard treatment, including corticosteroids. Positive feedback comes from the use of baricitinib in combination with remdesivir, better than baricitinib alone, in accelerating recovery time and improving the clinical condition of COVID-19 patients dependent on high-flow oxygen or non-invasive ventilation, with fewer adverse events (82). The FDA recently approved baricitinib for the emergency treatment of COVID-19 (July 2021). On the other hand, it should also be considered that baricitinib, as a potent immunosuppressant, may lead to an additional risk of infection in critically ill patients.

One concern with the use of pan-JAK inhibitors for COVID-19 is that such inhibitors may interfere with host responses mediated by type I and type II interferons, which have important antiviral effects through their ability to inhibit viral replication in infected cells (84,85). Because JAK2 is not involved in cell signalling that regulates type I interferons and is not essential for type II and III interferons in host immunity, selective JAK2 inhibitors might be preferred over other JAK inhibitors to block signalling by cytokines such as IL-6 and GM-CFS, leading to suppression of COVID-19-associated CRS. The hypothesized benefits of JAK2 inhibition in the treatment of COVID-19-associated CRS are currently being investigated with FDA-approved inhibitors. Fedratinib is an FDA-approved JAK2 inhibitor that has nanomolar activity in the treatment of myelofibrosis (MF) (86); it has also been reported to prevent the worsening outcomes that follow Th17 cell differentiation and the associated cytokine storm, helping to control pulmonary oedema in COVID-19 (87). Several other JAK2 inhibitors are currently under investigation for the treatment of various human diseases, including acute myeloid leukemia, MF, psoriasis, GvHD (graft versus host disease) (88, 89). Given that JAK2 inhibitors likely do not interfere with the type I interferon response in immunity but inhibit cytokines including IL-6 and GM-CSF in COVID-19 associated CRS, JAK2 inhibition should be an attractive therapeutic option for blocking the cytokine storm in COVID-19.

The need to find effective therapies against COVID-19 in the shortest possible time has forced the entire scientific community to make great efforts. The experience accumulated so far suggests that host-specific therapy is a rather complex approach and that the heterogeneity of the immunological milieu of COVID-19 patients must be taken into account. It is now clear that not all patients benefit from the same immunomodulatory treatment and that the same patient may respond differently depending on the stage and severity of the disease. In particular, the experience with the IL-1 antagonist anakinra points to the need to evaluate and use biomarkers to guide patient-specific immunotherapy.

ANTI-SARS-CoV-2 MONOCLONAL ANTIBODIES

Soon after the discovery that SARS-CoV-2 enters the cells, after binding the human angiotensin-converting enzyme 2 (ACE2) receptor through the spike protein (90) the idea of preparing monoclonal antibodies (MoAb) capable of binding the Spike protein in the receptor-binding domain (RBD) and inhibiting the

Spike/ACE2 binding was pursued. Neutralizing MoAbs (NMoAbs) would inhibit viral replication and cure patients. However, in the following months, it became progressively clear that what might seem a simple and successful idea presented some critical issues: 1) the role of antibodies in the fight against SARS-CoV-2 infection, 2) the decreasing efficacy of the MoAbs during the development of the infection so that the treatment has become half a way between cure and prevention, 3) the cost of antibodies considering the relatively low mortality rate also in patients at high risk of death, 4) the need to use the parental route, making more difficult the administration, 5) relevant changes in the Spike protein over time.

Point 1. The role of antibodies in COVID-19 remains to be fully defined. After SARS-CoV-2 appearance, it was clear within months that the more severe the COVID-19 disease, the higher the anti-Spike antibody titers (91), possibly suggesting that naturally arising antibodies were not protective. The idea seemed to be confirmed when several studies demonstrated that administration of polyclonal antibody-containing sera of patients who recovered from COVID-19 did not cure patients (92-94). In addition, some data suggest that certain patient-produced antibodies may lead to antibody-dependent potentiation (ADE) of the disease, favoring the entry of the virus into cells, as observed for other viruses, including coronavirus (95-98). On the contrary, some studies suggested a protective role of naturally arising antibodies. For example, hospitalized patients with no anti-Spike antibodies showed a mortality rate almost twice that of patients with anti-Spike antibodies (99).

Thus, it seemed reasonable to conclude that antibodies inhibiting ACE2/Spike binding and the entry of virus in the cells are protective if devoided of ADE effect. Indeed, the first clinical studies using one monoclonal antibody (MoAb) or two MoAbs in association demonstrated a relevant protective activity (100, 101).

A second crucial issue (point 2) was the timing of antibody administration relative to the evolution of the infection. Some studies demonstrated that antibodies were effective when administered early (*e.g.*, in the patient positive for SARS-CoV-2 but with few symptoms) and inactive when the patient is hospitalized and/or in intensive care (102,103). Therefore, all antibodies entered in the clinical use must be given as soon as possible, even if the patient does not have a serious disease. The need for early administration made it necessary to establish the type of patients that need to be treated. Indeed, it was and is still impossible to treat all the COVID-19 patients with antiviral MoAb, due to the shortage of the drugs (particularly soon after their approval) and their cost. Moreover, considering the very low mortality rate of COVID-19 in a large portion of the young-adult population, the administration may be non-ethical due to the very low benefit versus the potential risk of adverse events. Therefore, each Health Organization established the patient categories that should be treated, including old patients and those with co-morbidities known to increase the mortality rate (see below). Nonetheless, when treating paucisymptomatic patients, the NNT of antiviral monoclonal antibodies is quite high, ranging between 25 and 29 in the hypothesis of 5% risk of hospitalization (104). Therefore, the cost of the treatments is rather high (point 3).

The need for MoAb administration as soon as possible means that they are given to patients still at home and in a relatively good condition (see below for details). Considering that they must be given through the endovenous route, home administration of the drug to a patient positive for SARS-CoV-2 was a critical issue (point 4), considering the susceptibility of specialized personnel to CoViD-19 (especially before vaccination) and the lack of available medical and paramedical personnel, especially during the pandemic peaks.

The emerging SARS-CoV-2 variant: the most relevant issue

A crucial issue concerning antibodies efficacy is the appearance of variants of concern of

SARS-CoV-2 (point 5). Errors (point mutation) in RNA viruses such as SARS-CoV-2 are a rule. Despite SARS-CoV-2 codes for a polymerase with proofreading activity (105, 106), SARS-CoV-2 variants are quite frequent.

The issue was well known at the beginning of the pandemic. Now we know that the frequency of mutation in the viral RNA coding the Spike is much higher than the frequency of mutation in the RNA coding the other viral proteins (107). In particular, comparing 303,250 human SARS-CoV-2 spike protein sequences with the reference sequence of Wuhan-Hu, authors found mutations of each of the 195 amino acid residues forming the RBD, including the amino acid residues crucial for ACE2 binding (8 residues), which is somewhat surprising. We can conclude that: 1) no amino acid residues are indispensable to bind ACE2, 2) more importantly, we cannot bet on the efficacy over time of neutralizing MoAbs binding the RBD. Reasonably, the high frequency of mutation is due to a selective advantage for the virus having a Spike with a higher affinity for the ACE2 receptor, more able to favor virus entry or not recognized by anti-SARS-CoV-2 Abs produced by the host following infection with another variant of SARS-CoV-2 or the vaccination with a vaccine expressing the Spike of Wuhan-Hu virus.

In theory, the same use of monoclonal antibodies favors the appearance of variants, but we believe that their use in the population had been so infrequent that it did not exert sufficient selective pressure. Moreover, most MoAbs are administered as an association of two antibodies, making unlike the appearance in one virus particle of mutations conferring resistance to both antibodies (108). The consequence of the appearance of specific variants on the efficacy of the antibodies in clinical use will be discussed later.

Interestingly, forty-four invariant residues are present in the Spike protein outside the RBD and correspond to ten domains/regions in the SARS-CoV-2 Spike protein (107), possibly suggesting that MoAbs binding these amino acid residues may be effective not only against the present but also future SARS-CoV-2 variants.

Patients for which MoAbs treatment is indicated

As reported above, not all patients affected by COVID-19 are treated with MoAbs. Treatment is indicated soon after the occurrence of COVID-19 symptoms in non-hospitalized patients with laboratory-confirmed SARS-CoV-2 infection who are at high risk for progressing to severe disease and/or hospitalization. The patients must be aged > 64 years or aged 12-64 years with relevant comorbidities or conditions, such as obesity (BMI > 25), diabetes, cardiovascular and chronic lung diseases, including hypertension. Other patients poorly represented in the study leading to MoAb authorization but considered to be at high risk when infected with SARS-CoV-2 are patients under immunosuppressive treatment or immunocompromised, with chronic kidney disease, pregnant, with neurodevelopmental disorders, conditions that confer medical complexity and dependant on medical-related technological devices. Even infants with less than 1 year are considered at high risk. For sure, the anti-SARS-CoV-2 MoAbs are not authorized for use in the patients hospitalized for COVID-19 and/or who require oxygen therapy due to COVID-19, because MoAbs do not improve any parameter, including survival.

More recently, some anti-SARS-CoV-2 MoAbs have been found to be effective in reducing the risk of infection when used as pre-exposure prophylaxis (109) and as post-exposure prophylaxis in a household and other high-risk settings (110, 111).

The list of patients who are to be treated with anti-SARS-CoV-2 monoclonal antibodies overlaps with that of patients that should be treated with anti-SARS-CoV-2 small molecules (see paragraph Antiviral small molecules). Future studies will indicate which drug class has to be preferred in a specific category of patients also regarding the safety, the cost, and availability of the drugs.

Anti-SARS-CoV-2 MoAbs with emergency use authorization/full authorizations from EMA/FDA

Eight anti-SARS-CoV-2 MoAb products have received emergency use authorizations from EMA and/or FDA. They are bamlanivimab plus etesevimab given in association (previously called LY-CoV555 and LY-CoV016, respectively), casirivimab plus imdevimab given in association (previously called REGN10933 and REGN10987, respectively), regdanvimab (previously called CT-P59), tixagevimab and cilgavimab (previously called COV2-2196 and COV2-2130, respectively), and sotrovimab (previously called VIR-7831, the parent MoAb of S309).

Bamlanivimab, etesevimab, casirivimab, imdevimab, regdanvimab, tixagevimab, and cilgavimab are neutralizing mAbs binding to the RBD of SARS-CoV-2 Spike protein. Bamlanivimab and etesevimab bind to different but overlapping epitopes, whereas casirivimab/imdevimab and tixagevimab/cilgavimab bind to non-overlapping epitopes. Regdanvimab is not given in association.

Phase 3 BLAZE-1 trial had demonstrated that bamlanivimab plus etesevimab, compared to placebo, was associated with 4.8% absolute reduction and 70% relative reduction in COVID-19-related hospitalizations or all-cause deaths (112). Casirivimab plus imdevimab, compared to placebo, was associated with 7.5% absolute reduction and 70% relative risk reduction in COVID-19-related hospitalizations or all-cause deaths (113). Regdanvimab, compared to placebo, was associated with 2.2% absolute reduction and 78% relative risk reduction in progression to severe COVID-19 disease (114).

In March 2021 EMA's Committee for Medicinal Products for Human undertook the review of data on bamlanivimab plus etesevimab as part of a rolling review and supported the use at the National level before market authorization. On November 2, 2021, the manufacturer informed the EMA of the decision to withdraw from the approval process. The broad distribution of bamlanivimab plus etesevimab has been paused in the United States because the Omicron variant has markedly reduced in vitro susceptibility to this mAb regimen (see below) (115). In Italy, the authorization for the temporary use of bamlanivimab as monotherapy was revoked in May 2021, while, on March 1, 2022, the authorization for the use of the association has not yet been revoked.

On November 11, 2021, EMA's CHMP has recommended authorizing regdanvimab and the association of casirivimab with imdevimab for treating patients with COVID-19. The recommended dosage of regdanvimab in adults is a single IV infusion of 40 mg/kg within 7 days of developing symptoms of COVID-19. Casirivimab and imdevimab are administered at the dose of 600 mg each by IV infusion or by SC injection within 7 days of developing symptoms of COVID-19. Moreover, Casirivimab and imdevimab can be used to prevent COVID-19 after contact with an infected person or even when no contact has occurred. Moreover, a recent study demonstrated that hospitalized patients receiving high doses of casirivimab plus imdevimab (4,000 mg each) showed a significant reduction in 28-day all-cause mortality when seronegative for the anti-spike protein antibody (24% mortality in the mAb-treated group *vs.* 30% mortality in the standard care group) (99). However, the treatment of hospitalized patients is authorized by neither EMA nor FDA.

Tixagevimab and cilgavimab are in rolling review at EMA and have received emergency use authorization by FDA for the pre-exposure prophylaxis of COVID-19. In Italy, its temporary distribution was authorized for the prophylaxis of COVID-19 on 28 January 2022. Tixagevimab and cilgavimab were optimised using a proprietary half-life extension technology, which could afford up to 12 months of protection. An interim analysis of the PROVENT phase III trial having as the primary efficacy endpoint the first case of any SARS-CoV-2 RT-PCR positive symptomatic illness occurring post-dose prior to 6 months, demonstrated a reduced risk of

developing symptomatic COVID-19 (HR 0.23 with a median follow-up 83 days and HR 0.17 with a median follow-up 6.5 months) (116). Moreover, there were no severe or critical COVID-19 events in the antibody group compared to 5 in the placebo group. Tixagevimab and cilgavimab should be given as separate, sequential IM injections at different injection sites, preferably one in each of the gluteal muscles. The recommended dosage is 150 mg of each mAb every 6 months. The incidence of serious cardiac adverse events (*e.g.*, myocardial infarction, cardiac failure, arrhythmia) was higher in the antibody group than in the placebo group (0.6% *vs.* 0.2%) (116).

Sotrovimab is a neutralizing mAb binding to SARS-CoV-2 Spike protein outside the RBD. In particular, it recognizes an epitope that is highly conserved within the Sarbecovirus subgenus and prevents the virus from entering the cell by inhibiting the mechanisms downstream of the spike/ACE2 bond (117). Interestingly, it was derived from a parent antibody (S309) isolated for the first time in 2003 from an individual who recovered from SARS (118). Sotrovimab was designed to possess an Fc LS mutation (M428L/N434S) which confers greater binding to the neonatal Fc receptor resulting in prolonged half-life. Sotrovimab also demonstrated antiviral activity through antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell phagocytosis (ADCP) of virus-infected cells. In the first studies demonstrating the efficacy of Sotrovimab, three patients (1%) in the sotrovimab group, as compared with 21 patients (7%) in the placebo group had disease progression leading to hospitalization or death with a relative risk reduction of 85%. Moreover, only in the placebo group, five patients were admitted to the intensive care unit, including one who died (119). On December 17, 2021, EMA's CHMP has recommended sotrovimab for treating patients with COVID-19. The recommended dosage of sotrovimab in adults is a single IV infusion of 500 mg within 5 days from the developing symptoms.

Safety

The safety of anti-SARS-CoV-2 antibodies is quite high. Anaphylaxis and infusion-related reactions have been reported in a few patients who received anti-SARS-CoV-2 mAbs. More frequently, it is observed nausea, vomiting, diarrhea, dizziness, hyperglycemia, rash, and pruritis (120-124).

Neutralizing activity of MoAbs on SARS-CoV-2 variants

The above-mentioned MoAbs have been tested in clinical studies when the SARS-CoV-2 variant of concern Omicron was not present and most of them were effective in the treatment of patients infected with variants other than the Omicron variant. The Omicron variant encodes 37 amino acid substitutions in the Spike protein, 15 of which are in the RBD, and represents a major antigenic shift in SARS-CoV-2. Indeed it determines a marked reduction in neutralizing activity in plasma from convalescent patients and individuals who had been vaccinated against SARS-CoV-2. Due to the high infectivity of Omicron, currently, most patients are infected by this variant.

Some studies evaluated whether the above-described MoAbs retain neutralizing activity against Omicron variant (125, 126). For all the MoAbs binding the RBD of the Spike protein, a significant drop in the neutralizing activity was described (in practice, loss of activity), with the only exception of cilgavimab, which showed a slight drop only (about 12 fold decrease). Interestingly, the neutralizing activity of sotrovimab, binding to the Spike protein outside the RBD, was minimally affected. Consequently, FDA assessed that "the broad distribution of bamlanivimab plus etesevimab and casirivimab plus imdevimab has been paused because the products have reduced activities against Omicron variant of concern" (115, 127).

Anti-SARS-CoV-2 MoAbs in the clinical study

Several MoAbs are still in the clinical study. Two approaches appear very interesting: 1) MoAbs

binding to the Spike protein outside the RBD and active against Omicron variants (128); 2) The MoAbs MAD0004J08 showing an extremely high affinity for the RBD of the S protein and being one of the most potent antibodies selected by screening 453 neutralizing antibodies produced by B lymphocytes from 14 COVID-19 survivors (129). Its potency allows administration by i.m. injection and lower production cost.

ANTIVIRAL SMALL MOLECULES

At the outbreak of the pandemic, the available antiviral drugs seemed the obvious choice to fight the SARS-CoV-2 virus responsible for COVID-19. The virus was new, but it was an RNA virus of which much was known about biological and pathological characteristics. The pathology caused by SARS-CoV-2 infection, that is COVID-19, indeed showed entirely new and unexpected characteristics. We were therefore faced with a new virus and a new pathology. Obviously, neither against the first nor against the second there were already specific drugs available. The biological characteristics of the virus, in particular being an RNA virus, have however suggested the possibility of contrasting it with anti-retroviral drugs developed for similar viruses, such as those against HIV. This is why the WHO immediately suggested carrying out a multicenter study using the Lopinavir-Ritonavir combination, a drug capable of inhibiting viral- RNA-dependent RNA-polymerase. Also, the fact that SARS-CoV-2 was a member of the beta coronavirus family suggested that it could be contrasted with other drugs such as those developed for the treatment of the less-lethal but very widespread influenza viruses. Thus, antiviral drugs such as Favipiravir, Oseltamivir, Umifenovir and Ribavirin have been studied in different combinations. Obviously, antiviral drugs with activity on liver RNA viruses, such as those for hepatitis B (Remdesivir) and for hepatitis C (Sofosbuvir), have not been ignored.

The results obtained using these antiviral drugs have often been very disappointing. These drugs were expected to reduce the spread of the virus in the body and, consequently, the severity of COVID-19. Indeed, in the various clinical studies of which the outcomes have been reported, there have been no significant advantages both in reducing the severity of the disease and even less in mortality. In some studies, the lack of therapeutic success was attributed to the viral load, while in others to the advanced state of the disease. Ultimately, regardless of the drug combinations used, the state of the temporal course of the infection or the state of the pathology, with the exception of Remdesivir, which, in some cases, has reduced the risk of aggravation of the disease and consequent hospitalization of the patient, all other approaches have reported negative or unsuitable results for planning the use of these drugs in an appropriate and more extensive manner.

Of these antiviral drugs, their pharmacological and therapeutic characteristics and their effects in patients with COVID-19 have been revised in an exhaustive review that summarizes their value in controlling COVID-19 and in the progression of this disease to more severe stages leading to hospitalization and/or death (130). Indeed, another review (131) written at the end of 2020, already anticipated the often discordant and almost always negative results of the use of these drugs in patients with different statuses of COVID-19 severity. In this work, the reader can find the tables that summarize the results of clinical studies, often well-controlled, which highlight Remdesivir, among all the antiviral drugs examined, for which a certain response, expressed as a reduction in hospitalization and the risk of disease progression, was found in 3 of the four studies examined.

In the present review we will focus on the three antivirals currently authorized by regulatory agencies, remdesivir, molnupiravir and paxlovid; the second with a mechanism similar to that of remdesivir, and the third totally new and with a new and different molecular target.

Remdesivir

Remdesivir is the first antiviral medicine to be authorised by the European Medicines Agen-

cy (EMA) with specific indication for the "treatment of coronavirus disease 2019 (COVID-19) in adults and adolescents (aged 12 years and over and weighing at least 40 kg) with pneumonia requiring supplemental oxygen therapy". In December 2021, the EMA authorized an extension of indication relating to the treatment of coronavirus disease 2019 (COVID-19) in "adults who do not require supplemental oxygen therapy and have an increased risk of progression to severe COVID-19".

Remdesivir is a monophosphoramidate nucleoside analogue prodrug that was originally developed for Ebola virus and utilized in response to the 2014–2016 outbreak in West Africa (132, 133). It displayed broad-spectrum activity against different coronaviruses in preclinical models and has been suggested for COVID-19 clinical trials (132,134,135). It competes with endogenous nucleotides for incorporation into replicating viral RNA through the RNA-dependent RNA polymerase (RdRp) and inhibits viral replication (132). The RdRp is an attractive target for antiviral drugs, as it is highly conserved across coronaviruses. As a prodrug, remdesivir undergoes intracellular conversion by kinases to its active nucleoside triphosphate metabolite. Remdesivir and its metabolites display higher selectivity for RdRp compared to human polymerases (132).

Coronaviruses express a unique exoribonuclease (ExoN) which functions as a proofreading enzyme correcting errors in the growing RNA chain (136). The development of effective nucleoside analogues is, therefore, particularly challenging. Remdesivir is able to partly evade proofreading and maintain potent antiviral activity in the presence of ExoN. The reason remdesivir's activity is only modestly decreased by ExoN relates to two unique properties: i) it is incorporated into replicating RNA more efficiently than natural nucleotides (136-138); ii) it functions as a non-obligate or delayed RNA chain terminator (136-138). The incorporation of the delayed chain terminators perturbs the RNA structure, and synthesis is halted at some point downstream (138). In SARS-CoV-1, SARS-CoV-2, and MERS-CoV, remdesivir consistently induces chain termination after the addition of three nucleotides (136, 137), thus escaping ExoN excision.

Remdesivir is administered intravenously and is a substrate of several cytochrome P450 enzymes in vitro, however clinical implications are unclear since the prodrug is rapidly metabolized by plasma hydrolases (139). Consequently, hepatic impairment has little effect on remdesivir plasma levels, although specific studies have not been conducted in patients with hepatic impairment, and the drug is contraindicated in patients with severe hepatic impairment (139). Remdesivir exhibits low renal excretion (< 10%) (140). To date, there are no recommendations for dose adjustments in patients with mild to moderate renal impairment. There are no PK data available for children or women who are pregnant or breastfeeding.

The main randomized studies that evaluated the clinical efficacy of remdesivir in the treatment of hospitalized subjects, albeit open and with different primary endpoints, consistently did not show clinical benefit of remdesivir regarding mortality (141-145), with the exception of a clinical trial carried out among non-hospitalized patients who were at high risk for COVID-19 progression (146). In this study, a 3-day course of remdesivir had an acceptable safety profile and resulted in an 87% lower risk of hospitalization or death than placebo (146). Beneficial effects on time to recovery are confirmed in a single study, especially in the lower-risk population (subjects receiving low-flow oxygen therapy and starting treatment within 10 days of the onset of symptoms) (147).

Remdesivir is generally well tolerated and adverse effects are rare. However, since early reports, transient asymptomatic alanine amino-transferase (ALT) elevations were observed in most subjects in PK studies (148, 149). Transaminase increases have also been reported in COVID-19 patients treated with compassionate use remdesivir (150-152). Although transaminase elevation has been reported as a feature of COVID-19, there is a concern for possible

hepatotoxicity associated with remdesivir (152, 153). Based on the data regarding the adverse effects of remdesivir on hepatic function, caution must be taken by evaluating baseline liver function, avoiding the use of potentially hepatotoxic drugs, and monitoring liver function when using remdesivir in patients hospitalized with COVID-19 (153).

Molnupiravir

On 19/11/2021, the EMA's Committee for Medicinal Products for Human Use issued an opinion on the use of Lagevrio (the trade name of molnupiravir) for the treatment of COVID-19. The medicine can be used to treat adults with COVID-19 at high risk of developing severe forms of the disease.

Molnupiravir is an oral antiviral also known by the names EIDD-2801 and MK4482. The drug was originally developed by Drug Innovation Ventures at Emory University and subsequently acquired by Ridgeback Therapeutics in partnership with Merck & Co, USA. It belongs to the class of ribonucleoside analogues with broad-spectrum antiviral activity against a series of RNA viruses, including coronaviruses. MK-4482 was first developed as a flu shot and later "repositioned" as an oral treatment for adults with COVID-19 in a mild to moderate form. MK-4482 is a prodrug that is rapidly absorbed in the intestine and hydrolyzed into the ribonucleoside analogue N-hydroxycytidine (NHC) (154), which is widely distributed to tissues (including lungs and brain) and, similarly with remdesivir, converted to the pharmacologically active triphosphate form (NHC-TP).

The mechanism of the antiviral activity of MK-4482 is a two-step process that inhibits the RdRp through an accumulation of viral mutations beyond a biologically tolerable threshold, with consequent impairment of the normal fitness of the virus, leading to its death (154, 155). In fact, coronaviruses use the RdRp for the replication and transcription of their RNA genomes and it is therefore clear that this enzyme represents an important target for hitting the virus (156). This mechanism is distinct from that of remdesivir in which its incorporation into nascent RNA causes premature termination of RNA synthesis, stopping the growth of the RNA strand after the addition of some nucleotides. Because of this, MK-4482 has demonstrated *in vitro* activity against remdesivir-resistant SARS-CoV-2. Given its unique mechanism of action, NHC is expected to be active against viruses resistant to other antiviral agents.

At the start of the pandemic, MK-4482 was in the preclinical phase as an anti-flu drug, but a number of factors helped to move the molecule quickly into phase 1. These include: i) the favorable characteristics of the molecule to meet public health needs, 2) the in-depth non-clinical program that included model testing of various viral diseases and 3) collaboration between sponsors, multinational CROs and regulatory agencies in the US and UK (157,158). Based on the results of the planned interim analysis of the Phase 3 MOVe-OUT study (NCT04575597), Merck has discontinued patient enrollment and sought approval from the FDA. The planned interim analysis evaluated data from 775 patients enrolled in the Phase 3 MOVe-OUT study through August 5, 2021. Specifically, molnupiravir significantly reduced the risk of hospitalization or death in non-hospitalized at-risk adult patients with COVID-19 mild to moderate. In the interim analysis, molnupiravir reduced the risk of hospitalization or death by approximately 50%; 7.3% of patients receiving molnupiravir were hospitalized or died until day 29 after randomization (28/385), versus 14.1% of patients treated with placebo (53/377); p = 0.0012. Up to day 29, no deaths were reported in patients who received molnupiravir, compared with 8 deaths in patients who received placebo. The incidence of any adverse events was comparable in the molnupiravir and placebo groups (35% and 40%, respectively). Similarly, the incidence of drug-related adverse events was also comparable (12% and 11%, respectively). Fewer subjects discontinued study therapy due to an adverse event in the molnupiravir group

(1.3%) compared to the placebo group (3.4%). On the recommendation of an independent data monitoring committee and in consultation with US FDA, recruitment into the study was terminated early based on these positive results. In England, on November 4, 2021 molnupiravir was approved by the UK drug regulatory agency (Mhra) under the trade name of Lagevrio.
Molnupiravir displays in vitro activity against SARS-CoV-2 variants of concern such as B1.1.529 (omicron) (159-164), and B.1.1.7 (alpha), B.1351 (beta), P.1 (gamma) and B.1.617.2 (delta) (Merck Sharp & Dohme (UK) Limited. Lagevrio 200 mg hard capsules: UK prescribing information 2021) (165).

Paxlovid

Paxlovid, is the combination of Pfizer's investigational antiviral PF-07321332 (nirmatrelvir) and a low dose of ritonavir, an antiretroviral drug traditionally used to treat HIV. On April 6, 2021, Pfizer released the structure of an inhibitor of the 3-CL^{PRO} enzyme of the SARS-CoV-2 virus, named PF-07321332, which has been shown to be able to suppress the replication of the virus in human cells at submicromolar concentrations (166-168). PF-07321332 is the first molecule to target the SARS-CoV-2 main protease (3-CL^{PRO}). 3-CL^{PRO} is responsible for the cleavage of SARS-CoV-2 polyproteins 1a and 1b. Without the activity of SARS-CoV-2 3-CL^{PRO}, non-structural proteins 1a and 1b (including proteases) cannot perform their functions and, consequently, viral replication is inhibited (169-170). In particular, PF-07321332 is an inhibitor of a cysteine residue of 3-CL^{PRO} responsible for the enzymatic activity of the protease. The co-administration of a low dose of ritonavir (a drug used to treat HIV) helps slow down the metabolism, in which cytochrome p450 enzymes are involved, and breakdown of PF-07321332 and, consequently, to maintain higher concentrations for longer times resulting in a prolongation of its activity. A Phase 1 study (NCT04756531), conducted in double-blind and in which both single and multiple doses were tested, evaluated the safety, tolerability and pharmacokinetics of PF-07321332 in healthy individuals (171).
On November 5, 2021, Pfizer announced the first results of the NCT04960202 EPIC-HR (Evaluation of Protease Inhibition for COVID-19 in High-Risk Patients) trial. The EPIC-HR trial is a quadruple-blind study (NB: double-blind is specified in the title of the trial, but under MASK it is reported that the study was conducted so that the "Participant, Care Provider, Investigator, Outcomes Assessor" the type of treatment proposed was masked (171)) on non-hospitalized adult patients with COVID-19, who are at high risk of developing severe disease. The interim analysis assessed data from 1219 adults enrolled by September 29, 2021. By the time of the decision to stop patient recruitment, enrollment had reached 70% of the expected 3,000 patients from clinical trial centers throughout North and South America, Europe, Africa and Asia, with 45% of patients in the United States. Enrolled individuals had a laboratory-confirmed diagnosis of SARS-CoV-2 infection within a five-day period, with mild to moderate symptoms, and must have had at least one medical condition associated with an increased risk of developing COVID-19 severe. Each patient was randomized (1:1) to receive orally Paxlovid or placebo every 12 hours for five days. The scheduled interim analysis showed an 89% reduction in the risk of hospitalization or death from any cause related to COVID-19 compared to placebo in patients treated within three days of symptom onset (primary endpoint). On day 28, 0.8% of patients treated with Paxlovid went into hospitalization (3/389 hospitalized and 0 deaths), compared with 7.0% of patients who received placebo and were hospitalized or died (27/385 hospitalized with 7 subsequent deaths) ($p < 0.0001$). Similar rates of COVID-19-related hospitalization or death have been observed in patients treated within five days of symptom onset. Specifically, 1.0% of patients treated with Paxlovid were hospitalized (6/607 hospitalized, 0 deaths), compared to 6.7% of pa-

tients who received placebo (41/612 hospitalized with 10 subsequent deaths). ($p < 0.0001$). Overall, in the global population, no deaths were reported in patients who received Paxlovid compared with 17 (1.6%) deaths in patients who received placebo.

The review of the safety data included a larger cohort of 1881 patients in EPIC-HR, whose data were available at the time of the analysis. Treatment-associated adverse events were comparable between Paxlovid (19%) and placebo (21%), most of which were mild in intensity. Among patients evaluable for av events, fewer serious adverse events (1.7% *vs.* 6.6%) and fewer study drug discontinuation (2.1% *vs.* 4.1%) were observed in patients treated with Paxlovid versus those receiving placebo, respectively.

Paxlovid will be administered twice daily for five days at a dose of 300 mg (two 150 mg tablets) of PF-07321332 with one 100 mg tablet of ritonavir.

CONCLUSIONS AND PERSPECTIVES

The pharmacologic approach to control the SARS-CoV-2 diffusion in humans and the consequent COVID-19 pathology has been challenging the scientific community in the last couple of years. Here we focus on the two main aspects governing the pharmacological approach to this pandemic: i) the possibility of using drugs already available and ii) the need for new and appropriate drugs for this specific virus. After two years of a considerably high number of experiences (clinical trials of different kinds with a number of drug candidates, mainly based on the concept of "try-and-error" research) we can conclude that we have selected and adapted old drugs (treatment of COVID-19) and we have developed new drugs for the SARS-CoV-2 (prevention of COVID-19). It is reasonable to think that the results obtained are the best we could get in this short time-lapse.

The main medical aspects of the SARS-CoV-2 infection are a strong inflammation, variably distributed in different organs but with a particular propensity for the respiratory system, associated with the risk of blood coagulation. We were prepared for treating such diseases since anti-inflammatory drugs were available either from the panel of anti-cytokine medicines (small molecules or MoAbs) or with corticosteroids. By generalizing the observed results, we can admit that corticosteroids helped COVID-19 patients much more than the anti-cytokine drugs. The anticoagulants were the other family of drugs that made the difference between life and death in COVID-19 patients. All major international Societies on thrombosis rapidly produced and diffused the guidelines for the best use of anticoagulation in high-risk patients.

These approaches can be considered the best treatment options for patients with COVID-19. It must be said that all the other drugs tested on COVID-19, all of them selected on the basis of their mechanism of pharmacological action, almost failed or showed minimal effectiveness, often because of the low degree of the trial with which they were examined.

Better results, considering the appearance of new drugs, were observed with the prevention of the COVID-19, namely the control of SARS-CoV-2 infectivity. In this case, we have two separate approaches being developed: MoAbs directed to control the virus's ability to bind to the target cells and small molecules (conventional antiviral drugs) hampering the viral replication inside the infected cells. The knowledge of the virus's chemical structure and the molecular biology of its replication has considerably helped the research for optimal treatment options.

Similar to what was shown with anti-COVID-19 drugs, also these approaches suffered from successes and failures. At the beginning of the pandemic, when the only available data simply indicated that SARS-CoV-2 was a RNA coronavirus, the idea of the control of the infection lead to the use of antiviral agents already in our hands and known to be active against viruses with similar replicative steps. Unfortunately, among all the antiviral drugs tested, only

remdesivir showed some activity, sufficient to convince the regulatory agencies to suggest its use to contain the viral diffusion inside the body of the infected patients. Similar success has been documented with another repositioned anti-viral small molecule, molnupiravir. The target is the same as remdesivir, *e.g.* the RdRp, but the consequences on the viral replication, at least from the molecular aspect of this interaction, are dramatically greater, leading to the accumulation of mutations ending with a sort of replicative catastrophe. However, the most real advantage in the control of viral diffusion in the body is given by the new anti-viral small molecule nirmatrelvir that, when used in combination with ritonavir (namely Paxlovid), warrants a greater than 90% protection against the development of a severe COVID-19. The target of nirmatrelvir (the viral protease) is a specific locus of the protein that is relevant for SARS-CoV-2, and this makes the difference from the other re-positioned drugs that were tested and found inactive.

On the other hand, the control of patient's infection towards a severe COVID-19 with MoAbs showed the most intense activity by the pharmaceutical companies. A number of MoAbs became rapidly available, mostly targeting the viral proteins responsible for the viral attack on the target cells. The chapter on these drugs is exhaustive and here we simply remark the advantages and limitations of these therapies. The most important advantage is the high specificity of the MoAbs therapy and the rapid washout of the viruses from the body. At the same time, the high specificity of these drugs is also their weakness, given the high rate of mutations of their target operated by the SARS-CoV-2. In fact, the experience with these drugs showed how the virus mutated the target proteins without losing its ability to infect the target cells. This viral behaviour made the MoAbs to rapidly reduce their effectiveness with the appearance of the new variants of SARS-CoV-2, a process that forced the use of combinations of these MoAbs to prevent the viral escape.

In conclusion, the take-home messages of the pharmacological experience for the control of the SARS-CoV-2 pandemic, a very important message also for the pharmacological discipline as a whole, can be summarized as follows. Drug repositioning cannot be successful simply based on the knowledge of their molecular mode of action and the new drugs, even though based on a specific and selective target, may need a continuous arrangments in order to fulfill a complete therapeutic success.

Nonetheless, the experiences gained during these two years in the pharmacological treatment of the virus responsible for the pandemic and COVID-19 has demonstrated the possibility of significantly accelerating the development of new drugs with measurable innovation.

The MoAbs have highlighted the rapid versatility of their curvature on the targets of the virus in constant evolution and, in perspective, solve the pharmacokinetic problems of the earlier preparations with measures that significantly extend the therapeutic range. It is hoped that the new MoAbs under development will meet the needs of prescribers and patients and, together with the development of new vaccines, will prevent the spread of the virus in the body, hospitalizations and deaths of patients. In this context, an important role is attributed to the new antiviral drug nirmatrelvir, whose most intriguing advantage over existing antivirals is that it has a peculiar mechanism of action on a specific target of SARS-CoV-2 and can be easily taken orally compared to MoAbs. Considering that the SARS-CoV-2 pandemic may stabilize with an annual frequency very similar to winter flu, it is desirable that this molecule provides the inpetus for the development of other specific agents against SARS-CoV-2. Thus, this virus can be expected to offer a range of therapeutic options for its control, regardless of the type of mutations that will occur in the future. Indeed, in addition to nirmaltrevir, other products could be developed that target conserved viral pathways or whose mutations are

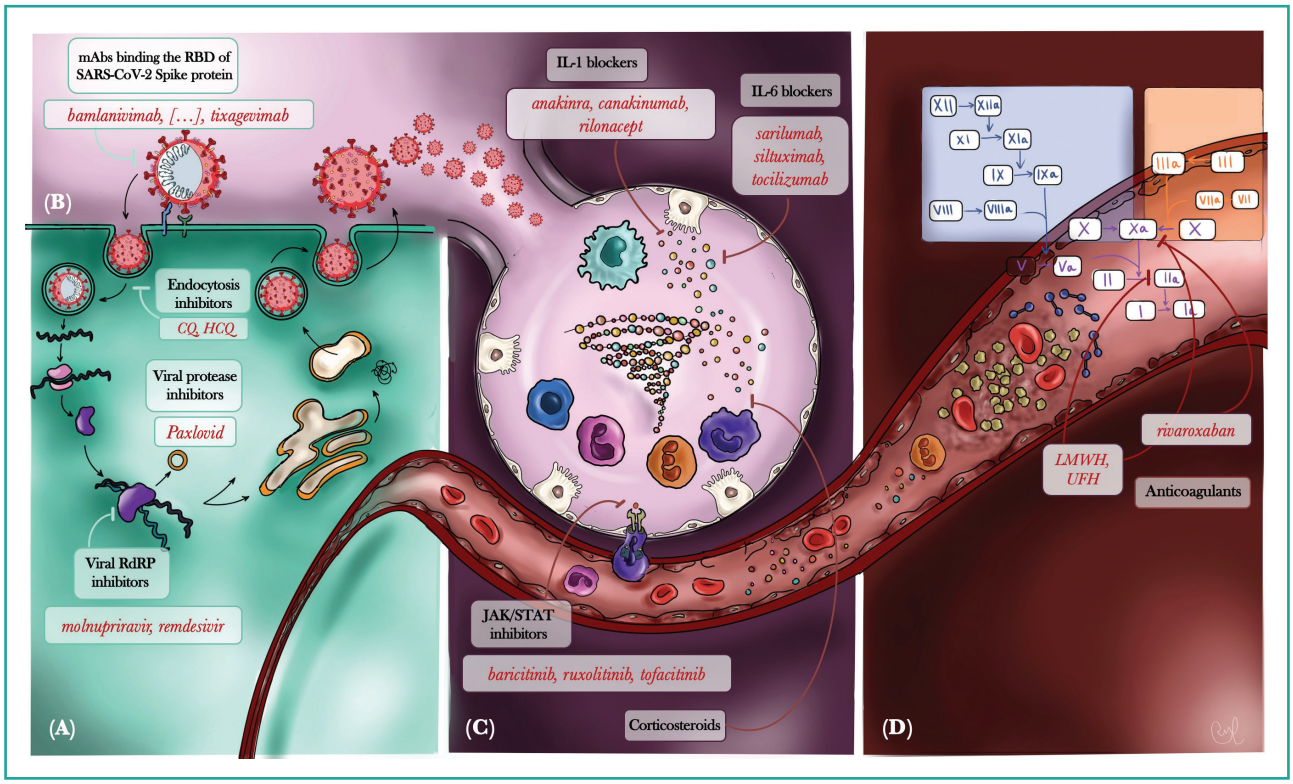

Figure 1. The main drugs studied and/or recommended for the treatment of CoViD-19 and their therapeutic targets. This figure summarizes the available or proposed medications for COVID-19 prevention or treatment and their sites of action.
(A) Drugs that inhibit viral replication. Remdesivir, paxlovid (nirmatrelvir/ritonavir) and molnupiravir are small antiviral molecules recommended by AIFA for the treatment of adults with CoViD-19 at high risk to develop severe disease. These drugs inhibit viral replication. Chloroquine (CQ) or hydroxychloroquine (HCQ) are not recommended. **(B) Monoclonal antibodies (mAbs) to prevent virus attachment to cellular proteins**. Monoclonal antibodies directed towards the RBD of the SARS-CoV-2 Spike proteins are approved as an early treatment or as pre-exposure or post-exposure prophylaxis in high-risk patients (see text for further details). **(C) Drugs that modulate the host inflammatory response**. A number of IL-1 blockers, IL-6 blockers, Jak-Stat inhibitors have been tested as repurposed drugs to dampen the host inflammatory response and the "cytokine storm" that might lead to severe complications such as acute respiratory distress syndrome (ARDS) in the lungs, intravascular coagulation, multiorgan failure, and ultimately death. Corticosteroids are also standard therapy for hospitalised patients requiring supplemental oxygen therapy (with or without mechanical ventilation) and are also recommended for home management of patients with severe CoViD-19 disease requiring supplemental oxygen. **(D) Anticoagulants**. Unfractionated heparins (UFH) or low molecular weight heparins (LMWH) are approved for the prophylaxis of thromboembolic events in patients with an acute respiratory infection and limited mobility. Oral anticoagulants such as rivaroxaban have also been tested in clinical trials but showed no evidence of efficacy.
CQ: chloroquine; HCQ: hydroxychloroquine; LMWH: low molecular weight heparins; UFH: unfractionated heparins.

extremely rare, ensuring the stability of the product in therapy.

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ETHICS

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

Authors' contributions
AB contributed to Anticytokine agents for COVID-19 treatment, ABi contributed to Repurposed drugs for the control fo COVID-19, AG contributed to Anticoagulation treatment for COVID-19, GN contributed to Anti-SARS-CoV-2 monoclonal antibodies, SP contributed to the Indroduction, MP contribute to Antiviral small molecules, GS contributed to Introduction, Antiviral small molecules, Conclusions and to the complete revision of the review, and GR contributed to the revision of the review.

Availability of data and materials
The data underlying this manuscript are available in the article.

Ethical approval
N/A.

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PERSPECTIVE

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TRANSLATION OF BERGAMOT ESSENTIAL OIL IN CLINICAL TRIAL FOR CONTROL OF BEHAVIOURAL AND PSYCHOLOGICAL SYMPTOMS OF DEMENTIA

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SUMMARY

The essential oil of bergamot (BEO; *Citrus bergamia Risso et Poiteau*) is endowed with analgesic activity in inflammatory and neuropathic pain models. Modulation of endogenous peripheral and central opioid system and morphine dose rescue are among the main pharmacological activities of the phytocomplex. Due to the tight link between undertreated pain and agitation in patients suffering from severe dementia, aromatherapy can turn out to be a useful approach if an essential oil with powerful analgesic activity is used. Methodological limitations of most aromatherapy trials hamper any conclusion about its effectiveness in dementia. Based on the strong preclinically proven antinociceptive and anti-allodynic activity of BEO, a nanotechnology-based delivery system consisting of odorless alpha-tocoferyl stearate solid lipid nanoparticles loaded with BEO deprived of furocoumarins (NanoBEO; patent EP 4003294), has been engineered and tested, confirming the previously demonstrated efficacy of the phytocomplex. Thus, the actually active BRAINAID (NCT04321889) double-blind, randomized, placebo-controlled, clinical trial has been designed to assess the effectiveness of NanoBEO on agitation and pain in severely demented patients to offer a safe tool able to provide relief to this fragile population.

Key words
Bergamot essential oil; agitation; pain; dementia.

Impact statement
BEO is the first essential oil to be deviced in a nanotechonology delivery system (NanoBEO) to allow double-blind, randomized, clinical trial (NCT04321889) for the control of agitation in patients with severe dementia.

INTRODUCTION

Bergamot is a citrus fruit classified as *Citrus bergamia*, Risso belonging to the *Rutacee* family, genus *Citrus*. The essential oil of bergamot (BEO) is obtained by cold pressing of the epicarp and, partly, of the mesocarp of the fresh

fruit, according to what is reported in the *Farmacopea Ufficiale Italiana* (1). It is world widely known for its great demand by perfumery and cosmetic industries but also employed by pharmaceutical, food and confectionery industries. BEO is composed of a volatile (93-96% of total) and a non-volatile (4-7% of total) fraction. Among these, the volatile contains oxygenated compounds, as linalool and linalyl acetate, and monoterpenes and sesquiterpenes, as limonene, mainly responsible for its pharmacological activity (2). On the other hand, the non-volatile contains coumarins and psoralens (3), within which bergapten is responsible for phototoxicity (4). Essential oils extracted from different organs of aromatic plants, have been extensively used in aromatherapy for mood disturbances for inhalation or massage, although their effectiveness remains controversial due to the lack of adequate methodology in preclinic and clinic studies originating poor quality evidence, mainly in dementia (5). In fact, insufficient methodological quality of clinical trials has been highlighted already two decades ago (6); indeed, despite the increased amount of clinical trials (**figure 1**) the critical appraisal cannot be considered remarkably improved, as demonstrated by Cochrane analyses concerned with aromatherapy confirming conduct or reporting problems in half of the studies or inconsistent results, preventing from drawing any convincing evidence (5, 7).

With all the above in mind, over the last two decades a series of controlled preclinical researches have led to the characterization of the pharmacological profile of BEO, that now provides the rationale for its clinical translation. BEO has been proven to interfere with basic mechanisms finely tuning synaptic communication, modulating excitatory amino-acids release and affording neuroprotection (**figure 2**). In particular, brain microdialysis and synaptosomes superfusion have demonstrated that BEO modulates hippocampal synaptic amino acid neurotransmitters: at low concentrations it causes exocytosis of glutamate from presynaptic nerve endings, while at high concentrations it may induce glutamate release *via* a Ca2+-independent, carrier mediated, process (9). Moreover, it induces neuroprotection in focal cerebral ischemia preventing glutamate accumulation (10), along with blockade of spinal Extracellular Signal-Regulated Protein Kinase

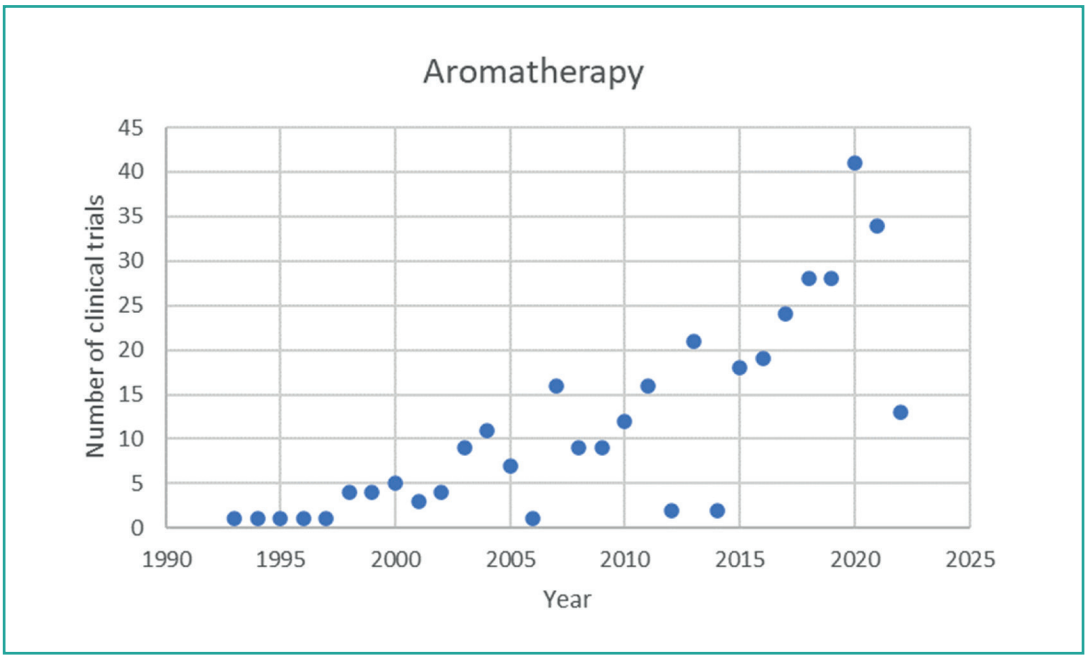

Figure 1. Studies concerned with aromatherapy clinical trials since PubMed/MEDLINE inception (date of last search May 16th, 2022).

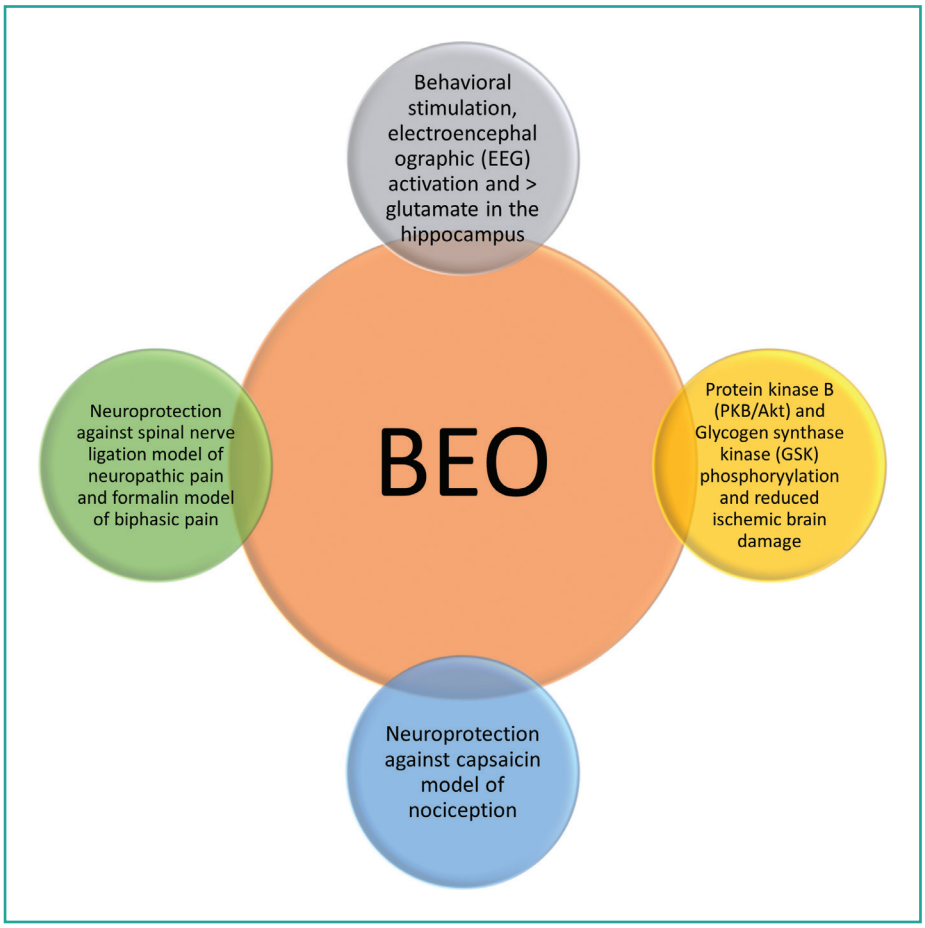

Figure 2. Neuropharmacological and behavioral effects of BEO (adapted with permission from (8)).
EEG = electroencephalography; Akt = Protein kinase B, PKB; GSK = Glycogen synthase kinase).

(ERK) activation, and it enhances autophagy (11), an evolutionarily conserved process undergoing derangement in chronic pain (12). Finally, BEO exerted anxiolytic-like effects, not superimposable to those of diazepam, thus devoid of sedative action, and involving serotonergic neurotransmission in the animal behavioural tasks Open Field Test, Elevated Plus Maze Test and Forced Swimming Test (13).

PHARMACOLOGICAL ACTION OF BEO IN PAIN

The potential for analgesic efficacy of BEO has been investigated in models of inflammatory (capsaicin model in mice), neuropathic [mice subjected to spinal nerve ligation (SNL) or partial sciatic nerve ligation (PSNL)] and biphasic pain (formalin test in mice). In particular, BEO has proven to reduce the time spent in licking/biting induced by the intraplantar (i.pl.) administration of capsaicin, an acute inflammatory algogen input (14, 15). Interestingly, BEO resulted effective also in the formalin test, reputed relevant to clinic conditions due to its biphasic nature (16), characterized by: 1) an early phase of about 5-10 minutes since formalin injection, resulting from the direct activation of nociceptive primary afferents; 2) a late phase, following the recovery interphase and up to 30 min following the administration of formalin, produced by sensitization of the dorsal horn neurons (17). In particular, BEO exerts analgesia in both phases of the formalin test (18), also when adminis-

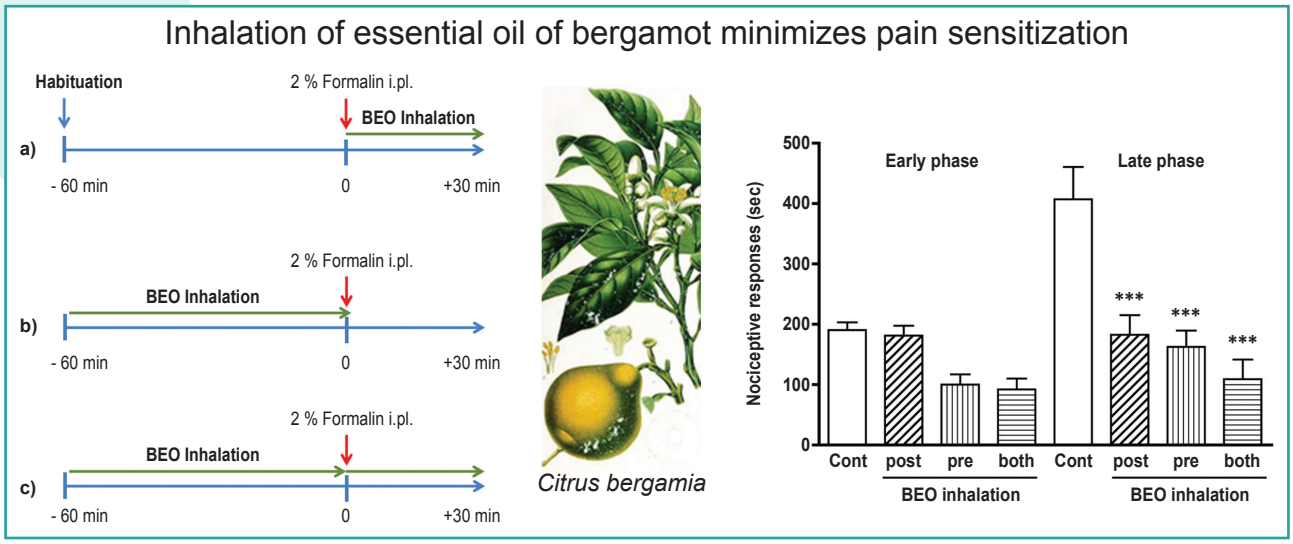

Figure 3. Analgesic efficacy BEO after inhalation in formalin test as pre-, post- or pre + post treatment (reproduced with permission from (19)).

tered *via* inhalatory (19) (**figure 3**) or transdermal route, as it occurs in aromatherapy (20, 21). Pretreatment with the opioid receptor antagonist naloxone methiodide decreases the antinociceptive effect of BEO, supporting the involvement of opioid system together with the evidence of enhancement of morphine-induced antiallodynic effect in the partial sciatic nerve ligation (PSNL) model of neuropathic pain (22); under the latter experimental conditions, BEO is also active by means of continuous administration through an osmotic pump mimicking chronic pain treatment (23). Moreover, the subcutaneous (s.c.) administration of BEO for 7 days attenuates long-lasting tactile allodynia induced by spinal nerve ligation (SNL) of the spinal nerve L5 (8). The first systematic review and meta-analysis investigating the preclinical evidence in favor of the working hypothesis of analgesic properties of the essential oils (24), following the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) criteria, highlighted that: 1) studies present different experimental design and rise serious concerns in terms of selection, performance and detection biases, not following the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines for accurate *in vivo* preclinical research (25); 2) BEO is the most suitable candidate for clinic translation since proving strong analgesic properties in the most reliable models of pain relevant to clinic in a methodologically rigorous manner. Accordingly, the preclinically demonstrated antinociceptive and antiallodynic properties of BEO provide a robust rational basis for its translation in clinical settings in which pain has a pivotal role.

DEMENTIA AND PAIN-LINKED NEUROPSYCHIATRIC SYMPTOMS (NPS)

Patients suffering from severe dementia often receive insufficient treatment of pain, particularly neuropathic, (26, 27) and this undertreatment is associated with widespread use of antipsychotics and antidepressants (28), usually due to their lack of communication skills preventing self-reporting (29). In particular, age-related comorbidities are responsible for chronic pain in up to 80% demented patients living in nursing homes (30). A tight link between unrelieved pain and the development of agitation, one of the most challenging and resistant neuropsychiatric symptoms (NPS) of dementia, has been demonstrated (31, 32) pointing at the priority of analgesia in the management of agitation (33). In fact, the latter can be significantly reduced by adequate pain treatment

and regular review of therapy (34, 35). There is growing evidence in favor of the significant correlation of pain intensity with dementia severity, NPS and antipsychotic prescriptions (36). However, the treatment of agitation often consists in the off-label use of antidepressants and atypical antipsychotics, known to increase up to almost doubling the risk of death for cardiocerebrovascular accidents (37). Therefore, pain control plays a fundamental role to decrease the use of unnecessary and potentially harmful atypical antipsychotics (33, 38), often used without evidence of the benefits and even increasing mortality risk after initiation of treatment (39). In this complex frame, aromatherapy with the melissa and lavender has proven efficacy for agitation in dementia (34), but the quality of the evidence has been downgraded (5, 7), as for all clinical trials in aromatherapy, due to sources of methodological biases (5). One of the major causes of poor quality is linked to strong aroma of essential oils hampering adequate allocation masking and double-blinding and to the lack or reproducibility and active principle titration in inhalatory systems. Moreover, because of the link existing between pain and agitation (40) the essential oil investigated for clinic treatment of agitation needs to be endowed with analgesic activity (41), as it is the case for BEO.

ENGINEERING BEO FOR TRANSLATION IN CLINIC

The illustrated limitations leading to poor quality clinical research in the field of aromatherapy have been overcome by the production of a nanotechnology delivery system, *i.e.*, NanoBEO, consisting in solid lipid nanoparticles (SLN) encapsulating BEO-bergapten free to avoid phototoxicity (European Medicine Agency [EMA], September 13rd, 2011 EMA/HMPC/56155/2011 Committee on Herbal Medicinal Products [HMPC]), and developed in the pharmaceutical form of a cream for transdermal application (42). Quite importantly, the antinociceptive and antiallodynic properties of NanoBEO have been studied in the capsaicin, formalin and PSNL pain models, demonstrating that it keeps all the pharmacological activities of BEO. Nano-BEO shows efficacy on scratching behavior, a typical neuropsychiatric symptom associated to dementia. This nanotechnology delivery system prevents the content in the active ingredients from declining after two and six months of light exposure over 10% and 18%, respectively, with no further degradation at 12 months. The prolonged physicochemical stability and titration in its main components (linalool, linalyl acetate, and limonene) are remarkable advantages allowing reproducible antinociceptive and anti-itch responses to be measured. Added to this is the possibility to perform double-blind clinical trials, impossible so far because of the strong smell of essential oils used in aromatherapy. The present invention has been recently patented (EP4003294) and this makes it possible to effectively test NanoBEO deprived of furocoumarins in clinical trials for the treatment of acute and chronic pain and the prevention or treatment of NPS. Furthermore, the presence of anxiolytic activity and the documented absence of sedative effects is very relevant for the use of NanoBEO in cognitively impaired patients (43). In fact, the actually ongoing clinical trial BRAINAID (NCT04321889) (44) has been designed with the purpose to study the effectiveness and safety of NanoBEO on the NPS agitation and on related pain in 134 patients (n = 67 per arm) over 65 affected by severe dementia (Mini-Mental State Examination ≤ 12) (44). The primary outcome is reduction of the Cohen-Mansfield Agitation Inventory (CMAI) (45), used to assess agitation. The secondary outcome is represented by decrease of the score of the recently translated, adaptated and validated scale in the Italian setting for the assessment of pain in non-verbal, severe demented patients Italian Mobilization–Observation–Behavior–Intensity–Dementia (I-MOBID2) (46). The latter is able to unravel even concealed musculoskeletal and visceral pain states by means of active guided movements

(47, 48). Incidentally, the clinical trial BRAIN-AID (NCT04321889) could provide rational basis for use in e-health setting, important during the pandemic (49-51).

ETHICS

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Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

All authors have contributed equally and they have read and agreed to the final version of the manuscript.

Availability of data and material

The data presented in this study are available within the article.

Ethical approval

N/A.

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