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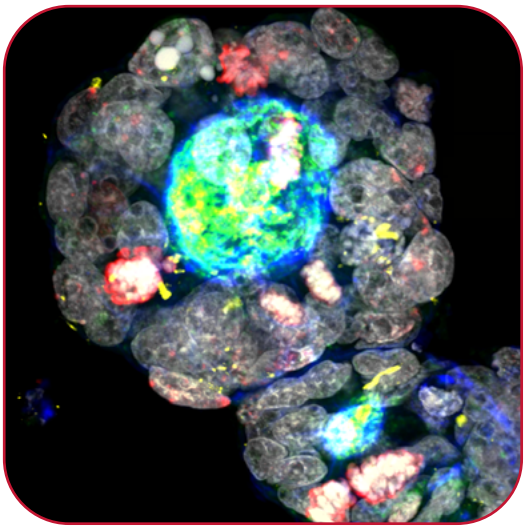
The cover image shows a maximum intensity projection of four planes, acquired by confocal microscopy, of colorectal cancer stem cell spheroids grown in vitro. *Prior to imaging, cells were fixed and stained to mark nuclei (grey), ganglioside (green), cortical cell membrane (blu), proliferating cells (red) and a stemness marker (yellow).*
The image is provided by Michele Signore in collaboration with Ruggero De Maria and Lucia Ricci-Vitiani, Istituto Superiore di Sanità, Rome, Italy.



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COMMENTARY
When enhanced games outpace public health and ethics

Ilaria Palmi, Simona Pichini and Renata Solimini

Centro Nazionale Dipendenze e Doping, Istituto Superiore di Sanità, Rome, Italy

Abstract

The enhanced games, a new sports event scheduled to take place in the United States in the spring of 2026, propose a competition model in which the use of performance-enhancing drugs (PEDs) is openly permitted and encouraged. This initiative represents a radical departure from long-established sporting norms and is strongly criticised by major international institutions, due to its potential public health consequences and ethical implications. PEDs use poses significant risks, ranging from severe physical damage to long-term mental health effects. The enhanced games risk normalising these substances, particularly among vulnerable populations such as youth, who are highly influenced by elite athletes and media narratives. This commentary examines the conceptual frameworks underpinning sport, performance enhancement, and doping as well as the main issues that may arise in relation to these games. Particular emphasis is placed on the possible implications for public health, from both an ethical and a health perspective.

Key words

- doping in sports
- physical enhancement
- public health
- ethics

INTRODUCTION

The enhanced games is a proposed multi-sport event that would allow athletes to use performance-enhancing drugs (PEDs). The inaugural enhanced games were announced in May 2025 and the event is scheduled to take place in Las Vegas, USA, from 21-24 May 2026. The event will feature swimming, athletics, and weightlifting. The enhanced games aim to transcend the restrictions present in traditional competitions, such as the Olympics, by eliminating bans on PEDs and encouraging the use of biotechnology and pharmacology to push human performance beyond natural limits. The event offers substantial prize money for competition winners, particularly for those who can surpass current world records. Organizers state that a team of scientists and medical specialists rigorously designs the medical profiling protocols, taking into account the potential use of performance enhancements (https://www.enhanced.com/science).

This event represents a significant departure from traditional sports norms and has drawn considerable criticism from major international institutions like the World Anti-Doping Agency (WADA) (https://www.wada-ama.org/en/news/wada-condemns-enhanced-games-dangerous-and-irresponsible). Enhanced games have also raised deep concern in the International Federation of Sports Medicine (FIMS) that adamantly opposed to them [1]. This commentary outlines some ethical and public health issues arising from the development and marketing of these games.

SPORT, PEDS AND DOPING

Sport is one of the most remarkable expressions of human endeavour. It serves as a source of recreation and enjoyment, but rules are in place to ensure fairness and equal opportunity for everyone. Sport enables individuals to push the boundaries of their physical and mental capacities while fostering the sharing of collective values and experiences. Sport originated as an individual pleasure derived from physical activity and competition is a natural consequence of this, a way to measure oneself against others.

Can enhanced games be of interest to elite athletes and, at the same time, amateur or recreational athletes, i.e., be a topic of interest for the protection of public health? Athletes are commonly classified into categories such as elite, amateur, and recreational, based on various criteria including competitive level, degree of commitment, purpose, legal status, and access to resources. Yet, those who compete at the highest international levels – such as the Olympics, World Championships, or professional circuits – almost always begin their athletic journey as amateurs, often starting at a young age. From this perspective, elite sport is not viewed as a separate domain, but as an endpoint that begins in the context of amateur sport. Accordingly, certain public health

Address for correspondence: Ilaria Palmi, Centro Nazionale Dipendenze e Doping, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: ilaria.palmi@iss.it.

ethics principles – such as the protection of vulnerable populations like youth – can be reasonably extended to the domain of sport.

Doping, defined as the use of specific substances or medical methods to artificially increase an athlete's physical performance, affects sports participants at all levels (i.e., elite, amateur and recreational athletes). It ranges from neighbourhood gym-goers exchanging tips on "sculpting" muscles to young exercisers achieving desired results faster, by pushing themselves to their physical limits and recovering faster after exercise/training [2, 3]. The use of PEDs is becoming increasingly prevalent among amateur athletes and the general population engaging in sports, as well as those simply attending gyms and sports facilities [4].

A number of authors have explored the motivations behind athletes' decisions to resort to doping. They have concluded that the problem is extremely complex and multifaceted, arising from a combination of individual, relational, social and situational factors [5, 6]. The integrated model of doping behavior explains that regulative, normative, and cognitive systems shape an athlete's choices about performance enhancement, including both legal and prohibited methods. Together, these systems influence the athlete's mindset – their attitudes, values, beliefs, and behaviors toward doping, essentially their orientation to cheating. The extended model considers both system-level and individual perspectives, aiming to align anti-doping policies, education, and personal behavior [7].

At an international level, the World Anti-Doping Agency (WADA), the international independent agency that leads a collaborative worldwide movement for doping-free sport, currently defines which drugs or practices violate the World Anti-Doping Code (WADC). The WADC is the fundamental document that harmonises antidoping policies, rules and regulations within sports organisations and among public authorities worldwide. WADA states that there is an intrinsic value about sports that is the celebration of the human spirit, body and mind, and is reflected in values including fair play and honesty, respect for self and other participants, respect for rules and laws, and health.

WADA considers including a substance or method on the prohibited list if the substance or method meets any two of the following three criteria: it has the potential to enhance athletic performance; it poses an actual or potential risk to the athlete's health; or its use is deemed to violate the spirit of sport as defined by the WADC [8].

PEDs are banned because they violate these criteria. In particular, the health risks to athletes are a matter of public health. Depending on the substance, the dosage and the duration of use, some PEDs have been proven to have severe side effects and can cause irreversible damage to an athlete's body, including mental health issues [9]. Indeed, most PEDs are drugs developed with the aim to treat specific diseases, and the off-label use in healthy subjects can induce short- and long-term damages. For example, erythropoietin (Epo) employed to treat anaemia resulting from chronic kidney disease or in chemotherapy induced anaemia. The intake of Epo in healthy subjects leads to "thick blood" and the danger of thromboses, with the additional risk of heart attack [10]. Another example is that of anabolic-androgenic steroids (AAS). In recent decades, there has been an increasing interest in the long-term effects of AAS abuse, due to accumulating evidence of their adverse effect on physical [11-14] and mental health [15-17]. As described above, doping affects both elite and amateur (or recreational) athletes. The latter are arguably the most exposed to the health risks associated with the use of PEDs, as they do not have access to teams of experts (doctors, athletic trainers, physiotherapists, etc.) and often resort to Do It Yourself (DIY) methods or rely on hearsay. In line with public health principles, governments have a duty to educate people about the health risks of PED use. Combating doping is a public health priority, tied to promoting sport and physical activity to improve population well-being.

In 2014, the European Union (EU) Commission reviewed the evidence on policies to address doping in recreational sports, due to concerns about its use among amateur athletes [18]. Along with legislative measures and controls, this review concluded that doping prevention in recreational sports relies primarily on education and information. In EU, several countries have implemented bans on doping. For example, some European countries (e.g., Austria, France, Italy, Sweden, Germany) introduced national legislation that punishes the use of a substance included in WADA prohibited list, while many more countries have also enacted sports-specific legislation that punishes the possession, the supply or distribution and the administration or prescription of WADC prohibited substances (e.g., Finland) (https://www.coe.int/en/web/sport/adq-reports).

ENHANCED GAMES AND PHYSICIANS

In the field of public health, healthcare professionals have a fundamental role to play in terms of their professional responsibilities, such as acting with competence, honesty and integrity, and complying with regulations. They also play a key role in health promotion, educating and raising awareness among the population about the importance of health and healthy behaviours. One of the key challenges in diagnosing and treating doping-related conditions is the clandestine and illegal nature of substance use, which can result in severe disciplinary consequences for athletes. Consequently, individuals rarely voluntarily disclose such use, even when experiencing adverse effects that prompt them to seek medical attention from an endocrinologist or another specialist. Physicians should be highly vigilant towards patients engaged in intense physical training, regardless of whether they are amateurs or professionals. Given this population elevated risk profile, the possibility of doping-related complications should always be considered as part of the differential diagnosis, regardless of the initial reason for consultation [19].

The health risk is arguably the most significant concern in the context of enhanced games, as it conflicts with a cornerstone of medical ethics: the principle of *primum non nocere* (i.e., first, do no harm). Since no drug is entirely free of risk, physicians must, above all, avoid causing harm when selecting a therapy. Accordingly, in

treating any illness, they should prioritize interventions associated with the fewest adverse effects, based on a careful assessment of the risk-benefit ratio. This principle applies to the treatment of sick patients. However, PEDs are not used to cure illness; rather, they are drugs taken by otherwise healthy individuals with the sole aim of improving athletic performance. This creates an ethical paradox for physicians who agree to assist athletes in doping. In this context, the position of FIMS is unequivocal: the medical care of athletes must be grounded in three core principles, i.e., scientific analysis, assessment of the individual physical condition, and the protection of health (www.fims.org/about/code-ethics/).

ENHANCED GAMES AND THEIR IMPACT ON YOUTH

It is important to note that encouraging elite athletes to publicly endorse the use of prohibited and potentially harmful substances sends a dangerous message, particularly to young people. This message suggests that success in sport is not achieved through hard work and dedication, but rather through pharmacological shortcuts. This narrative poses serious ethical concerns. Sports physicians are especially alarmed by the health risks associated with drug use among young, aspiring athletes: they are particularly concerned that young people will be exploited in the quest for fame and fortune, and by the allure of the enhanced games [1].

Elite athletes can exert a powerful influence on adolescents' purchasing decisions, lifestyle choices, and overall engagement in sport. Teenagers who regard athletes as role models are more likely to emulate their behaviours, including mimicking consumption patterns and expressing greater interest in sport-related activities. This influence is amplified by adolescents' sensitivity to trends and their active engagement with media and advertising featuring sports celebrities [20].

The motivational climate shaped by elite athletes, coaches, and peers plays a central role in determining adolescents' motivation, self-esteem, and enjoyment in sport. Coaches and peers are particularly influential in adolescence, affecting effort, enjoyment, and perceived competence, while parental influence diminishes with age [21]. The broader social environment – including the attitudes and behaviours of elite athletes – can thus contribute to the development of either adaptive or maladaptive motivational patterns among youth [21, 22].

DISCUSSION AND CONCLUSIONS

Physical activity and exercise participation are associated with a wide range of benefits to mental and physical health. Nonetheless, the health-enhancing properties of physical activity and exercise can be offset by certain behaviours, such as the use of PEDs. Whereas the use of PEDs is highly regulated by sports authorities governing competitive sports and has been considered as a cheating behaviour and a danger for health, the enhanced games appear to challenge and overturn long-established norms and ethical standards traditionally associated with sporting conduct. The issue of athletic enhancement, whether pharmacological or technological, raises complex and open questions that cannot always be answered unequivocally, even by experts. For instance, who should be held responsible for the use of PEDs? Should the athlete be held solely responsible, or should coaches, doctors, federations, sponsors and the sports system itself also be held responsible? In many cases, doping is structural in nature, meaning it is encouraged by a system that prioritises victory over ethical limits [6]. A modern whole rounded approach needs to consider doping not only as a violation of sporting rules, but also as a threat to one's own health. It is somehow a form of addiction that affects individuals and communities and is supported by crime. In this context, doping is not only considered a sporting violation or a risk factor for an individual's health, but also a disease of society, affecting society and acting against it [23].

Initially planned for Australia in 2024, following Paris 2024 Olympics, enhanced games have now been scheduled to take place in May 2026 in USA. Reactions from the sporting world have been extremely negative, highlighting the dangers of encouraging PEDs. However, unless the event is cancelled in the coming weeks, it is set to take place. On their website, the organisers state: "We are on a mission to redefine super humanity through science, innovation and sports" effectively opening the door to transhumanism and raising ethical, social and philosophical questions about what it means to be human. Should we see the body as a limit to be overcome, or as a boundary to be respected? This is the first time that sporting competitions have been proposed, promoted and implemented in which the use of doping is openly accepted and, indeed, it is actively encouraged. The potential impact on civil society and the world of sport is unknown.

It is known that a model for health promotion focuses attention on both individual and social environmental factors as targets for health promotion interventions. This model assumes that appropriate changes in the social environment will produce changes in individuals, and that the support of individuals in the population is essential for implementing environmental changes [24]. Conversely, it is highly likely that inappropriate changes in the social environment can produce inappropriate changes in individuals. What impact do enhanced games have on social perceptions of the body and health? Can they exert cultural pressure on young people and amateur athletes, thereby influencing risky behaviour or drug use? It is well known, for example, that AAS are associated with addiction [25]. However, it is doping itself that can induce phenomena similar to addiction in those who practise it. Indeed, the positive effects of a victory and the negative effects of a failure make the practice of doping almost like a drug itself [6]. In light of the promotion and spread of enhanced games, what effective awareness-raising measures could health professionals implement?

As described above, the WADA exercises preventive control and imposes disciplinary sanctions on athletes who violate the provisions of the WADC but regarding enhanced games, should states intervene to counteract the idea that the use of PEDs is socially acceptable for improving sporting performance or body image?

Enhanced games raise several unresolved questions related to the protection of public health in general, and athlete health in particular. What should health promotion professionals do in light of the advertisement of an event like this? It is essential that medical professionals, psychologists and healthcare workers in the broader sense, as well as institutions at various levels, consider the potential social and health risks associated with the practice of enhanced games, with the aim of understanding its impact on population and implementing appropriate countermeasures.

Authors' contributions

IP conceived and wrote the manuscript with support from RS. RS and SP carefully revised the final draft of the manuscript. All Authors have read and approved the last version of the manuscript.

Conflict of interest statement

The Authors declare no conflict of interest.

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COMMENTARY

Seroepidemiological studies: a cornerstone of public health strategies

Anita Muglia[1] and Paola Stefanelli[2]

[1]*Dipartimento di Sanità Pubblica, Medicina Sperimentale e Forense, Università degli Studi di Pavia, Pavia, Italy*
[2]*Dipartimento di Malattie Infettive, Istituto Superiore di Sanità, Rome, Italy*

Abstract
Well-designed population-based seroepidemiological studies can be useful to refine estimates of infectious agent transmission and disease severity, thus representing an important component of epidemiological surveillance. However, the interpretation of results may be hampered by heterogeneous data quality and the lack of standardized methodology in some cases.
The development and use of highly sensitive and accurate tools, refined over the past decades, have facilitated the study of current and past exposure to infectious agents in target populations. In particular, seroepidemiological studies provide a detailed overview of past and current infection burdens (i.e., respiratory infectious diseases), helping to identify high-risk groups and biomarkers of disease severity. They also help to understand the evolutionary dynamics of the disease over time, providing crucial information on its spread and community impact. Investing time and resources in seroepidemiological studies is essential, not only for monitoring the population's immunological status, but also for implementing strategies that promote public health and predict future infectious diseases scenarios.

Key words
- seroepidemiology
- infectious diseases

Emerging and re-emerging infectious diseases pose a global threat to public health (https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death). Every year, frail individuals, elderly, and people with comorbidities are the main victims and carriers of serious complications [1]. Epidemiologic investigations, combined with the use of laboratory techniques, are essential to study the transmission of pathogens causing infections in humans, the emergence of new viral variants, host-virus interactions, and the influence of the environment on the spread of pathogens, for example, viruses. They are also important to identify the main risk factors in the population and for implementing targeted intervention strategies [2, 3]. The systematic collection and analysis of blood samples from a target population sample may permit the study of the distribution and determinants of infection or the impact of vaccination. Seroepidemiological data can reveal the prevalence and/or the incidence of infections in a population, in order to monitor the emergence of new pathogens and/or the re-emergence of old pathogens and their transmission rate, and to evaluate immunization programs and the level of community immunity [4].

One of the main advantages of seroepidemiological techniques is their ability to utilize biological samples, such as serum samples, routinely collected for clinical or diagnostic purposes. This approach permits the minimization of both costs and logistical complexity because, by creating databases with clinical and demographic data on a large number of individuals, it is possible, for example, to retrospectively identify and select the desired target population without the need to prospectively recruit participants exclusively for the study.

The opportunity to collect and analyse serum samples has also facilitated the conduct of more in-depth studies, since the analysis of the level of IgG and IgM antibodies in response to exposure to specific pathogens may enable to distinguish between an acute or recent infection and a response to a previous infection [5]. In general, the immune response to pathogens is initially expressed with an increase in IgM antibody titer and subsequently with an increase in IgG antibody titer. The strength of using antibody-based techniques is represented by the high sensitivity and specificity of the methods, as antibodies rapidly detect the presence of antigens, even at

Address for correspondence: Paola Stefanelli, Dipartimento di Malattie Infettive, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: paola.stefanelli@iss.it.

low concentrations, and their molecular structure allows them to precisely recognize and bind to specific parts of a pathogen's antigen with high affinity.

Subjects are classified as seropositive or seronegative based on antibody levels above or below a specified threshold, or as seroconverted following increases in antibody levels above a predefined threshold between two time points. A bias in the interpretation of serological status data relative to a defined threshold could be due to sensitivity and specificity in defining seroconversion, declining antibody levels over time, or cross-reactivity within or between pathogens of the same species [6].

Furthermore, high IgG titers allow the detection of a past infection regardless of the development of symptoms, and the presence of different types of antibodies, some directed against a vaccine target and others against the target of natural infection, permits the differentiation between post-infection and post-vaccination. The ability to test for different antibodies on the same sample facilitates the simultaneous examination of antibody response [7].

The tests employed in the seroepidemiological studies can present limitations, including low specificity that can lead to false-positive results, or even low sensitivity that can lead to false-negatives, and cross-reactivity. The analytical limitations related to test performance have been partially overcome by technological advances.

To obtain good results from a seroepidemiological study, a solid study design and a proper selection of the target population are necessary. Defining the study's objectives is another prerequisite, as it is crucial to decide whether to choose a longitudinal or a cross-sectional study design. The former, i.e., seroincidence study, involves the follow-up of a given cohort of individuals for months or years, allowing to determine the rate at which they develop antibodies over time. These studies are useful for estimating infection rates and identifying some of the factors that may contribute to an increase in the risk of infection. This latter design is also called a "seroprevalence study", and is used to determine the percentage of people with antibodies against a specific pathogen at a given time [8]. Obviously, longitudinal studies are more expensive and have the disadvantage of potentially losing a percentage of participants over time. However, cross-sectional studies can be useful for obtaining a snapshot of population immunity at a specific time, although they do not provide information on cause-effect relationships or changes in individuals' immune status over time.

Another possible limitation of seroepidemiological studies may be due to bias in the selection of the population sample [9]. It is important to emphasize that the sample must be representative of the target population, possess certain demographic and clinical characteristics, and be randomly selected. Furthermore, determining the appropriate sample size is also of fundamental importance in the design of any seroepidemiological study, as it can influence both the level of precision and the statistical power of the study results. An inadequately sized sample can lead to inaccurate estimates and a reduced ability to detect real associations; conversely, an excessively large sample size can lead to an unnecessary increase in overall cost and complexity of the research [10]. A well-designed seroepidemiological study can offer significant advantages.

For example, a seroprevalence study conducted in five municipalities of the autonomous province of Trento, Northeastern Italy, during the COVID-19 pandemic, provided a better understanding of the extent of viral circulation and contributed to the estimate of the proportion of asymptomatic infections. Its purpose was to estimate the number of people exposed to SARS-CoV-2 virus and assess the spread of the infection. This study revealed a higher number of seropositive individuals compared with reported cases, likely due to a high proportion of people with mild or asymptomatic disease or who had not been tested. It also demonstrated that the youngest age groups had higher seroprevalence, and that symptoms such as anosmia and ageusia were strongly associated with the presence of antibodies [11].

In another study, data on IgG antibodies against SARS-CoV-2 spike protein suggested that children were significantly less likely to be infected than adults, useful data for providing infection control policy planning [12].

Seroprevalence studies can be used to study various pathogens and can support the formulation of public health strategies. In the context of vaccine-preventable pathogens, an example can be found in a study conducted on an unexpected increase in meningococcus serogroup C, Sequence Type (ST)11 and clonal complex (cc)11, in the Tuscany region of Italy few years ago [13]. In this case, the study was conducted to further investigate and obtain evidence that could explain the dynamics that led to the sudden increase in meningococcal serogroup C disease and to identify population groups at highest risk of infection. The results suggested a significantly higher incidence of cases compared to previous years with the C:P1.5–1,10–8:F3–6:ST-11(cc11) meningococcal strain as predominant and a particular incidence in the 20-29 age group. Information on vaccination status suggested that the protection provided by a single dose may be inconsistent in some cases.

In the United Kingdom (UK), a 2014 national serological survey of population immunity identified the rapid decline in immunity after infant and toddler immunization as well as the low proportion of teenagers protected against meningococci of serogroup C (MenC) invasive disease around the time the adolescent vaccination programme was introduced [14].

As described, seroprevalence studies provide a precise picture of past and recent infections, allowing estimates of the extent of spread of human pathogens such as viruses or bacteria. If properly designed and conducted, these studies can provide important support for public health strategies. To maximize their benefits, it is essential to follow standardized epidemiological and serological protocols that may facilitate comparisons between studies (over time and space), allow for accurate data analysis, and support the response to emerging and re-emerging infectious threats.

Conflict of interest statement

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COMMENTARY

New demands for digital and AI skills in health occupations: a few perspectives for the future of the Istituto Superiore di Sanità (ISS)

Alessandro Palombo[1], Enrico Alleva[2] and Carlo Petrini[3]

[1]*Centro Nazionale Intelligenza Artificiale e Tecnologie Innovative per la Salute (IATIS), Istituto Superiore di Sanità, Rome, Italy*
[2]*Centro di Riferimento per le Scienze Comportamentali e la Salute Mentale, Istituto Superiore di Sanità, Rome, Italy*
[3]*Unità di Bioetica, Presidenza, Istituto Superiore di Sanità, Rome, Italy*

Abstract
The OECD Report "Digital and AI skills in health occupations: What do we know about new demand?" published in 2025 analyses the impact of digital technologies, AI, and advanced robotics on health occupations across OECD (Organisation for Economic Co-operation and Development) countries, with a particular focus on Canada, United Kingdom, and United States. Drawing on the analysis of more than 55 million online job postings (2018-2023), the study examines trends in the demand for digital skills and classifies health occupations according to their susceptibility to automation or augmentation. Conclusions indicate that, while a limited share of roles faces automation risks, a variety of health professions are expected to benefit from forms of task augmentation. The report highlights the need for tailored policies on lifelong training, data governance, and human-centred bioethical approaches to ensure the sustainable integration of AI into healthcare systems.

Key words
- artificial intelligence
- ethics
- health occupations

The OECD Report "Digital and AI skills in health occupations: What do we know about new demand?" published in 2025 [1] explores the impact of digital technologies and artificial intelligence (AI) on health occupations across OECD (Organisation for Economic Co-operation and Development) countries, focusing on the way these innovations can support health workers amid rising demand for healthcare services, providing three key empirical contributions. It analyses about 55.5 million online job postings (OJPs) from Canada, UK and USA, tracking the demand for digital and AI skills in health-related jobs between 2018 and 2023. It also identifies specific digital and AI skill requirements for various health functions, revealing emerging priorities such as Health Information Management, Telehealth, and Cybersecurity. Finally, it evaluates the potential effects of Generative AI (GenAI) and Advanced Robotics (AR) on health occupations, classifying roles based on their susceptibility to automation or augmentation. Results indicate that while some job types face automation risks, most professions stand to benefit from productivity-enhancing advancements, depicting the relevance of targeted reskilling policies and continuous training to maximise AI benefits integration in healthcare, ensuring that technological progression complement rather than displace health professionals and their reasoned choices.

Such a volume categorises health jobs into four main groups based on their susceptibility to automation by these technologies (yet it misses some relevant perspectives, such as the Asian ones): low risk of automation, potential augmentation, potential automation, and high risk of automation. Low risk occupations consist in tasks mainly involving complex human interactions and decision-making, high-risk ones involve a large share of routine, automatable tasks (physical, cognitive or both).

Address for correspondence: Enrico Alleva, Centro di Riferimento per le Scienze Comportamentali e la Salute Mentale, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: enrico.alleva@iss.it.

Job roles where augmentation is or seems possible could witness efficiency gains in a limited number of tasks through automation, while other responsibilities remain difficult to render automatic. Potential automation, by contrast, refers to occupations where GenAI and/or AR can replace numerous tasks, potentially transforming the nature of the occupation at hand; however, some tasks still fully necessitate human expertise.

Based on information from the United States, occupations with potential for augmentation, such as Registered Nurses and Physician Assistants but also Family medicine physicians, seem to benefit significantly from digital technologies enhancing human capabilities. For these roles, continuous, long-last in training in advanced medical technologies, including EHR systems and telemedicine, is a basic to maximize the benefits of technological integration.

Other occupations as Orderlies and Medical Transcriptionists, face significant automation threats due to emerging developments that integrate GenAI into intelligent robots and other even less sophisticated automated systems. Policy measures should focus on reskilling these workers and providing career mobility to minimize displacement.

Following these results, this study further assesses the proportion of health employment in the

USA that would be susceptible to augmentation or automation. The study indicates significant variation in automation potential across health roles, with 32% of employment stratified under potential augmentation. Approximately 4.3% of roles, such as Pharmacy Technicians, are on the contrary identified as potentially automatable. The high automation risk category, including roles like Orderlies and Medical Transcriptionists, range at around 0.6% of the health workers as a whole. The results highlight how the integration of GenAI and Advanced Robots will likely reshape employment patterns but that most of such a relevant impact may be channeled through a positive enhancement of the individual capabilities of existing health workers.

As a whole, the analysis attempts to monitor the evolving landscape of digital and AI related skills in the health sector across Canada, UK and the USA. The study leverages data from millions of OJPs to track the longitudinal change of the demand for digital competencies, including GenAI and those related to the interaction with Advanced Robots, and their progressive integration into the various typologies of health occupations.

Results for the USA indicate that its health sector exhibits a significant demand for digital skills, mainly in areas such as Health Information Management/Medical Records, Computer Science, and Data Analysis.

The adoption of AI in niche roles, such as the very often mentioned AI-driven diagnostic tools in radiology, also underlines the transformative potential of such new technologies. The UK demand for digital skills like Cybersecurity, Data Analysis, and Clinical Informatics reflects the health sector effort to integrating advanced technologies in order to ameliorate patient care and operational efficiency. The demand for clinical informatics skills underlines the importance of managing and analyzing clinical data to profitably support decision-making processes. In this integration resides the core problem today. Canada has a more stable (yet growing) demand for digital skills in health occupations, with a notable emphasis on Health Information Management/Medical Records, Computer Science, and Data Analysis. The increased need for data collection and clinical informatics competencies points to the focus on leveraging data.

The analysis identifies several health occupations with high potential for augmentation and automation through AI and the use of Advanced Robotics. However, some USA tasks of Registered Nurses and Physician Assistants are likely to be augmented by AI and AR, increasing their productivity and efficacy. These roles could also benefit from enhanced digital literacy and competencies related to advanced technologies, such as AI-integrated diagnostic tools and telehealth platforms. In particular, robotic systems, theoretically can handle tasks such as cleaning, sterilising, and preparing medical instruments, thereby reducing manual workload and enhancing operational efficiency. These technologies may possibly enable health staff to focus on more complex, patient-centred activities, (including psychological empathic bonding) improving overall health delivery, but the steady advancement in AI and AR will fruitfully lead to further automation which could bring significant impacts on employment prospects.

The need for targeted policy interventions facilitating the current digital transformation of the health workforce will in final conclusion entail:

- investment in training and education remains paramount;
- if the digital transition and the adoption of new AI technologies has to benefit the largest number of people everyone, policymakers must address the digital divide by providing resources and support to underserved areas, ensuring that all health providers can leverage the benefits of digital and AI technologies; the same applies to taking care of minorities, disabled and in general vulnerable sets or stratified subsets of human population;
- data protection and security are also a priority.

The document fits into a time and context in which AI is the focus of attention worldwide and its applications are pervasive in every field. Various authoritative international institutions have recently addressed similar topics, including, for example: the World Health Organization (WHO), the International Labour Organisation (ILO), the Council of Europe (CoE), the World Medical Association (WMA) and others.

From an ethical point of view, there are recurring issues in the various perspectives, such as: responsibility, dignity, fairness, equality, non-discrimination, confidentiality, sustainability, self-determination, inclusiveness, safety, etc. These cross-cutting criteria must then find appropriate specific concrete applications for the various areas of application in the health professions: it is clear that data analysis software and a robot used in personal care are not equivalent and require different considerations.

Particular attention must be paid to the impact of new technologies in the health professions, which typically involve direct contact with the people being cared for. In such circumstances, one of the main recommendations expressed by the World Medical Association in the WMA Statement on Artificial and Augmented Intelligence in Medical Care (adopted by the 76th WMA General Assembly in October 2025) is particularly significant. The WMA places the notion of "augmented" among the key principles of the Declaration: "The term signals a human-centred approach to AI – one that reinforces the physician's role as the final decision-maker. Rather than viewing AI as a replacement, augmented intelligence frames these tools as extensions of clinical expertise, designed to support – not replace – professional judgement, empathy, and responsibility".

These are issues in which legal, deontological, and ethical considerations are closely intertwined. Significant in this regard, for example, is the "right to human interaction", which is currently the subject of particular attention.

One of the report's main strengths lies in its methodological design. The use of granular job posting data allows for cross-country comparability and the identification of fast-growing skill clusters, such as Health Information Management, Telehealth, Cybersecurity, and Clinical Informatics. The distinction between clinical health occupations and other occupations within the health sector further enhances analytical clarity, highlighting how digital transformation is progressing more rapidly in non-clinical and support functions than in direct patient care roles.

The report also makes a notable contribution by addressing the potential impact of Generative AI and advanced robotics on health occupations. The findings suggest that, while a limited share of roles may face automation risks, the majority of health occupations are more likely to experience forms of task augmentation, with digital technologies enhancing productivity rather than replacing human labour.

At the same time, the review highlights several conceptual and interpretative limitations. Most notably, the reliance on OJPs as a proxy for technological transformation captures only part of the ongoing changes in healthcare work. AI adoption increasingly occurs within existing workflows, embedded in clinical decision-making tools, monitoring systems, and administrative processes, without being explicitly mentioned in recruitment requirements. As acknowledged in the report itself, this dynamic risks underestimating the actual penetration and influence of AI in clinical practice, particularly in public health systems and regulated care environments.

Furthermore, while the automation *vs* augmentation framework represents a methodological choice, it remains largely abstract and insufficiently contextualized. Moreover, it poses significant ethical issues: all the major reference documents recommend great caution towards the complete replacement of human activity with AI. Convergent agreement states that man has to remain "in the loop", not "on the loop" and even less "out of the loop". The analysis does not fully engage with key healthcare-specific dimensions such as professional accountability, regulatory constraints, ethical considerations, and the cognitive workload associated with human-AI collaboration. In clinical settings, the feasibility and acceptability of automation are shaped not only by technical capability, but also by medico-legal responsibility, trust, and the centrality of professional judgement and other factors that are difficult to capture through task-based scoring alone.

From a policy perspective, the report's recommendations are necessarily broad. Emphasis on continuous training, reskilling, and skills development is appropriate, yet the discussion would benefit from deeper engagement with system-level governance issues. These include health data governance, integration of AI into care pathways, organisational redesign, and differentiated strategies across hospital, primary care, community-based, and long-term care settings. Without such integration, skills-based analyses risk remaining disconnected from the operational realities of healthcare delivery.

Looking forward, the main trajectories of clinical technological development point towards the progressive integration of AI-enabled decision support systems, predictive analytics, digital diagnostics, and remote monitoring tools directly into clinical workflows. These technologies are expected to support earlier diagnosis, personalised treatment, continuity of care, and preventive approaches, while increasing the need for systemic capabilities rather than isolated digital skills. In this context, the challenge is not merely technological adoption, but ensuring that innovation reinforces clinical reasoning, ethical responsibility, and human oversight within increasingly complex socio-technical systems.

The recent establishment of the National Centre Artificial Intelligence and Innovative Technology for Health (Centro Nazionale Intelligenza Artificiale e Tecnologie Innovative per la Salute, CN-IATIS – Istituto Superiore di Sanità, ISS) emerges within this evolving landscape as a response to the need for integrated, human-centred innovation in healthcare. The Centre is positioned as a platform for applied research, experimentation, and capacity building at the intersection of clinical practice, digital technologies, and health system governance. By focusing on the responsible development, evaluation, and implementation of AI-driven solutions, the IATIS Centre aims to bridge the gap between technological potential and real-world clinical and organisational impact, supporting health systems in translating innovation into sustainable improvements in quality of care and system resilience.

Overall, the OECD Report represents a robust and informative contribution to the analysis of digital and AI-related skills in healthcare. As a descriptive labour market study, it provides valuable insights for workforce planning and training policies. Its findings, however, are best interpreted as a starting point rather than a comprehensive account of healthcare transformation, and should be complemented by process-oriented, clinical, and governance-focused analyses to fully capture the implications of AI for health systems.

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COMMENTARY

The double-edged sword of automation and the risks of AI's uneven impact on healthcare professions: a comment on the OECD artificial intelligence papers report

Marcello Di Pumpo[1,2], Francesco Andrea Causio[1,2], Stefania Boccia[1,3] and Walter Ricciardi[1]

[1]*Sezione di Igiene, Dipartimento di Scienze della Vita e Sanità Pubblica, Università Cattolica del Sacro Cuore, Rome, Italy*
[2]*Società Italiana di Intelligenza Artificiale in Medicina (SIIAM), Rome, Italy*
[3]*Dipartimento di Scienze della Salute della Donna e del Bambino, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy*

Abstract
The integration of artificial intelligence (AI) is rapidly transforming healthcare, enhancing accuracy, personalizing care, and streamlining administrative tasks. The report published in May 2025 by the OECD (Organization for Economic Co-operation and Development) offers a taxonomy of health roles based on their susceptibility to automation through GenAI and robotics, ranging from low risk (e.g., physicians) to high risk (e.g., orderlies, transcriptionists). While AI augments many routine clinical tasks, roles requiring complex judgment and empathy remain largely human-driven. Key concerns arise from the potential uncritical adoption of these evaluations, which fragment healthcare roles and risk overlooking their integrative, relational nature. Additionally, industry-led implementation of AI may undervalue frontline clinical expertise and ethical considerations. To ensure responsible integration, multi-stakeholder strategies such as the European Pact for Skills and targeted upskilling initiatives are essential. Policymakers must guide AI adoption to redesign roles and education in ways that empower the workforce without sacrificing the core values of care.

Key words
- artificial intelligence
- healthcare professions
- automation
- risks

The integration of artificial intelligence (AI) into healthcare is rapidly transforming practice. AI enhances accuracy, enables treatment plans personalization, and administrative tasks streamlining. AI-driven tools have demonstrated potential in reducing the administrative and cognitive burdens that contribute to clinician burnout [1].

Despite these advancements, the adoption of AI in healthcare raises significant concerns. A study highlights the importance of addressing these ethical and safety challenges to ensure that AI technologies are developed and deployed responsibly [2]. A paper exploring the implications of AI for the nursing workforce emphasizes the need for strategic planning to manage these transitions effectively [3]. The Food and Drug Administration (FDA) recently announced the adoption of "Elsa", a GenAI tool designed to "help employees, from scientific reviewers to investigators, work more efficiently" (https://www.fda.gov/news-events/press-announcements/fda-launches-agency-wide-ai-tool-optimize-performance-american-people).

In this context, the last OECD Report from the artificial intelligence papers series (May 2025) [4] presents a data-driven taxonomy of health roles based on their susceptibility to automation through GenAI and advanced robotics (AR). Jobs are classified into four risk

Address for correspondence: Marcello Di Pumpo, Sezione di Igiene, Dipartimento di Scienze della Vita e Sanità Pubblica, Università Cattolica del Sacro Cuore, Largo F. Vito 1, 00168 Rome, Italy. E-mail: marcello.dipumpo@unicatt.it.

categories: low risk, potential augmentation, potential automation, and high risk.

Low-risk occupations include roles that demand complex decision-making, interpersonal care, and ethical judgement. These are exemplified by physicians in general practice, psychiatry, and oncology. The inherently human dimensions of empathy, diagnostic reasoning, and shared decision-making seem to spare these roles from full automation, even as AI tools increasingly support clinical workflows.

Reportedly augmentable roles such as registered nurses and physician associates are characterized by a blend of routine and high-stakes tasks. While elements like triage, documentation, and remote monitoring are already enhanced by AI systems, many tasks like patient interaction still demand a human presence.

On the other hand, roles with reported high potential for automation include pharmacy technicians, radiologic technologists, and laboratory technicians. These positions involve highly structured, repetitive tasks susceptible to automation by AI-based image recognition, diagnostic platforms, and robotic dispensing systems. The OECD estimates that about 4.3% of the US health workforce falls into this "potential automation" category.

Finally, reportedly high-risk occupations such as orderlies and medical transcriptionists are described as replaceable by process automation tools and speech-to-text systems, with 0.6% of such health roles present in the US in 2025.

Some considerations seem necessary. The reasoning and methodology behind automatability scores assigned to healthcare occupations, while allowing for a detailed analysis, risk fragmenting healthcare roles into discrete functions and ignoring the integrative, relational, and holistic nature of care, potentially underrepresenting many critical aspects of healthcare work, such as clinical intuition, ethical decision-making, and emotional intelligence. If not properly translated into healthcare workforce skilling and employment processes, this scoring could produce more harm than benefit.

Another significant concern regards how AI adoption appears predominantly driven by private technology developers and healthcare solution providers, potentially undervaluing key experiential insights and ethical considerations from frontline healthcare professionals. This industry-led adoption risks prioritizing technological capabilities over patient care instances and workforce well-being. It is therefore imperative for policymakers to proactively ensure responsible and inclusive AI integration in the healthcare sector.

In this regard, initiatives such as the European Pact for Skills [5], promoting a multi-stakeholder approach to upskilling and reskilling, offer a valuable model. The decision of the European Public Health Association (EUPHA) to create a dedicated "digital health and artificial intelligence" (https://eupha.org/digital-health-and-artificial-intelligence), for instance, is a commendable effort to enhance the AI-readiness of a health workforce sector. This is true especially considering how Preventive Medicine Physicians are reported to have one among the highest GenAI automatability scores (0.45) [4]. Similarly, in the US, programs like the National Initiative for Cybersecurity Education (NICE) [6] or broader federal investments in STEM education and workforce development, including AI-focused traineeships and reskilling programs promoted by the National Science Foundation or Department of Labor, aim to equip the workforce for technological shifts. Adapting and expanding such frameworks specifically for the healthcare sector could ensure that the AI transition is guided by a broader coalition of stakeholders, including professional bodies, educational institutions, and workers themselves, thereby aligning technological advancement with the core values and practical needs of healthcare.

In conclusion, the digital transformation of healthcare is real, present, and risks becoming uneven. The Report provides crucial empirical evidence that health occupations are experiencing divergent exposure to automation and augmentation. These differences, deeply rooted in task complexity and clinical context, must inform all future policy on health workforce development. Avoiding obsolescence is not enough. We must invest in redesigning roles, workflows, and education to build a digitally empowered, AI-ready health workforce, one that harnesses the power of technology without sacrificing the human complexities or health care.

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Generative use AI disclosure

During the preparation of this work the authors used Generative AI to ease the writing process. After using this tool, the authors reviewed and edited the text as needed and took full responsibility for the content of the publication.

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Expert Consensus on the use of autologous platelet-rich plasma in the context of regenerative medicine: moving forward to good clinical practice

Massimo Danese[1], Vittoria D'Esposito[2], Rosario Miranda[3], Vincenza Dolo[4], Ilaria Giusti[4], Alessio Borean[5], Donato Rosa[6], Bruno Amato[6], Anna Rughetti[7], Valentina Del Greco[7], Laura Mazzucco[8], Antonio Aversa[9], Valter Cassutti[10], Francesco Casabona[11], Alfonso Maria Irollo[12], Carlo Setacci[13], Eugenio Caradonna[14], Andrea Ascoli Marchetti[15], Gaetano Caloprisco[16], Michele Angelo Farina[17], Pietro Formisano[18], on behalf of SIMCRI Expert Consensus Group*

[1]Medicina Rigenerativa, Azienda Ospedaliera San Giovanni Addolorata, Rome, Italy
[2]Istituto degli Endotipi in Oncologia, Metabolismo e Immunologia "G. Salvatore" (IEOMI), Consiglio Nazionale delle Ricerche, Naples, Italy
[3]Azienda Sanitaria Locale Napoli 3 Sud, Naples, Italy
[4]Dipartimento di Medicina Clinica, Sanità Pubblica, Scienze della Vita e dell'Ambiente, Università degli Studi dell'Aquila, L'Aquila, Italy
[5]Servizio di Medicina Trasfusionale, ULSS 1 Dolomiti, Belluno, Italy
[6]Dipartimento di Sanità Pubblica, Università degli Studi di Napoli Federico II, Naples, Italy
[7]Servizio di Immunoematologia e Trasfusionale, Azienda Sanitaria Locale 1 Abruzzo, L'Aquila, Italy
[8]SC Medicina Trasfusionale, AOU SS Antonio e Biagio e Cesare Arrigo, Alessandria, Italy
[9]Dipartimento di Medicina Sperimentale e Clinica, Università degli Studi di Catanzaro "Magna Graecia", Catanzaro, Italy
[10]Società Italiana di Medicina e Chirurgia Rigenerativa Polispecialistica, Terni, Italy
[11]Chirurgia Plastica Ricostruttiva ed Estetica, Gruppo Multimedica, Milan, Italy
[12]Unità di Medicina Rigenerativa Ginecologica, Chianciano Salute, Chianciano Terme, Italy
[13]Dipartimento di Scienze Mediche, Chirurgiche e Neuroscienze, Università di Siena, Siena, Italy
[14]Dipartimento di Medicina di Laboratorio, Centro Diagnostico Italiano, Milan, Italy
[15]Dipartimento di Biomedicina e Prevenzione, Università di Roma "Tor Vergata", Rome, Italy
[16]Poliambulatorio Bellunomedica, Belluno, Italy
[17]Centro di Medicina Rigenerativa, Studio Medico Farina, Caserta, Italy
[18]Dipartimento di Scienze Mediche Traslazionali, Università degli Studi di Napoli Federico II, Naples, Italy
**The members of the SIMCRI Expert Consensus Group are reported in the Acknowledgments*

Abstract

Introduction. This paper summarizes the conclusions of the Expert Consensus (EC) on the use of platelet-rich plasma (PRP) in regenerative medicine. The initiative aimed to promote appropriate and effective clinical use of PRP based on current scientific evidence and expert experience. The main objectives are: to define a flexible operative range for PRP, allowing for personalized application; to establish principles for standardized clinical practice.

Methods. The methods used to develop this EC involved the scientific literature review, the data quality assessment, the evaluation of practical experiences, the critical summary of reviewed literature, the development of questions for expert panel and the drafting of consensus statements.

Results. Recommendations emphasize compliance with legislation, proper device use and operator training. Adequate platelet load is essential for therapeutic efficacy; platelet

Key words
- regenerative medicine
- platelet-rich plasma
- Expert Consensus

Address for correspondence: Pietro Formisano, Dipartimento di Scienze Mediche Traslazionali, Università degli Studi di Napoli Federico II, Via Pansini 5, 80131 Naples, Italy. E-mail: fpietro@unina.it.

recovery should be considered as quality standard. PRP has demonstrated effectiveness in managing diabetic and venous ulcers, osteoarthritis, and tendinopathies, although variability in protocols and inconsistent reporting limit the strength of evidence. Emerging applications include vasculitis, mucositis, and urogenital conditions, although further research is needed.

Conclusions. The EC encourages multicentre research, strict publication standards, and international collaboration to strengthen scientific foundations for PRP use in regenerative medicine.

INTRODUCTION

In recent years, the use of platelet-rich plasma (PRP) has seen significant advancements and has been applied across numerous clinical scenarios. Platelets serve as a critical reservoir of soluble mediators, including cytokines and growth factors. Upon activation, platelets release these molecules, which act locally to promote tissue repair processes and modulate immune and inflammatory responses. Recently, it has been shown that activated platelets also release extracellular vesicles rich in various molecules, including miRNAs, and transfer functional mitochondria to other cells, thus sustaining all the processes of tissue regeneration [1-4]. This has formed the basis for the use of platelet concentrates in various medical and surgical applications.

In Italy, the use of PRP is regulated by law (Ministry of Health Decree, published in the Official Gazette "*Gazzetta Ufficiale*" on December 28, 2015, n. 300, and amended on August 1, 2019). The decree mandates a platelet concentration of 1×10^6/µL ±20% in the final product.

In 2012 Italian Society of Transfusion Medicine and Immunohematology (Società Italiana di Medicina Trasfusionale e Immunoematologia, SIMTI) guidelines referred to this concentration range (Raccomandazioni SIMTI sugli emocomponenti per uso non trasfusionale, available from: https://www.aovr.veneto.it/documents/20182/286958/Raccomandazioni+SIMTI.pdf/93e9ba10-931b-49b7-a26d-412b78f56f73); however, based on reported references, the concentration is not explicitly indicated as a mandatory standard, but rather as an observed value.

One of the earliest documented uses of PRP was in maxillofacial surgery [5], where a minimum platelet concentration of 1×10^6/µL ±20% was established as the baseline threshold for efficacy.

In 2016, the Cochrane Database [6] on platelet concentration mentioned: *"Because most individuals have a baseline blood platelet count of 200,000 (±75,000)/µL, a platelet count of 1 million/µL has been postulated as the ideal therapeutic dose of PRP"*. This statement refers to Marx's 2004 work as the sole bibliographic source [7]. *In vitro* studies have demonstrated a correlation between platelet concentration and activation, and the resulting levels of growth factors [8]. Other studies have explored the efficacy of various platelet concentrations on different cell types (endothelial cells, fibroblasts, mesenchymal stem cells) in terms of proliferation, vasculogenesis, and motility [9-12]. Nevertheless, specific studies on tenocytes have indicated better outcomes at lower platelet concentrations (500,000/µL) [13]. Thus the "regenerative" effect of platelet appears to be dependent on cellular targets.

It is important to emphasize that PRP is a biological product classified as Substance of Human Origin (SoHO), and treatment based on SoHO have been recently regulated by the European Union (Regulation EU 2024/1938 of the European Parliament and of the Council of June 13, 2024, on standards of quality and safety for substances of human origin intended for human application, which repeals Directives 2002/98/EC and 2004/23/EC). PRP is characterized by significant variability in platelet content and soluble factors. Inter-individual variability depends on platelet endowment and possible pathological conditions (e.g., platelet dysfunctions or systemic diseases) that may affect platelet features. Despite potential platelet dysfunction, several cases of patients with such conditions have benefited from autologous PRP treatment, suggesting that altered platelet function does not necessarily compromise treatment efficacy [14]. Additionally, plasma molecule concentrations (e.g., growth factors, cytokines) may vary depending on lifestyle, metabolic conditions, and inflammatory states [15, 16]. Finally, the clinical context in which PRP is applied (e.g., tissue type, damage extent, and local inflammation) represents another important variable, needing personalization of therapeutic plans. Factors such as delivery method, concentration and volume, number of applications, intervals between treatments, and synergistic approaches (e.g., hyaluronic acid, antibiotics, ozone therapy, photobiomodulation) should be carefully tailored.

While encouraging results in PRP applications have been reported, key issues remain:

- variability in platelet production and activation processes due to different commercial devices;
- difficulty comparing studies in the literature due to methodological inconsistencies;
- insufficient sample sizes in many studies, limiting robust statistical comparisons;

  poorly designed studies that are often incomparable.

Thus, acknowledging the complexity of PRP applications in regenerative medicine, this Expert Consensus (EC) aims to:

- define operational "freedom" within minimal evidence-based ranges for current indications;
- establish clear methodological models for significant and comparable scientific studies to enrich autologous PRP use as an adjunct therapy;
- address production, biological qualification, and storage challenges to suggest practices aligned with existing regulations;

- guide the development of standardized operating procedures based on best clinical practices.

This document seeks to serve as a rigorous and shared reference for practitioners in the field. It does not claim to be definitive but rather represents a step in the ongoing evolution of regenerative medicine and its integration with other synergistic methodologies under development.

METHODS

The methods used to develop this EC involved the following steps:

- scientific literature review: examination of recent publications using scientific databases such as PubMed, Scopus, and others;
- data quality assessment: evaluation of data quality based on critical analysis of study results and bibliometric indices (e.g., citation count, journal Impact Factor);
- evaluation of practical experiences: consideration of real-world applications and expert experiences in the field;
- critical summary of reviewed literature: synthesis of findings from the analysed studies into a comprehensive critical summary;
- development of questions for expert panel: formulation of targeted questions to be addressed by a panel of experts;
- drafting of consensus statements: preparation of statements reflecting agreement among the experts based on evidence and discussion.

RESULTS

Critical evaluation of PRP preparation techniques

Clinical PRP preparations enable the local delivery of active molecules (e.g., >300 growth factors and cytokines) to injured tissues. Preparation methods significantly influence PRP quality and efficacy, particularly platelet yield. Double-spin techniques typically yield higher concentrations and superior platelet capture rates than single-spin systems [12, 17]. Recent approaches emphasize the load of platelets applied, measured as absolute platelet count, enabling better standardization and outcome reproducibility.

These considerations might explain why conflicting results have been published regarding PRP therapy: specifically, in studies that used protocols with low collection volumes, single-spin methods, and low capture rates, the insufficient number of administered platelets might have compromised adequate management. Another complication for standardization is the deliberate or accidental inclusion or exclusion of the leukocyte component, as it introduces highly active biological variables in the repair process, such as involvement in angiogenic and inflammatory processes, which are critical for initiating and maintaining the reparative process [18].

Recent scientific evidence has also demonstrated the involvement of other blood components in coagulation processes, inflammation modulation, and wound healing. Many studies have evaluated the clinical effects of fibrin scaffolds, monocytes/macrophages [19, 20], and stem cells [21, 22]. Some authors have clinically used the application of whole blood clots, in which all blood corpuscular elements are incorporated into a fibrin network that facilitates cellular migration and modulates the release of growth factors. Red blood cells, which are almost completely excluded during centrifugation to obtain PRP, play a role in coagulation, but little is known about the potential therapeutic effects of whole blood red clots and the role of red blood cells in the healing process [23-26]. This highlights the need to introduce additional parameters for classifying and assessing the quality of PRP, particularly in terms of presence of blood cell populations. A brief mention is warranted regarding the long-term preservation of PRP, which facilitates repeated treatments over time in authorized facilities. Although platelet-derived growth factors have a relatively short half-life, some studies have demonstrated the efficacy of PRP in the form of platelet lysates stored for nine months at –80 °C [27]. Future research should focus on identifying the optimal temperature and time range for preserving sufficient regenerative activity in PRP components.

Another aspect to evaluate is the type of anticoagulant. EDTA alters platelet structure and function, causing irreversible damage with the destruction of platelet granules [28]. Citrate-dextrose solution (ACD) has been widely used in clinical practice for years. It has an acidic pH [29, 30], which may induce pain and reduce platelet functionality, and contains D-glucose, which could facilitate inflammatory reactions [31]. However, its dilution in blood and removal from PRP (particularly in its platelet gel form) renders it safe, with no significant pain reported from its use. Sodium citrate (SC) has a basic pH [32, 33] and shows better platelet recovery efficiency [34].

Overall, the literature reports mainly encouraging findings with PRP in a wide spectrum of clinical conditions; however, studies with negative or inconclusive outcomes should not be disregarded. Despite the complexity of the underlying biological mechanisms and the heterogeneity of the treated pathologies, it is noteworthy that studies reporting limited efficacy frequently utilized PRP preparations characterized by a low total platelet content and, in most cases, a leukocyte-poor composition.

Critical evaluation of PRP treatment in diabetic foot ulcers

The use of autologous PRP in the treatment of diabetic foot ulcers has become standard practice and represents a significant therapeutic tool. The literature is rich in studies, most of which have been conducted in developing countries, primarily India, Pakistan, Iran, and Egypt. This trend likely reflects the high interest in low-cost, self-prepared treatments in these regions, in contrast to high-income countries, where the availability of advanced wound care products reduces the incentive for similar research. The literature includes [35-51]: a double-blind randomized controlled trial (RCT) using platelet lysate [37], open-label RCTs [39, 40, 44, 46-49], non-randomized controlled studies [35, 36, 51], a non-randomized controlled study comparing two PRP

administration methods [43], case reports [45], case series [38], and prospective comparative studies between patient subgroups [41, 42].

All studies included adult patients of both sexes. In some cases, lower and upper age limits were specified. To be included, ulcers had to be non-responsive to conventional therapy. The definition of non-responsiveness varied across studies and included ulcer durations exceeding from 4 weeks to 6 months [35-41]. In some studies, the criteria for defining non-responsiveness were not clearly specified [42], or the inclusion was based solely on the presence of diabetic ulcers [43]. Some studies established additional inclusion criteria, such as ulcer diameter [39, 43], area [37, 44], number [36, 40], or anatomical location [44]. Most studies excluded ulcers involving tendons, bones, or ligaments, as well as those with gangrene. Notably, some studies also excluded patients undergoing anticoagulant or antiplatelet therapy [38, 39, 42]. Thrombocytopenia or platelet disorders were also exclusion criteria in several other studies [37-41, 43].

Blood volumes used for PRP preparation ranged from 20 to 50 mL. All studies used sodium citrate or ACD as anticoagulant. A two-step centrifugation process was employed in nearly all studies, except for one that used a single centrifugation step [45]. Most studies [35, 38, 41, 43] reported centrifugation time and rpm, or, less frequently, time and acceleration (g-force). In some cases, these parameters were not specified [36, 40]. One study [39] used a specialized kit with syringes that fit directly into a dedicated centrifuge and specified both the duration and speed for double centrifugation. When performed, activation was achieved using calcium gluconate [39, 40] or chloride [35, 45]. However, most studies did not report the activation method or explicitly stated that platelet concentrate was used without preactivation. Only a limited number of studies reported platelet concentration in the final PRP product, noting a low leukocyte content and thus clearly identifying the product as P-PRP. Among these, one uncontrolled prospective study [38] and two case reports [41, 45] documented a platelet concentration slightly above 1×10^6/µL. The remaining studies did not specify either the final platelet concentration or leukocyte content.

In some studies, PRP was injected perilesionally and intralesionally without being used as a dressing over the wound [36, 38, 42, 46]. One study involved only perilesional injection [37], although it used platelet lysate rather than PRP. The exclusive use of PRP as a topical dressing – without injection – was reported in a large group of studies [40, 41, 44, 47-50]. Other publications described combined application methods, with PRP used both as a dressing and injected intra- and perilesionally [39, 45]. A single prospective comparative study included two non-randomized arms – one receiving perilesional injections and the other receiving topical PRP application (method of topical application not specified). The study concluded that injected PRP was superior [43]. A limited number of studies [38, 43] specified the injected volume, which ranged from 0.4 to 10.2 mL. Another study that mentioned the volume (5 mL) used a platelet lysate instead of PRP [37].

Treatment is generally performed over multiple sessions, with only one study reporting a single administration [41]. In studies where PRP was used exclusively via injection, sessions were scheduled every two weeks [36, 37] or every three weeks [38]. Only one study reported a more frequent injection schedule – on days 1, 5, 10, and 20 [35]. Another study included two injections spaced three days apart [42]. In studies employing both injection and topical dressing, the treatments were administered concurrently, either weekly until complete healing [45] or for up to four [43] or ten sessions [39]. In studies using PRP exclusively as a dressing, applications were typically twice per week for up to three [48], eight [47], or twenty weeks [40]. One study did not specify treatment duration [50], and only two studies used weekly dressing applications [41, 49]. Another study did not indicate the timing at all [44]. The scoping review by Kunder *et al.* [50] analysed this aspect and found that twice-weekly applications appeared to be more effective than once-weekly.

Excluding case reports, some studies did not clearly define primary endpoints [36, 39, 41]. When specified, primary endpoints were typically wound healing rates, assessed either by the number of wounds completely healed at the end of the observation period or by the percentage reduction in wound area at study completion or during follow-up [35, 40, 42, 43, 47]. In one study involving platelet lysate [37], the primary endpoint was safety, while secondary endpoints included complete healing or wound status at 12 weeks.

Only a limited number of studies compared PRP with a standard reference treatment. Studies comparing PRP with other innovative wound therapies (e.g., adipose tissue grafts) or combined grafting techniques were excluded. The reference treatment included: platelet-poor plasma (PPP) [37], silver sulfadiazine [48], dressings with or without collagenase [46], saline-moistened gauze [36, 39, 40, 44], which corresponds to the outdated *wet-to-dry* technique. Other studies generically referred to "standard treatment" [35, 51] or "antiseptic dressing" [50]. None of the studies used modern advanced dressings based on wound bed preparation principles as a comparator.

Most controlled studies concluded that PRP treatment was significantly superior to the control in terms of number of wounds completely healed and degree of wound surface area reduction (in non-healed wounds or at specific observation times). One open-label RCT [44], however, found no significant difference between PRP and saline-moistened gauze.

The literature also includes several meta-analyses and reviews [50, 52-59], all confirming the utility of autologous PRP in treating diabetic foot ulcers.

Su's meta-analysis of 17 studies on diabetic foot ulcers found that PRP was significantly more effective than standard of care (OR: 2.11; 95% CI: 1.55 to 2.86). PRP also appeared to significantly shorten complete healing time (mean duration: –19.04 days; 95% CI: –20.46 to 17.61) [52], Deng's meta-analysis confirmed higher efficacy of PRP (RR: 1.42; 95% CI: 1.30 to 1.56; $p<0.001$) [54], OuYang's review supported this (OR: 4.37; 95% CI: 3.02 to 6.33; $p<0.001$) [55]. Meznerics' meta-

analysis, which included ulcers of different aetiologies, showed PRP superiority in the diabetic ulcer subgroup (OR: 2.26; CI: 1.50 to 3.41; I^2=12%), though the effect was less marked than in venous ulcers. No significant difference was observed between injectable and topical PRP (though this was based on all ulcer types) [57]. Qu's meta-analysis, also covering ulcers of various aetiologies, reaffirmed PRP's superiority [58]. Thanigaimani's network meta-analysis, which included three trials on diabetic ulcers of various types, also supported PRP efficacy (RR: 9.69; 95% CrI: 1.37 to 103.37) [56]. Finally, Platini *et al*., confirmed the superiority of topically applied PRP gel over controls, identifying Wagner grade 2 (RR=2.12, 95% CI: 1.01 to 0.44; p=0.05) and grade 3 (RR=1.36, 95% CI: 0.70 to 2.64; p=0.37) ulcers as the stages where PRP treatment is most beneficial [53].

The review by Napit *et al*. [59], which analysed 7 studies (4 of which were on diabetic ulcers), noted that none of the studies reported hazard ratios (HR). Deng's meta-analysis [54], which included 19 studies, found a statistically significant reduction in healing time compared to conventional treatment (MD=–3.13 days, 95% CI: –5.86 to –0.39, p<0.001). Similarly, Ou Yang's review [55] reported a significant result (MD=–3.21 days, 95% CI: –3.83 to –2.59, p<0.001).

Napit [59] highlighted the heterogeneity in how healing rates were reported across the 7 studies analysed. While a trend toward faster healing in the PRP group emerged, the heterogeneity prevented statistical pooling or definitive conclusions. Platini [53], in his meta-analysis on topical platelet gel, found a 16.97-day reduction in wound duration (95% CI: –32.64 to –1.29; p<0.00001). However, no statistically significant difference was found when wound duration was expressed in weeks (MD=–5.60 weeks, 95% CI: –18.92 to 7.72; p=0.41). The authors concluded that PRP accelerates tissue growth, but its effect on healing time may not emerge clearly due to high study heterogeneity.

Napit's review [59] noted that, except for one study, no healing occurred in control groups, making statistical comparison infeasible. Deng's meta-analysis [54] showed a significant reduction in wound area with PRP compared to controls (MD=1.02, 95% CI: 0.51 to 1.53, p<0.001). Meznerics *et al*. [57] found PRP to be superior to controls in the diabetic ulcer subgroup (SMD=–0.68, 95% CI: –1.31 to –0.06; I^2=93.64%). Qu's meta-analysis [58], which included both autologous and homologous PRP, also found a significant reduction in ulcer area (SMD=1.37, 95% CI: 0.91 to 1.82; I^2=22.1%) and no difference between autologous and homologous PRP. In contrast, OuYang [55] did not find a statistically significant reduction (MD=5.67, 95% CI: –0.77 to 12.11; p=0.08). Platini [53] concluded that no consistent evidence exists for a significant wound area reduction using topical PRP gel.

Babaei *et al*. [41], in a prospective comparative study, assessed PRP efficacy on ulcers of varying sizes. All wound sizes benefited from PRP, but statistically significant differences in healing times were observed between smaller wounds (2 to 5.5 cm^2) and larger wounds (8.5 to 12.5 cm^2). Thus, supporting the use of PRP especially in smaller lesions.

Overall, both controlled and uncontrolled studies indicate that PRP is safe when used either by injection or as topical dressing. Deng's meta-analysis [54] found no significant difference in adverse events [35-37, 39, 42, 44-46]. Only one study [48] noted a minor local injection-site irritation. A study using injectable platelet lysate [47] reported minor adverse events such as injection site pain, local oedema, infection, bleeding, redness, and warmth. No adverse events were reported in studies using topical PRP as a dressing [40, 41, 44, 47-50].

Meznerics [57] hypothesized a protective effect of PRP against infection, though no quantitative analysis was conducted. Finally, Platini *et al*. [53] found standard treatment to be associated with higher infection risk than PRP at week 1 (RR=0.56, 95% CI: 0.34 to 0.91; p=0.02) and week 2 (RR=0.13, 95% CI: 0.02 to 0.04; p=0.01). However, over time, differences became non-significant.

In addition, no significant difference in amputation rates was found in most of the studies analysed.

However, Deng found PRP to be associated with reduced risk (RR=0.35, 95% CI: 0.15 to 0.83; p<0.001) [54]. In Platini's meta-analysis [53], the amputation rate was significantly higher in the control group (MD=0.36, 95% CI: 0.16 to 0.84; p=0.02).

Overall, the available literature on the use of PRP in diabetic foot ulcers is characterized by substantial heterogeneity, particularly in the criteria used to define chronic or non-healing ulcers. Inclusion thresholds vary widely, encompassing lesions as recent as four weeks to ulcers persisting for up to six months, thus limiting the comparability of study outcomes. Patient selection also differs, with some studies excluding individuals on anticoagulant or antiplatelet therapy, or those with platelet disorders, likely due to concerns about interference with platelet activation or the risk of hematoma formation, even though such medications are common in the diabetic population due to associated cardiovascular risk. The preparation of PRP is inconsistently reported and varies significantly, ranging from single centrifugation procedures reliant on operator expertise to commercial kit-based protocols. This procedural variability undermines reproducibility and reduces the evidentiary strength of the findings, though the safety profile remains generally favourable. Crucial methodological details such as initial and final platelet counts and injection volumes are often omitted, preventing accurate calculation of the administered platelet load, which is increasingly recognized as a fundamental parameter influencing biological activity. The mode of PRP administration also differs, with injectable formulations currently appearing more effective than topical ones, although comparative data are limited and frequently lack key technical specifications, such as platelet concentration and activation status. The frequency and volume of administration are inconsistently reported, further complicating assessment of therapeutic impact. Notably, no studies have incorporated platelet-rich fibrin, which could offer enhanced fibrin stability. The overall quality of study designs remains suboptimal, with a scarcity of double-blind randomized controlled trials; most

evidence derives from non-blinded or non-randomized trials, which carry higher risk of bias. Moreover, control group treatments often rely on outdated standards such as saline-soaked gauze, especially in low-resource settings, and the specifics of standard care are frequently underreported. Endpoints and outcome measures vary widely across studies, ranging from ulcer healing rates and area reduction to healing time, without standardized definitions. This variability significantly hampers cross-study comparisons and the synthesis of data in systematic reviews or meta-analyses.

Critical evaluation of PRP treatment in venous ulcers

The use of PRP in the treatment of venous ulcers is generally reserved for hard-to-heal wounds, which show no signs of healing despite the application of all therapeutic procedures established by international guidelines.

The types of studies reviewed included [60-72]: randomized controlled trials (non-blinded) [63, 67], randomized controlled trials comparing two administration routes [62, 70], non-randomized controlled studies, where the control was a second ulcer in the same patient [65], and case series [66, 72]. Additionally, three studies [60, 61, 64] labelled as prospective controlled trials provided detailed individual patient descriptions but lacked statistical analysis; they can therefore be considered extended case series (dual case series).

One study [60] included only patients with reflux limited to the superficial venous system, but it focused on comparing two treatment approaches to venous insufficiency – "modern" (latest advances in dressings and surgery) *vs* "traditional" – thus its findings are not suitable for comparison with other studies. Another study [61] included ulcers measuring >2 cm² and <100 cm². Some studies also reported ulcer age as an inclusion criterion: 6 weeks [62], 12 weeks [61], or 6 months [63].

Exclusion criteria, when specified, included thrombocytopenia [61] (<150,000 PLT/mm³), treatment with anticoagulants or antiplatelet agents [63], immunosuppressants [64], corticosteroids [61], concomitant arterial disease, diabetes, infection [63, 64], exposed bone or tendon [63], among others.

Blood volumes for PRP preparation are often unspecified; when reported, the standard amount is around 30 ml. Sodium citrate or ACD is used in all studies (though not always explicitly stated). A double-spin protocol is employed in all studies that describe the method [62-64], usually reporting duration and revolutions per minute (rpm) or relative centrifugal force (*g*). Two studies reported 10% calcium chloride at a 1:4 ratio [62, 64] for PRP activation; one study did not activate the PRP [63], and others did not specify activation protocols. Only one prospective comparative study [64] reported the platelet concentration in PRP, which was 5.4 times the baseline level, although leukocyte content was not measured despite labelling the product as P-PRP. In this study, platelet concentration was slightly above 1×10^6 PLT/µl. All other studies did not report final platelet or leukocyte concentrations.

In several studies PRP was injected perilesionally and intralesionally, without topical application [60, 63, 65]. Exclusive use of PRP as a topical dressing without injection was commonly reported [61, 64, 66-69]. No study investigated combined use (injection+dressing). One prospective comparative study [62] included two non-randomized arms: one received perilesional injections, the other topical dressings (topical application method not specified). Results showed a trend toward better outcomes with injections. Notably, dressing changes were performed biweekly in both groups, which may have negatively affected the topical PRP group. Another study [70] comparing the two administration methods found no significant difference between injection and topical groups overall, but topical PRP was more effective in diabetic patients, while injection was preferable for chronic, fibrotic ulcers. Histopathological findings were similar between the two groups [70]. None of the included studies reported the volume of PRP administered. In studies using topical PRP, dressing changes were generally performed weekly for varying durations: up to six weeks [70, 71], nine weeks [68], or one year [64]. One study [61] performed a single application. Many studies did not specify timing. In a comparative study [62], treatment was given every two weeks for a total of 8 sessions, which may have limited the effectiveness of the topical PRP.

Excluding case reports, some studies did not explicitly specify the endpoints [61, 63]. When endpoints were defined [60, 62, 65, 68, 69, 71], primary outcomes included wound healing rate, the number of wounds healed by the end of the observation period, and the percentage reduction in wound size during or at the conclusion of the study. One comparative study between injectable and topical administration based its endpoint on histopathological characteristics of the lesions [70].

It should be noted that studies comparing PRP with other innovative dressings such as adipose tissue or those associating PRP with grafting techniques were excluded. The standard comparator treatment was specified only in some cases: saline-soaked gauze [70], petroleum jelly gauze [67], hydrocolloids [69], while many studies generically referred to a standard treatment without further detail [62, 63, 65, 68, 71]. One study used a calcium chloride solution as control [64]. PRP treatment demonstrated superiority over controls in all studies, for both injectable and topical forms. Direct comparisons between these two methods yielded mixed results: one study [63] found them substantially equivalent, while another [62] reported superiority of the injectable form. However, the latter study performed biweekly dressing changes, which may have disadvantaged the topical method.

A recent review selectively analysed data from randomized controlled trials on venous ulcers, including a total of 20 studies focusing on ulcers rather than patients [73]. It showed that PRP-treated groups had higher complete ulcer closure rates than controls (OR 5.06, 95% CI: 2.35 to 10.89, I^2=58%, p<0.01). Geographic subgroup analysis revealed no significant differences between PRP and control in Asia-Pacific (OR=3.94, 95% CI: 0.53 to 29.59), Europe (OR=1.46,

95% CI: 0.63 to 3.37), or America (OR=1.00, 95% CI: 0.17 to 5.77), but significantly favoured PRP in Africa (OR=14.26, 95% CI: 4.33 to 46.95). The positive effect of PRP remained significant at ≤3 months (OR=9.40, 95% CI: 2.93 to 30.22) and >3 months (OR=2.66, 95% CI: 1.27 to 5.58). Moreover, both injectable (OR=6.69, 95% CI: 3.14 to 14.24) and topical PRP (OR=4.24, 95% CI: 1.75 to 10.26) outperformed controls without significant difference between them (χ^2=0.59, df=1, p=0.44). PRP was more effective for both small (≤10 cm²: OR=8.72, 95% CI: 1.85 to 41.14) and large ulcers (>10 cm²: OR=4.72, 95% CI: 1.68 to 13.29). Few studies assessed pain via VAS scale and only two were included in the meta-analysis [73], with no significant difference between PRP and controls (MD=1.19, 95% CI: –0.67 to 3.04, I^2=52%). In addition, based on two studies, PRP shortened healing time compared to controls (MD=–3.25 days, 95% CI: –4.06 to –2.43, I^2=49%). Recurrence rates, based on limited data, were not significantly different (OR=0.16, 95% CI: 0.05 to 0.50, I^2=18%), nor irritant dermatitis was found (OR=0.38, 95% CI: 0.08 to 1.90, I^2=0%) [73].

Overall, the inclusion criteria for venous ulcer studies are variable and generally do not exclude ulcers associated with post-thrombotic syndrome, while exclusion criteria are more uniform and commonly encompass pregnancy, breastfeeding, coagulation abnormalities, corticosteroid use, immunosuppression, suspected neoplastic ulcers, recent transfusion, and circular ulcers. The frequent exclusion of patients on antiplatelet or anticoagulant therapy, often with the option of temporary suspension, lacks a clearly stated rationale, despite the high prevalence of such therapies in the elderly population typically affected by venous ulcers. The methods used to prepare PRP are frequently underreported, and while double centrifugation appears to be the most adopted technique, insufficient methodological detail weakens the strength of evidence. Crucial parameters such as baseline and final platelet counts, as well as the volume of PRP administered, are rarely specified, preventing accurate estimation of platelet dose per session or across the entire treatment course. This gap may reflect limited expertise in blood component handling, especially when commercial kits are used without critical assessment. Although maintaining a minimum platelet concentration is important, the total platelet dose administered is increasingly regarded as a more relevant indicator of the biological effect. No substantial efficacy differences have yet emerged between injected and topical PRP, although data remain limited. Topical PRP typically requires activation to form a fibrin gel, which is relatively unstable and may necessitate more frequent applications. A comparison with platelet-rich fibrin, which generates a more stable matrix via spontaneous activation, would be of interest. Two non-randomized comparative studies have attempted to evaluate injection versus topical use but lacked essential details such as platelet concentration and delivery mode, underscoring the need for well-designed, double-blind randomized trials. Most studies administered PRP once weekly, though in one comparative study, biweekly injections were employed – potentially disadvantaging the topical group due to rapid degradation of activated PRP gels. The overall quality of the studies is limited by a lack of high-level evidence, with most trials being non-blinded or non-randomized. In several studies, saline-soaked gauze was used as the control treatment, despite its misalignment with contemporary wound care standards, likely due to resource constraints in lower-income settings. Furthermore, standard treatments were often poorly defined and failed to explicitly adhere to principles of moist wound healing or wound bed preparation. Study endpoints were inconsistently reported, further limiting the comparability and interpretability of outcomes.

Critical evaluation of PRP treatment in musculoskeletal disorders

The use of PRP in musculoskeletal disorders has been widely debated over the years for various reasons, including preparation methods, indications for treatment, its efficacy compared to other biological and/or synthetic preparations (hyaluronic acid – HA – and corticosteroids), the number of administrations, and regulatory issues related to the manipulation of blood components. Nevertheless, numerous randomized clinical trials and systematic literature reviews almost unanimously support the efficacy of this treatment, particularly in pain relief and functional restoration in patients with osteoarthritis (OA) and tendinopathies (*Supplementary Table 1 available online*).

In 2023, the ORBIT group of ESSKA (European Society of Sports Traumatology, Knee Surgery and Arthroscopy) established a consensus regarding the use of PRP in patients with osteoarthritis [74]. The consensus includes level I and II studies, as well as prospective studies and meta-analyses, supporting the safety and benefits of PRP compared to both placebo and other infiltrative treatments such as hyaluronic acid and corticosteroids. PRP has proven effective in patients with mild to moderate knee OA (Kellgren-Lawrence – KL ≤3) but offers less benefit in patients with KL 4 OA, for whom it serves mainly as a palliative option in case of refusal or contraindication to surgery.

The consensus also investigated the superiority of PRP over glucocorticoid (GC) infiltrative therapy and the different types of PRP preparations. Regarding comparison with GCs, PRP demonstrated a longer-lasting effect and a better safety profile on chondrocytes, since GCs, especially with repeated injections, exhibit high toxicity. Compared to HA therapy, PRP has a more prolonged efficacy, although the variability of commercially available HA formulations may introduce bias in the evaluated studies.

Based on several meta-analyses, the consensus was unable to reach agreement on the optimal platelet concentration and/or quantity in the preparation or whether Leukocyte-Rich PRP (LR-PRP) or leukocyte-poor PRP (LP-PRP) yields greater patient benefit, suggesting that the beneficial effects of PRP are multifactorial, complex, and not yet fully understood [74].

Thus, in Europe, ESSKA sought to clarify certain aspects of PRP use, while in 2021, the AAOS (American Academy of Orthopaedic Surgeons) published an over-

review confirming the clinical efficacy of PRP compared to placebo but emphasized that PRP outcomes are comparable to other infiltrative therapies, highlighting the need for higher-quality studies to affirm PRP's superiority [75, 76].

Critical evaluation of PRP treatment in vasculitis, mucositis, andrological and gynaecological disorders

Only few studies are available regarding the use of PRP in vasculitis and mucositis. In 2021, a case report described a 46-year-old female patient with leukocytoclastic vasculitis, a vasculitis with various aetiologies affecting the skin and characterized by polymorphic rash with erythema, purpuric skin lesions, necrosis, and ulceration [77]. After 6 months of ineffective conventional treatments, PRP therapy was started, and lesions healed within one month. The frequency and application modalities of PRP were not reported in this study.

Another study [78] is a pilot trial analysing the efficacy of LP-PRP therapy in a group of patients with Behçet's Disease (BD), a chronic immune-mediated vasculitis characterized by mucocutaneous ulcers, ocular involvement, and vascular and neurological alterations. Of 77 patients, only 12 were enrolled because patients with ocular or neurological involvement, those on anticoagulants or immunobiological drugs, or without consent were excluded. Enrolled patients were treated for oral ulcers with nine 3 ml applications over 6 months, with one-year follow-up. Results showed PRP promoted an anti-inflammatory profile characterized by increased regulatory T cells (Tregs) in plasma, decreased activated NK cells, and cytokine profile changes. Clinical improvement was observed with reduced number and faster healing of ulcers.

Several studies [79-81] analysed the efficacy of homologous PRP/Platelet Gel in treating oral mucositis induced by chemotherapy and radiotherapy in oncologic patients. Chemo- and/or radiotherapy can cause oral complications that severely affect quality of life by interfering with essential activities such as eating and communication. Oral mucositis is a frequent and severe complication. PRP use in oral mucositis treatment appears effective in improving oncologic patients' quality of life.

A recent review [82] analysed numerous studies on PRP, alone or combined with adipose-derived stem cells (ADSC), for the treatment of genital female localized Lichen Sclerosus (LS). Safety of PRP and/or ADSC therapy was assessed, with adverse events limited to mild and transient symptoms such as pain and oedema at the injection site. Significant improvements in subjective symptoms (itching, burning, dyspareunia, and sexual function) were reported. Symptom improvement allowed reduction or cessation of topical corticosteroids.

Although few studies exist, available evidence suggests PRP therapy in inflammatory skin and mucosal lesions with difficult healing is effective. Literature shows PRP, delivered in various ways, can rapidly repair oral and oesophageal mucosal damage from the first applications, halt mucositis progression, reduce pain intensity, and restore patients' ability to feed (*Supplementary Table 2 available online*).

PRP use for erectile dysfunction (ED) and Peyronie's Disease (PD) remains still investigational. Regarding ED, 11 studies have enrolled men aged 30 to 75 years, mostly poor responders to conventional oral pharmacotherapy. Only three were placebo-controlled [83-85], with contradictory efficacy results but excellent safety profiles. Despite methodological variability, current studies show promising results in restoring responsiveness to phosphodiesterase type 5 (PDE5) inhibitors. However, the absence of standardized operating procedures renders outcomes highly dependent on the investigators' expertise. Quantification of the platelet concentration administered per session remains necessary, as both treatment intervals (ranging from 4 to 8 weeks) and number of injections (ranging from one to three or more) lack standardization. Notably, only the study by Francomano *et al*. [86] identified mean platelet volume, measured preoperatively, as a potential biomarker of therapeutic efficacy.

Patient selection deserves special attention, with careful evaluation of ongoing therapies and comorbidities to maximize treatment efficacy. The main inclusion criterion for ED should be the presence of a vasculogenic form, confirmed by dynamic penile colour Doppler ultrasound following established protocols [87]. Most studies feature relatively short follow-up periods.

Regarding PD, few studies exist [88-93] and enrolled heterogeneous populations in terms of age, plaques, and deformity. Despite varying methodologies, studies showed promising results in curvature improvement, though techniques varied among authors. The main inclusion criterion should be the absence of anticoagulant therapy, recommending suspension for a suitable period before the procedure if clinically safe. Currently, evidence on PRP effects for all forms of ED is limited and very limited for PD (*Supplementary Table 3 available online*).

The use of PRP in female genital pathologies is also still investigational. Regenerative medicine, particularly PRP with its well-established regenerative, anti-inflammatory, immunomodulatory, and angiogenesis-stimulating properties, is well-suited for application in gynaecology. However, recent studies describing PRP use are limited. The main proposed indications include: chronic pelvic pain (regardless of aetiology), stress urinary incontinence, ovarian insufficiency (e.g., poor ovarian reserve, symptomatic menopause, assisted reproduction scenarios) and endometrial disorders (e.g., failed implantation, recurrent early miscarriage, endometritis).

In the last 5 years, several studies have been published (*Supplementary Table 4 available online*). Current literature and clinical experience highlight PRP's utility in gynaecology. Standardizing protocols and refining patient selection will further strengthen the evidence base.

Key clinical research questions

Q1. Does PRP preparation methodology affect product quality?

Q2. Is there sufficient evidence that platelet concentration affects clinical efficacy?

Q3. Are there robust data supporting specific PRP topical and injectable protocols for defined pathologies?
Q4. What clinical questions remain unanswered?

Expert answers

• *A1: Influence of PRP preparation method on product*

Based on a critical analysis of the literature and the field experience of the expert panel participating in the EC, it is considered that the type of procedure used for product preparation may influence its yield. Numerous devices are currently available for the preparation of PRP for non-transfusional use, most of which are based on the principle of centrifugal separation. Available evidence indicates that protocols involving double centrifugation ensure a higher platelet yield. It should be noted, however, that some of these methods are exclusively available at transfusion facilities. The medical devices (MD) available on the market and primarily used in outpatient or non-hospital settings may produce variable yields compared to the standard indicated in the current transfusion law, which is $1x10^6$/µl ±20%. It is emphasized that such medical devices must be compliant with the medical device class defined by law, and transfusion services are urged to verify the compliance and to employ all necessary measures to ensure the safe use of PRP by healthcare professionals.

Recent scientific literature has highlighted that, in addition to the well-known action of growth factors released by platelets, other mechanisms of intercellular communication are involved in the regenerative and anti-inflammatory effects of PRP. These include the release of extracellular vesicles and the transfer of mitochondria. The practical implication is that "viable" platelets may retain greater regenerative and anti-inflammatory potential. Nevertheless, practical experience and the need to obtain enough platelets suggest that freezing procedures for platelet-derived products can still result in a clinically satisfactory effect.

• *A2: Effect of platelet concentration on PRP efficacy*

Several *in vitro* studies have indicated that the proliferative and differentiative effects of PRP are cell-specific and dependent on the platelet concentration used. Some studies have reported that higher platelet concentrations may paradoxically result in reduced effects compared to lower concentrations, following a bell-shaped dose–response curve. With regard to human application, the concept of concentration should be harmonized with that of "absolute quantity." In other words, in individuals with lower baseline platelet concentrations, a larger blood volume may be required to achieve an effective dose. Although the scientific literature is not entirely consistent or easy to interpret – due to incomplete information on preparation methods and platelet concentrations – three interesting studies [94-96] analysing numerous reports, identified an effective average quantity of 3.2 $x10^9$ platelets, above which all positive results were achieved, while below this threshold, almost all results were negative. This could serve as a starting point for further targeted studies on effective platelet load, tailored to specific tissues/regions, to treat lesions based on their severity and chronicity.

• *A3: Evidence for protocol-specific PRP use*

With regard to diabetic foot ulcers, the extensive scientific literature – despite limitations in the overall quality of studies – is generally consistent in supporting the usefulness of PRP treatment, both when applied as a topical dressing and when administered via peri- and intra-lesional injections. Currently, limited evidence suggests a possible superiority of the injectable form.

For venous ulcers, more evidence is available supporting the use of PRP in topical form as a dressing, whereas fewer studies have evaluated its peri- or intra-lesional injection. These findings are consistent with the recommendations issued by the Italian National Blood Centre (Centro Nazionale Sangue, CNS) in the guideline *Therapeutic indications for the use of blood components for non-transfusional purposes – Third edition, June 2024* which supports the use of PRP in the treatment of both diabetic foot ulcers and venous ulcers (in treatment cycles of 12 applications), with a level of evidence rated 1B, without specifying a preferred route of administration (topical *vs* injectable). It remains to be determined whether PRF (platelet-rich fibrin, obtained through coagulation by contact with glass surfaces without additive agents) may offer similar or superior performance compared to PRP.

Regarding orthopaedic applications, a review of the scientific literature – further reinforced by recent consensus conferences from ESSKA and AAOS – has shown that PRP is effective in patients with mild to moderate knee osteoarthritis (KL grade ≤3) but offers limited benefit in KL grade 4 patients. PRP has also demonstrated superiority over corticosteroid injections, particularly in terms of the duration of therapeutic effects and safety profile with repeated treatments. Superiority over HA has also been reported, although this comparison is affected by the heterogeneity of HA formulations available. The role of leukocytes in PRP preparations remains to be clarified.

These conclusions align with the CNS guidelines, which recently recommended PRP for the treatment of grade 1-3 osteoarthritis of the knee and hip, in treatment cycles consisting of three applications, with a level of evidence rated 1B. The level of evidence for PRP use for other musculoskeletal conditions (ankle osteoarthritis, pseudarthrosis, anterior cruciate ligament lesion/reconstruction, patellar tendinopathy, epicondylitis infiltrative treatment, Achilles tendon inflammation, rotator cuff injury) has been rated 2B.

As for the use of PRP in andrological conditions (ED and PD), in the treatment of vasculitis and mucositis – including those induced by chemotherapy and radiotherapy in cancer patients – and in female genital disorders (chronic pelvic pain, stress urinary incontinence, ovarian insufficiency, endometrial pathologies), the scientific literature remains limited. Nevertheless, despite the small number of published studies, there is broad clinical practice supporting the efficacy and applicability of PRP in these fields. Further research is needed to establish standardized treatment protocols.

- *A4: Unanswered research questions*

The scientific literature shows significant heterogeneity regarding PRP preparation methods. The variability is largely attributable to the use of different devices developed by the industry. These systems, involving minimal manipulation, are designed to simplify the production of platelet concentrate, particularly for users who may lack the manual expertise of transfusion medicine specialists in handling blood products.

Several important issues remain to be clarified:

1. identification of patient-related predictors of clinical outcomes. For example, in the case of diabetic foot ulcers, outcome predictors should consider the relative contribution of ischemic, neuropathic, and infectious components – the three main pathogenic factors involved in lesion development and persistence. Regarding ulcer severity as a prognostic indicator, it would be important to confirm that Wagner grade 1 and 2 lesions show the best therapeutic response. For venous ulcers, it would be necessary to assess blood count parameters and the role of deep or superficial venous insufficiency in lesion development and maintenance;
2. identification of outcome predictors related to the PRP product itself, such as the concentration of specific growth factors or other bioactive mediators;
3. comparison of PRP efficacy with other innovative therapeutic approaches, including advanced wound dressings, synthetic and biological scaffolds;
4. determination of optimal platelet amount, both per session and across the entire treatment cycle, as no definitive clinical data are currently available to establish the most effective total platelet load;
5. clarification of the preferred mode of administration – injection versus topical application;
6. identification of the optimal interval between treatment sessions (both injectable and topical), and the total number of sessions required based on specific pathologies;
7. validation through robust clinical trials of PRP use in less-established applications such as mucositis, vasculitis, ED, PD, stress urinary incontinence, and fertility disorders.

Recommendations from the SIMCRI Expert Consensus Group

In line with previous recommendations and based on the clinical and laboratory experience of the working group members, it has been deemed appropriate to define shared criteria for clinical assessment and PRP preparation procedures. The dual objective is to enhance the therapeutic efficiency of PRP according to the underlying pathology and to promote personalized treatment strategies.

Inspired by the TIMERS framework used in wound care, a dedicated checklist protocol – TAMIS (Tissue, Area, Method, Inflammation/infection, Scheduling) – is proposed to support the clinical use of PRP. The TAMIS protocol guides clinicians in evaluating the following parameters:

- Tissue: identify as bony (e.g., orthopaedic, dental) or soft (e.g., skin, mucosa, muscle);
- Area: define the surface area to be treated;
- Method: choose application mode – e.g., topical gel, injectable (intra-tissue, intra-articular), aerosol, mucosal/ocular;
- Inflammation/infection: determine if adjunct medication is needed (simultaneous or sequential);
- Scheduling: define single dose *vs* cycle, number of sessions, intersession interval, and follow-up maintenance.

The TAMIS parameters serve both as a practical tool for guiding clinical decision-making and as a standardized framework for comparing outcomes across scientific studies.

Patient assessment must comply with national regulations concerning the preparation and use of autologous blood components (MoH Decree, 2 November 2015; updated 1 August 2019). For instance, local health authorities in Italy (e.g., USL Toscana, Emilia-Romagna, Veneto, Lazio) have established eligibility criteria, including:

- platelet count >100×10³/µL in prior 3 months (Toscana, EmiliaRomagna, Veneto);
- exclusion if platelet count <120×10³/µL (Lazio);
- must avoid platelet-interfering medications 5 days before sampling (EmiliaRomagna, Lazio);
- exclude poorly controlled diabetes, active chemotherapy, coagulopathies, immunodeficiencies, immunomodulatory treatment (Lazio).

It should be noticed that the total number of blood components remains constant in a closed system, regardless of processing. Therefore, while plasma volume may be reduced during PRP preparation, the absolute number of platelets remains dependent on the initial volume of whole blood drawn.

To standardize platelet evaluation, the efficiency of recovery should be calculated for each device:

$$Recovery\ efficiency\ \% = \frac{Vol.PRP \times PLT_{PRP}}{Vol.Blood \times PLT_{blood}}$$

An efficiency of 50% (0.5) is generally considered acceptable.

To obtain a desired quantity of platelets (Desired Platelet Quantity, DPQ), the following equation can be applied:

$$Volume\ to\ draw = \frac{DPQ}{PLT_{blood} \times Recovery\ efficiency}$$

Example:
To obtain 3.2×10⁹ platelets in a patient with 300×10³ platelets/µL (0.3×10⁹/mL) and assuming a 60% efficiency, the required blood volume is:

$$Volume\ to\ draw = \frac{3.2}{0.3 \times 0.6} = 17.77\ mL$$

This example, along with others, is outlined in *Table 1*.

Treatment area, application site, and method (topical *vs* injectable) should be considered when determining the desired platelet load, in accordance with the TAMIS protocol. For instance, 25 cm² HA sheets soaked with

Table 1
Practical example of calculation for whole blood volume to be drawn

Patient PLT count (PLT/µL)	Recovery efficiency (%)	Target PLT quantity ($\times 10^9$)	Required blood volume (mL)
150,000	60	3.2	35
200,000	70	3.2	18
300,000	60	3.2	18

1.8 mL of PRP have demonstrated therapeutic efficacy in ulcer treatment [8].

It should be noted, however that a desirable platelet content has to reach a concentration factor of 4-5x, in order to minimize the volumes, particularly for injective procedures in musculoskeletal disorders.

When using devices that employ thixotropic gel, attention must be paid to the gravitational weight and composition of the gel, which may influence the final PRP volume and concentration [97].

It is essential to promote a culture of regenerative medicine that encourages researchers to report all parameters potentially influencing treatment outcomes. Scientific journals should sensitize their peer reviewers to demand comprehensive reporting of platelet concentrate preparation protocols. Researchers must be encouraged to conduct randomized, multicentre, double-blinded trials capable of generating robust scientific evidence.

To this end, scientific publications should ideally include the following information:

1. device used, centrifugation time and temperature, and centrifugal force (g);
2. platelet concentration in the patient's baseline whole blood;
3. platelet concentration in the final product;
4. volume administered, including:
   – injectable form: volume injected in peri- or intra-lesional locations;
   – topical form (dressing): volume applied topically;
5. type of dressing used; for topical application, details of any secondary dressing;
6. additional devices applied (e.g., orthoses, low/high-stiffness bandaging, compression stockings with specified compression level).

Below are reported specific recommendations for PRP use in selected pathologies (schematically illustrated in *Figure 1*):

- the use of PRP for the treatment of diabetic foot ulcers is a safe procedure. For non-primarily ischemic diabetic ulcers without active infection and without bone or tendon exposure (Wagner grades 1-2), PRP treatment is recommended with a relatively high level of evidence. For more severe non-primarily ischemic diabetic ulcers (Wagner grades 3-4), the literature is scarce; however, the available evidence suggests potential efficacy of the treatment, so PRP use is not discouraged;
- the correct approach to treating venous ulcers is based on hemodynamic correction of venous insufficiency, both deep and superficial, typically via elastic compression (which applies counterpressure against transmural pressure) or varicose vein treatments in various forms. If the lesion fails to respond satisfactorily to these treatments, regenerative medicine techniques such as PRP become relevant. The use of PRP for venous ulcer treatment is a safe procedure. The predominant literature supports topical (dressing) use on lesions, while data on injectable treatment are currently too limited to establish comparative efficacy. Therefore, topical PRP use is recommended for venous ulcers, but injectable intra- or peri-lesional PRP is not contraindicated;
- a large body of scientific literature, supported by international consensus, currently supports PRP as a consolidated therapeutic option for various musculoskeletal conditions. Comparisons with other injectable therapies (corticosteroids and HA) do not disadvantage PRP. Its use is therefore recommended, particularly for mild to moderate osteoarthritis and various tendinopathies;

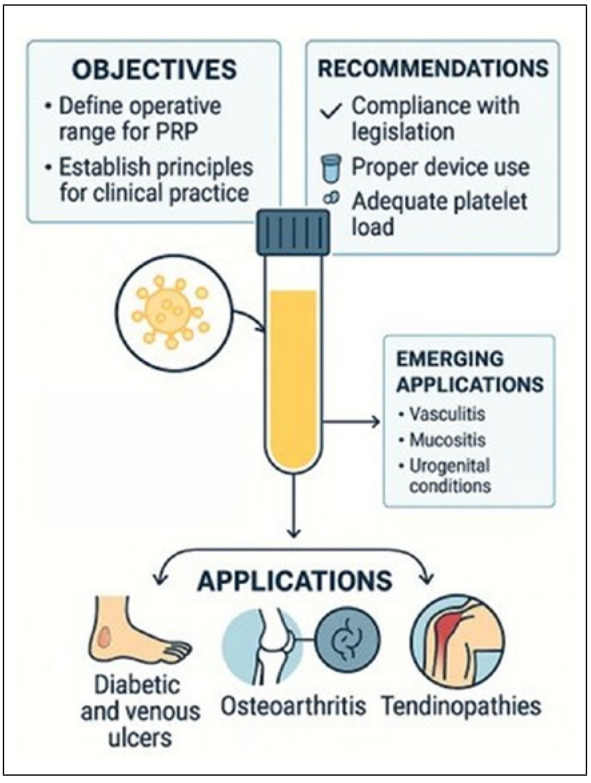

Figure 1
Platelet-rich plasma (PRP) in regenerative medicine. Schematic representation of objectives, recommendations and clinical applications. Image design was created with Artificial Intelligence support (ChatGPT, OpenAI).

- few studies in the scientific literature describe evidence of PRP use in problematic conditions such as various mucositis, all reporting satisfactory results. However, randomized controlled trials are lacking. Given the nature of lesions and the lack of definitive alternative therapies, PRP use is not discouraged if patient conditions permit;
- regarding andrological disorders, PRP use for ED and PD appears safe. It is not discouraged, especially for vasculogenic ED unresponsive to PDE5 inhibitors. Evidence for PD treatment is less clear; injection site, number of injections, and intervals are yet to be standardized. PRP use is not discouraged for stabilized PD without pain and/or ED;
- for some gynaecological disorders (chronic pelvic pain, stress urinary incontinence, ovarian insufficiency, endometrial pathologies, unsuccessful assisted reproduction or recurrent miscarriages), although randomized controlled trials are absent, evidence does not discourage PRP use.

CONCLUSIONS

Ethical considerations

The use of PRP within the context of regenerative medicine raises important ethical questions that require thorough reflection. First and foremost, it is essential to ensure that this therapy is applied in full respect of the principles of autonomy and informed consent, guaranteeing that patients receive clear and comprehensible information about the benefits, risks, and available alternatives. Furthermore, the standardization of procedures and the quality of PRP must be strictly monitored to avoid disparities in treatment and to ensure equity in access to care. Transparency in communicating clinical results and trial outcomes is also crucial, promoting rigorous research free from conflicts of interest and placing patient well-being at the center. Finally, the continuous evaluation of PRP efficacy and safety must be accompanied by ethical attention in clinical application, always considering the cost-benefit ratio and the appropriateness of therapeutic indications.

Limitations and areas for future research

This document, aimed at establishing a consensus on PRP use, originates from a critical review of recent scientific literature and the experience of the participants in the EC. It is a concise text that does not solve all the issues in an ever-expanding field and, therefore, has several limitations. The main limitation concerns the restricted scope of medical areas considered, since PRP is also widely used in dentistry, aesthetic medicine, and dermatology. Moreover, PRP can be combined with other regenerative medicine tools, such as natural or synthetic scaffolds, ozone therapy, photobiomodulation, stem cells, and leukocytes. These are all fundamental aspects requiring further investigation and may be addressed in future studies. The evidence and recommendations presented in this EC may be strengthened by future research based on collaborative and multicentre studies in the field of platelet-rich therapies.

Noteworthy, the implementation of SoHo EU regulation 2024/1938 and the adoption of good clinical practice will significantly impact the field of blood components for non-transfusion use, including PRP.

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Conflict of interest statement

The Authors declare that there are no conflicts of interest.

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Biomonitoring and toxicological assessment of Ochratoxin A and Citrinin in a sex-gender perspective: The OTABioTox Project

Lucia Coppola[1], Giuseppina Scialò[2], Francesca Debegnach[2], Gianluca Frustagli[3], Viviana Iacovelli[1], Gabriele Lori[1], Martina Enza Grieco[2], Francesca Maranghi[1], Alessia Tammaro[1], Roberta Tassinari[1], Enrica Fabbrizi[4], Patrizia Di Matteo[4], Lorella Ciferri[5], Marco Dolci[6], Barbara Gigli[6], Alessandra Di Stasio[6], Beatrice Murri[6], Giacomo Toffol[7], Alessandro Ciccotosto[7], Miriana Callegari[7], Margherita Guarnieri[8], Angela Pasinato[7], Barbara De Santis[2] and Cinzia La Rocca[1]

[1]*Centro di Riferimento per la Medicina di Genere, Istituto Superiore di Sanità, Rome, Italy*
[2]*Dipartimento Sicurezza Alimentare, Nutrizione e Sanità Pubblica Veterinaria, Istituto Superiore di Sanità, Rome, Italy*
[3]*Servizio Grandi Strumentazioni e Core Facilities, Istituto Superiore di Sanità, Rome, Italy*
[4]*Ospedale di Civitanova Marche, Unità Operativa Semplice di Pediatria, Civitanova Marche (MC), Italy*
[5]*Azienda Sanitaria Territoriale Fermo, Porto San Giorgio (FM), Italy*
[6]*Azienda Sanitaria Territoriale Macerata, Civitanova Marche (MC), Italy*
[7]*Associazione Culturale Pediatri, Narbolia (OR), Italy*
[8]*Società Italiana Pediatria, Rome, Italy*

Abstract

Introduction. Ochratoxin A (OTA) and citrinin (CIT) are toxic compounds produced by fungi that may contaminate a variety of food, posing significant health risks, particularly following chronic exposure. OTA, a potent nephrotoxin, is widely present in cereals, dried fruits, coffee and animal-derived products; CIT, which often co-occurs with OTA, also exhibits nephrotoxic effects.

Methodology. This project aims to evaluate the exposure to OTA and CIT in adults and children of both sexes, and in breastfeeding women, and to investigate OTA toxicological effects using a juvenile animal model. The biomonitoring study analyzed OTA and CIT in urine, and OTA in breast milk. It also involved the evaluation of biomarkers of effect and the administration of food frequency questionnaires to assess dietary intake. The toxicological study employed an innovative animal model to identify early biomarkers of renal and endocrine effects and to elucidate OTA's mechanisms of action.

Results. The findings contribute to a deeper understanding of risk characterization and support the development of public health strategies aimed at minimizing OTA and CIT exposure.

Key words

- mycotoxins
- children exposure
- human milk
- nephrotoxicity
- biomarkers

INTRODUCTION

Mycotoxins are toxic chemical compounds produced by certain fungal species under specific environmental conditions [1]. The toxigenic fungi colonize agricultural commodities, spreading mycotoxin contamination into the food chain. These contaminants persist through food processing, and the contamination is also transferred to the derived food products posing significant risk to both humans and animals [2, 3].

Dietary exposure to mycotoxins represents a significant health concern, especially in cases of chronic exposure, which has been linked to a range of adverse health effects [4]. Due to cumulative exposure over a lifetime, mycotoxins can profoundly impact human health, causing a broad spectrum of toxic effects including carcinogenicity, nephrotoxicity, hepatotoxicity, estrogenic effects, neurotoxicity, and disruption to reproductive and immune system functions [1, 5].

Address for correspondence: Martina Enza Grieco, Dipartimento Sicurezza Alimentare, Nutrizione e Sanità Pubblica Veterinaria, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: martina.grieco@iss.it.

Among the mycotoxins of greatest concern to risk assessors, ochratoxin A (OTA) is one of the most widely distributed. It is produced by ubiquitous fungal genera such as *Aspergillus and Penicillium* and several toxigenic species (e.g., *A. niger*, *A. ochraceus*, *A. carbonarius* and *P. verrucosum*) can occur in a broad range of plant derived foods products all entering human and animal diets. These commodities include cereals, dried nuts, spices, dried fruits, coffee, cocoa, licorice and food derivatives, including supplements (e.g., green coffee, gooseberry, and licorice derived supplements). Furthermore, OTA contamination has also been detected in certain cured animal-derived products (such as ham, salami). In these cases, contamination can occur during meat maturation and curing process, where storage conditions may be viable to OTA production. Like many other mycotoxins, OTA is highly resistant to processing and heat treatments, contributing to its persistence in the food chain [6].

In vivo studies showed that OTA has been associated with nephrotoxic, carcinogenic, teratogenic, immunotoxic and developmental effects, with kidney representing the critical target organ. OTA has been identified as a key causative agent in the development of porcine nephropathy, but the available epidemiological evidence remains insufficient to definitively classify it as a renal carcinogen in humans or directly link it to Balkan Endemic Nephropathy (BEN) or other unexplained endemic nephropathies [7-10]. Indeed, OTA is able to bindplasma-proteins, mainly albumin, with consequent reduction of excretion rate and accumulation in renal tissue. Albeit to a lesser extent, liver, muscle and fat are also involved in OTA toxicity in terms of signalling modulation of apoptosis, inflammation and stress responses [7, 11].

OTA showed also genotoxic effects both *in vitro* and *in vivo* studies, although the mechanisms are still unclear, and direct and indirect genotoxic and non-genotoxic modes of action may contribute to tumor development https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2020.6113. The International Agency for Research on Cancer (IARC) classified OTA as "possibly carcinogenic for humans" (Group 2B), with carcinogenicity being the result of interactions among several epigenetic mechanisms [12, 5]. Because recent studies have raised uncertainty regarding the mode of action for kidney carcinogenicity, the European Food Safety Authority (EFSA) deemed it inappropriate to maintain the tolerable weekly intake (TWI) established in 2006 as the health-based guidance value (HBGV) [13]. Indeed, the Margin of Exposure (MOE) approach was considered most appropriate, providing a safety margin (10000 for carcinogenic compounds) below which the public health concern can be considered low, even considering overall uncertainties [7]. The use of the Benchmark Dose Lower Confidence Limit ($BMDL_{10}$), the dose that causes a 10% increase in the incidence of a specific effect in an animal population, was proposed for neoplastic and non-neoplastic effects (BMDL10G 14.5 µg/kg body weight (bw) and BMDL10NG 4.75 µg/kg bw, respectively).

Citrinin (CIT), a mycotoxin with well-documented nephrotoxic properties, is commonly detected in food in the co-occurrence with OTA, and it is known to have synergistic effects with OTA, potentially exacerbating kidney damage [6, 14, 15]. It is produced by several species of *Aspergillus*, *Penicillium*, and *Monascus*, and it is commonly found in stored grains. A No-Observed-Adverse-Effect Level (NOAEL) of 20 µg/kg bw per day was identified from a 90-day study in rats. However, due to a lack of data, EFSA did not establish a HBGV for CIT. Instead, a level of no concern for nephrotoxicity was set at 0.2 µg/kg bw per day, reflecting the limited understanding of its potential effects. Despite this provisional threshold, concerns about CIT possible genotoxic and carcinogenic properties remain unresolved. Risk characterization for CIT has therefore focused on limiting its concentrations in grains to levels that would keep dietary exposure levels below the no concern threshold for nephrotoxicity. EFSA emphasized the need for further research to better define the risk associated with CIT and since then several studies have expanded the understanding of CIT toxic effect confirming its impact on multiple organs and systems [16]. In fact, in addition to nephrotoxicity, which remains the major effect, also hepatotoxic, cardiotoxic, neurotoxic, and reproductive effects have been reported [17-21]. Studies reported CIT-induced liver and heart toxicity, including fatty liver disease and cardiac tissue damage, linked to mitochondrial impairment and oxidative stress confirming hepatotoxicity and cardiotoxicity [17-19], while the genotoxic and mutagenic potential of CIT remains controversial. Some *in vitro* studies show DNA damage, cell cycle arrest, and autophagy induction [17]. However, IARC classifies CIT as "not classifiable as to carcinogenicity in humans" (Group 3) due to insufficient evidence [21].

EFSA has emphasized the need for more comprehensive data on human exposure to OTA and CIT, particularly in vulnerable populations like children and adolescents, who are more susceptible to the toxic effects of these contaminants [7, 13]. In this respect, human biomonitoring studies (HBM) using biological fluids are useful for assessing internal doses of contaminants because of combined exposure. Moreover, HBM together with dietary exposure assessment represent an integrated approach for a more accurate and comprehensive evaluation of human exposure.

The Italian National Committee for Food Safety, which addresses urgent and critical topics public health issues, has raised concerns about the exposure of younger age groups to OTA through the consumption of certain foods, particularly cereals, cheeses and pork derived cured meats [22]. National official control and monitoring plans have consistently reported the presence of OTA in a variety of foodstuff, in particular coffee, cocoa, ham and more recently certain varieties of hard cheese. Among the different exposure scenarios, particular attention should be given to breastfed infants, who may be exposed to mycotoxins such as OTA through lactation. Breast milk can serve as a potential exposure pathway, transferring OTA from mothers to their children and thus representing an additional risk for this especially vulnerable population group [7]. This is particularly concerning since children have higher

metabolic rates and immature detoxification system, making them potentially more vulnerable to the accumulation of mycotoxins in their developing organs.

In this context, exposure evaluation through biomonitoring studies in the general population and vulnerable groups, such as children, is essential to address existing gaps in risk assessment. In this framework, the project "Ochratoxin A: risk assessment in population groups through biomonitoring and toxicological characterization" (OTABioTox Project) aims to assess the exposure and toxicological effects of OTA and CIT through an integrated approach which includes: a) a biomonitoring study in adults and children of both sexes, as well as lactating mother-child pairs, including evaluation of selected kidney biomarkers; b) a dietary exposure evaluation, with a focus on foods that are particularly susceptible to OTA and CIT contamination, such as cereals, dried fruits, and cured meats; c) a toxicological study using male and female rats in the juvenile phase of life, corresponding to the childhood/peripubertal age in humans, treated at realistic dose levels of OTA derived from children exposure.

Upon the evidence of gender-based differences in the absorption and detoxification of contaminants, the project will possibly explore potential sex differences in human internal dose content and toxicological susceptibility [23, 24].

By combining biomonitoring, exposure assessment, and toxicological studies, this research seeks to bridge the knowledge gap in the exposure risks posed by OTA and CIT, considering adults and also vulnerable groups, such as young population and breastfeeding women.

METHODOLOGY

Biomonitoring study

The HBM in OTABioTox Project aims to measure the internal doses of OTA and CIT in urine and milk samples. The internal dose provides data at an individual level that helps to overcome the limitations associated with the heterogeneous contamination of food, sampling variability and constraints, and the availability of food and consumption data.

The elimination of OTA from the body is slow and incomplete, with variable rates of excretion through urine, blood, and breast milk collectively contributing to its persistence and potential for chronic health effects. OTA is typically excreted in urine with very low rates ranging approximatively 2-3% of the ingested dose. In kidney, OTA is partially reabsorbed due to its binding with albumin [25]. Importantly, OTA has also an excretion route via breast milk, albeit at low concentrations (lower than in blood, comparable with urine) and it provides a route of exposure for nursing infants. The presence of OTA in breast milk underscores the risk of early-life exposure, especially in regions where dietary contamination is common [26].

CIT and its main metabolite dihydrocitrinone (DH-CIT) exhibit a high urinary excretion rate with about 40% of the ingested dose eliminated within 24 hours. This makes urine a particularly reliable matrix for CIT exposure assessment [20, 27, 28]. There is currently insufficient evidence to confirm significant transfer of CIT to breast milk, so this pathway remains negligible.

To assess exposure to OTA and CIT, including their metabolites, a total of 350 subjects has been recruited across diverse demographic groups. The recruitment details are as follows:

- children aged 5-14 years: 70 boys and 70 girls;
- adults aged 18-65 years: 70 men and 70 women;
- breastfeeding women: 70 subjects.

In recent years, HBM studies have increasingly focused on evaluating biomarkers of effect, which can provide a link between internal exposure to contaminants and the related adverse health effects or diseases. They can increase the biological plausibility of the associations and indicate a possible mode of action. In this respect, the biomarkers of effect can improve the risk assessment of chemicals [29].

In OTABioTox project, albumin, creatinine and beta2-microglobulin are considered as kidney biomarkers associated to OTA and CIT exposure and measured in urine samples of the enrolled subjects. OTA is known binding serum albumin with a reduction of the clearance and a persistence of OTA in the body. However, a lower level of albumin increased OTA hepatoxicity and nephrotoxicity [30]. Higher levels of beta2-microglobulin in the urine, affecting renal reabsorption function, was associated to higher OTA level in human [31]. Creatinine values will be also used to normalize OTA and CIT concentration values.

Family pediatricians in the National Health System and hospital pediatricians collaborated in recruiting patients. This physician network was established under the European LIFE PERSUADED biomonitoring project (Phthalates and bisphenol A biomonitoring in Italian mother-child pairs: link between exposure and juvenile diseases; ENV/IT/000482) and the Finalized Research program (Integrated Approach to Evaluate Children Agricultural Pesticide Exposure and Health Outcome; RF-2016-02364628) therefore, the healthcare professionals possess extensive experience in conducting such studies across the national territory.

Healthcare professionals were provided with informative materials to instruct themselves, and subsequently recruited subjects and their families for enrollment in the study:

- food frequency questionnaire;
- description of the study for the physicians;
- description of the study for enrolled adults and children;
- a fact sheet for children and mothers;
- instructions on how to complete the FFQ;
- instructions for collecting urine and breast milk samples;
- privacy information and legal basis for processing personal data;
- informed consent for parents.

Food Frequency Questionnaire and dietary exposure

A paper-based questionnaire was designed to collect personal, residential information, as well as dietary habits. The first section requested personal details such as age, body weight and physical activity level. The second section consisted of a specially tailored Food Frequen-

cy Questionnaire (FFQ) aimed at capturing participant dietary habits. The FFQ included tables for each food category covered in national and European control activities [13]. In the FFQ, each participant was asked to specify the food items consumed, indicating the frequency (daily, weekly, monthly) and usual portion size (small, medium, large, or specific quantity). Once validated, the final version of the FFQ was distributed to the pediatricians overseeing participant enrollment.

The completed FFQs were reviewed for consistency, and the data were entered into an online database. This database was designed to efficiently manage the volume of data and ensure consistency during data entry. Due to the extensive number of fields in each questionnaire (more than 130), general-purpose software such as spreadsheets was considered inadequate. Therefore, a custom web application was developed to provide an optimized and user-friendly interface, specifically designed to minimize input errors and allow full data editing capabilities. The application was built using the HTML-Javascript-PHP-MySQL-Apache stack, utilizing open-source software. Data entered into the application were stored in a relational database on a server and subsequently exported serialized via dedicated functions into Tab-Separated Values (TSV) files, which could then be processed further using scientific statistical analysis software.

Analytical determination of OTA and CIT

Urine and breast milk are valuable biological matrices for assessing exposure; however, it is essential to have appropriate, validated and accurate analytical methods to reliably measure the targeted biomarkers [25, 32]. The most important challenge in the analysis of mycotoxins in biological fluids is the extremely low concentrations of the analytes in the specimen. The choice of suitable analytical protocol shall be focused on the identification of appropriate limits of detection (LOD) and quantification (LOQ) values [33]. Liquid chromatography coupled with tandem mass spectrometry (e.g., LC-MS/MS) enable a sensitive and accurate detection of biomarkers in biological fluids also in condition of low excretion rate, such as the one of the OTA in urine samples. These capabilities of modern analytical techniques are essential for reliable biomonitoring studies [34].

In OTABioTox Project, urinary OTA and CIT, as well as OTA in breast milk, were analyzed in a two-step analytical approach. In the first step, a screening method was employed to detect the presence of OTA, CIT and/or other biomarkers in the biological matrices. Upon obtaining a positive result in the screening, a second step for specific quantification was performed applying sample dilution in phosphate-buffered saline (PBS) followed by immunoaffinity columns (IAC) cleanup prior injection.

The IAC cleanup employs monoclonal antibodies to selectively isolate and concentrate the mycotoxin while removing matrix interferences. This approach is particularly well-suited for targeting and quantify single or a limited number of analytes in complex biological matrices such as urine and milk [35].

The determination was performed by LC-MS/MS; the triple quadrupole, set in positive mode with MRM (Multiple Reaction Monitoring) acquisition mode, enables high accuracy, selectivity and sensitivity which is necessary as the concentration of mycotoxins in biological matrices is generally lower than the contamination levels found in food. The method was validated and met satisfactory performance of precision and trueness in both urine and breast milk matrices. The LOQ was established at 0.004 ng/g for OTA and 0.009 ng/g for CIT in urine and 0.008 ng/g for OTA in breast milk.

Toxicological study

An *in vivo* toxicological study is proposed, using an innovative juvenile animal model that corresponds to the pre- and peri-pubertal developmental stages in children [36]. This stage of the life cycle is particularly vulnerable and susceptible to the potential adverse effects of chemical substances, as body systems are still developing and maturing, while dietary exposure is higher compared to adults. This model enables the early detection of alterations, the study of underlying mechanisms and the identification of early effect biomarkers through the analysis of tissue, serum and molecular markers, and transcriptomics [37, 38]. The animal study was executed in accordance with Directive 2010/63/EU, the Italian Legislative Decree n. 26 of 4 March 2014, the OECD Principles of Good Laboratory Practice. The design and sample size of the animal studies were chosen according to 3R principles and ARRIVE guidelines 2.0 [39].

18 dams of CD1 mice (Envigo, Italy) with offspring will be kept under standard laboratory conditions (22+0.5 °C room temperature, 50%-60% relative humidity, 12 h of dark-light alternation with 12-14 air changes per hour) with water and food available *ad libitum*. Specific rodent diet was select to minimize the OTA e mycotoxin exposure (AIN-76A Purified Diet NOT RADIABLE purchased from Mucedola, Italy). After seven days of acclimatizing, weaning F1 mice were separated by the dams and housed in cages to obtain 4 groups of 18 mice/sex/group, treated with 3 dose levels of OTA dissolved in corn oil and a control group treated with vehicle only for 28 days (5 days/week) *per os* by gavage, from weaning (postnatal day, PND, 22 to sexual maturity PND60).

The selected dose levels are 1 (low), 100 (medium) and 1000 (high) µg/kg bw per day. The low dose level was derived based on dietary exposure to OTA at the 95th percentile (i.e., among high consumers), which was estimated to reach up to 0.0517 µg/kg bw per day [7] that corresponds to 0.63 µg/kg bw per day in mice [40], rounded to 1 µg/kg bw per day as a practical value. The medium dose level derived from the LOAEL (Lowest Observed Adverse Effect Level) of 8 µg/kg bw day in pigs, corresponds to 93 µg/kg in mice, assuming interspecies extrapolation [40] and rounded to 100 µg/kg bw per day as a practical value. High dose level was obtained by a toxicological study on mice where OTA was administered orally for 28 days with toxicological effect in systems other than the renal one without clinical sign of toxicity [41].

Body weight, feed consumption, kidney, liver, thyroid, ovary, uterus, testis, hypothalamus absolute and relative weights will be measured. Serum clinical and biochemical parameters (albumin, globulin, total bilirubin, gamma-glutamyl transferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, creatine kinase, creatinine, glucose, triglycerides, calcium, phosphate, magnesium, potassium, and sodium) will be also assessed. In addition, a proteomic analysis of liver tissue will be conducted.

Specific effects of endocrine system will be analyzed by:

- hormones serum levels (e.g., thyroid-stimulating hormone, TSH, thyroxine, estradiol, testosterone);
- histopathological analysis (thyroid, reproductive organs and liver);
- gene expression of selected hypothalamus marker (TSH, luteinizing hormone, follicle-stimulating hormone).

Renal toxicity endpoints will be studied by:

- kidney DNA damage by comet assay;
- renal oxidative stress enzymes (e.g., catalase, superoxide dismutase, glutathione peroxidase 1);
- serum renal toxicity markers (e.g., proliferating cell nuclear antigen, vimentin);
- kidney expression of DNA repair and general toxicity genes (e.g., Ercc1, ERCC excision repair 1; Ogg1, 8-oxoguanine DNA-glycosylase 1; and Kim1, Kidney Injury Molecule 1; respectively);
- kidney histopathological analysis.

The sample size calculation is performed using the G*Power 3.1.9.7 software on a comparison between two groups (treatment dose *vs* control) using the Mann-Whitney test, with a one-tailed significance level of alpha=0.0167 (corresponding to alpha=0.05 with Bonferroni correction applied to the three comparisons between each treatment dose and the control) and a power of 1-beta=0.80. The key endpoint used for the sample size estimation was the change in the activity of renal oxidative stress-related enzymes, as reported in a previous study involving oral administration of OTA to male mice for 45 days [42]. *Table 1* shows the results of the sample size calculation (N) based on the selected enzymes.

Table 1
Calculation of sample size (N) according to decreased activity of renal oxidative stress enzymes

Endpoint	Control (0 mg/kg bw day) (means±SD)	OTA (1.5 mg/kg bw day) (means±SD)	N
Catalase activity (µmoles H_2O_2 consumed/mg protein/min)	32.72±1.26	26.82±0.72	4
Superoxide dismutase activity (units/mg protein)	3.34±0.15	2.79±0.06	4
Glutathione peroxidase activity (µmoles NADPH consumed/mg protein/min)	0.38±0.09	0.25±0.03	8

OTA: ochratoxin A.

GSH activity is most conservative condition and a 34% reduction in the mean value is observed, corresponding to a Cohen's d effect size of 1.94 (very large effect). However, considering the selected dose levels and the short time of administration, it is important to detect smaller differences, starting from 20%, which are plausibly expected to occur in the groups treated with lower doses. For example, comparing 0.38±0.09 (control group) *vs* 0.30±0.03 (20% reduction) to GSH activity, the sample size is N=15. The final sample size per sex/group is increased to 18, taking into account that the expected mortality rate is no more than 5% and high the number of biomarkers to be measured in mouse serum.

Data analysis plan

Statistical analyses will be performed using Stata 16.0 software (StataCorp), setting significance at $p < 0.05$. Descriptive statistics will be calculated for each group of population. Sex-stratified analysis will be performed both in adults and children using parametric or non-parametric tests, when non normal distribution is verified (Mann-Whitney test). The same tests will be used to compare the population groups. Confounders (e.g., age, breastfeeding frequency, renal function variability, diet reporting bias) will be considered in the analysis. Age will be considered as covariate to obtain age-adjusted estimates in all groups, whereas breast feeding frequency will be included in data from breastfeeding women. Urinary OTA and CIT concentrations will be normalized to creatinine concentration, measured in the corresponding urine sample.

If data are missing from the subject's questionnaire, the operators responsible for data quality control will identify the pediatrician who enrolled the subject through the alphanumeric code of questionnaire and will request the completion of the unanswered questions. The pediatrician will then contact the patient to obtain the missing information. This process ensures that all fields are completed, thus avoiding the exclusion of data. If no response is received, the incomplete data will be excluded. Correlations between OTA/CIT levels and renal biomarkers will be assessed using Pearson/Spearman coefficients and multiple regression models. The association between dietary intake and OTA/CIT levels will be analyzed through linear/logistic regression and multivariate analysis (PCA). Data from toxicological study will be analyzed using ANOVA with post-hoc tests. Risk assessment will be conducted by calculating the Margin of Exposure (MOE) for OTA and CIT comparing the estimated provisional dietary intake of OTA and CIT with established safety thresholds.

State of the art

Ethical approval was obtained by the National ethics committee for clinical trials of public research bodies (EPR) and other national public institutions at the Istituto Superiore di Sanità (AOO-ISS-30/06/2023-0030990 Class: PRE BIO CE 01.00). To date, the enrollment of the subjects was completed, obtaining samples and FFQs filled in. The FFQs were reviewed and, in case of missing data, the pediatricians involved in the en-

rolment asked the subjects. All the data has now been transferred to the online database.

The animal study protocol was approved by the Italian Ministry of Health (879/2023-PR). The study has been completed, and general toxicological data are summarized in the Excel file. Statistical analysis is currently ongoing. Animal serum and tissues have been stored.

Analytical tests of human biological fluids and serum and tissues of mice are currently in progress.

EXPECTED RESULTS

The main outcome of the OTABioTox Project will be the development of a model to evaluate the exposure and toxicological effects of OTA and CIT through an integrated approach. The integration of the scientific results will provide an estimate of the predictive risk of the potential effects of exposure to OTA and CIT on the health of different population groups, taking into account age and sex.

Relevant results will be achieved for OTA and CIT in the frame of risk assessment:

As for *human exposure*:

evaluation of CIT and OTA urinary levels in children and adults of both sexes, as well as in breastfeeding women;

- association of the internal levels with the food consumed according to data acquired from the FFQ to ascertain if group of foods are triggers for internal levels;
- estimation of OTA and CIT provisional dietary exposure upon standard daily urine volumes, excretion data and body weights values;
- association between the observed exposure levels and effect biomarkers of renal function to highlight potential health impacts in children and adults, considering sex differences;
- evaluation of the OTA levels in breast milk of the enrolled breastfeeding women;
- estimation of exposure to OTA and CIT in breastfeed children, who have breast milk as the sole food;
- correlation between OTA and CIT levels in breast milk with the corresponding values measured in the urine of the same breastfeeding woman to identify possible associations of critical ratios of contamination levels in the two biological matrices;
- calculation of the Margin of Exposure (MOE) for OTA, based on the comparison of BMDL10 for non-neoplastic and neoplastic effects with the calculated exposure values; whereas for CIT, comparison of the tolerable dose for nephrotoxicity with the calculated exposures.

As for *toxicological characterisation*:

- evaluation of OTA effects in a juvenile rodent model at dose levels compatible with estimated exposure levels in children;
- identification of early effect biomarkers for the renal and endocrine systems;
- evaluation of sex-related differences in susceptibility;
- study of the mechanisms by specific omics technologies.

The selected endpoints will primarily focus on the endocrine system. Additionally, the mechanisms of action and DNA damage induction by OTA at the renal level will be investigated to identify and characterize early markers of damage associated with carcinogenesis processes. Finally, the use of both male and female mice will allow the identification of sex-specific effects on the endocrine system and renal damage.

In conclusion, these findings will provide new insights into OTA and CIT exposure in adults and children, increasing the understanding of their toxicological effects and supporting the development of evidence-based strategies for risk mitigation and public health protection.

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Conflicts of interest statement

The Authors declare no conflict of interest.

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Advancing pesticide risk assessment: the role of adverse outcome pathways and new approach methodologies – what's next?

Teresa D'Amore[1,2], Emanuela Testai[2] and Emma Di Consiglio[2]

[1]*Dipartimento di Farmacia, Università degli Studi di Napoli Federico II, Naples, Italy*
[2]*Dipartimento Ambiente e Salute, Istituto Superiore di Sanità, Rome, Italy*

Abstract

Introduction. In recent years, the scientific community, together with regulatory authorities, institutions and stakeholders, joined in the common objective of changing the regulatory toxicology paradigm, moving from classical toxicological animal testing towards an exposure-driven risk assessment, based on mechanistic information derived from alternative methods, globally known as new approach methodologies (NAMs). The transition to the next generation risk assessment (NGRA) requires the collective effort of all involved actors. One approach addressing the needs of the NGRA is the adverse outcome pathway (AOP) concept. This construct describes a sequence of temporally and causally linked events at distinct levels of biological organization leading to an adverse health effect. The development, validation and implementation of AOPs at the regulatory level have become key objectives of several authorities.

Methods. In this paper, the AOPs and AOP networks as applied by the European Food Safety Agency are analyzed, alongside the proposed batteries of NAMs.

Results and discussion. Two case studies, (1) Parkinson's disease and pesticide exposure, and (2) exposure to substances with endocrine-disrupting properties and the development of uterine adenocarcinoma, are described. The analysis of the entire AOP construct for each case and the assembly of the specific NAMs allowed us to identify the strengths and limitations of the AOP strategy, as well as of each proposed test. Their integration and status are assessed within the current regulatory framework.

Key words

- next generation risk assessment (NGRA)
- new approach methodologies (NAMs)
- adverse outcome pathways (AOPs)
- pesticides
- regulatory toxicology
- chemical risk assessment

INTRODUCTION

Risk assessment (RA) is a rigorous, structured, and iterative process designed to address key questions regarding exposure to one or more chemical, physical or biological agents, and the associated potential risk for human, animal and environmental health. As a specialized discipline of applied science, RA involves the analysis and review of scientific information, whenever available and aligned with the regulatory framework, and/or new or existing data to evaluate the likelihood of adverse events occurring in organisms, following the exposure to these agents. The process considers the hazardous properties of the chemicals and quantifies risk, considering both hazard and exposure. If necessary, mitigation measures should be suggested and then implemented to reduce the potential risks, as part of risk management actions [1, 2].

Both scientific and technological progress, coupled with increased awareness among governments, regulatory bodies, academia and industries, have represented a great driving force for the knowledge and clarification of toxicological profiles, or "fingerprinting", of substances. This applies both to substances requiring approval before entering the market and to those already present in the environment (pollutants and contaminants) [3]. The "One Health" approach, a holistic perspective integrating the health of humans, animals, and the environment as a *unicum*, has also contributed to directing the RA towards a shared, multi-sectoral and multidisciplinary collaborative vision [4]. Similarly, the EC initiative "One Substance, One Assessment" was proposed to strengthen the chemicals strategy for sustainability (CSS), aiming to ensure coherent and transparent safety assessments of chemicals with diverse uses across various regulatory frameworks, and to build a comprehensive body of knowledge on chemicals [5]. It is im-

Address for correspondence: Emma Di Consiglio, Dipartimento Ambiente e Salute, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: emma.diconsiglio@iss.it.

portant to note, however, that the "One Substance, One Assessment" approach is most readily applicable to hazard characterization. In contrast, the broader RA process must also account for exposure, which can vary significantly, depending on how a substance is used across different sectors. For example, if the same chemical is used as a flavoring agent in candy or as an ingredient in a bath gel, the exposure scenarios, such as frequency, duration, route of exposure, and consequently the kinetics and internal dose, can differ substantially.

Historically, the classical RA process, particularly in the hazard identification and characterization phases, had extensively relied on animal testing to derive dose descriptors and, subsequently, reference values. However, the growing demand for more sustainable, ethical and species-relevant methods (i.e., those tailored for humans) has driven the scientific community to investigate, develop and implement "alternative" approaches, internationally known as new approach methodologies (NAMs). They include a range of innovative strategies, including *in vitro, in silico, in chemico, ex vivo* and *non-testing methods* or their combinations, designed to improve accuracy and relevance for human health assessments while reducing or replacing animal use (*Table 1*) [6, 7].

In addition to ethical issues, limitations of animal models, including interspecies physiological differences, varying toxicokinetic profiles and lack of interindividual susceptibility, often hinder the extrapolation of data to humans [8]. NAMs aim to address these challenges, increasingly designating animal testing as a "last resort", considering the principle of equivalent protection, meaning that the same risk assessment conclusions and risk management decisions would be reached by using NAMs instead of animals. Initially, they were developed to accelerate the screening and evaluation of the thousands of substances currently in use or under development. At the same time, their predictability, accuracy and reliability have been widely recognized [9]. The effort to develop, validate and make internationally accepted NAMs has been shared globally and reflected in initiatives starting from the Directive 2010/63/EU on animal experimentation, to EU CSS [5] and some specific EU regulations adopted for regulated chemicals and/or products before their marketing: examples include Regulation (EU) n. 283/2013, which requires that active substances used in plant protection products undergo a comparative *in vitro* metabolism study on cells derived from various species, including humans. These studies are pivotal for determining the relevance of toxicological *in vivo* data and for guiding the interpretation of findings or defining the testing strategy. Similarly, Regulation (EU) n. 1223/2009 prohibits the marketing of cosmetic products if either the ingredients or the final formulation has been subjected to animal testing.

In parallel, the European Chemicals Agency (ECHA) published on the use of NAMs within REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) and CLP (Classification, Labelling and Packaging) regulations almost 10 years ago, then set guidance updates aimed at improving the use of *in vitro*, *in silico* and read-across approaches [10], while the European Food Safety Authority (EFSA) has developed a Roadmap for NAM integration in RA [8, 11], reinforcing the convergence toward a mechanism-based, exposure-driven assessment framework. Alongside EU initiatives, similar trends toward NAM-based regulatory toxicology are underway internationally: the US-EPA is advancing the transition toward non-animal, mechanistic RA through programs such as ToxCast/Tox21 and its NAMs Work Plan [12], while the US-FDA Modernization Act 2.0 [13] formally recognizes NAMs as acceptable alternatives to animal testing in regulatory submissions.

For simple endpoints (e.g., skin and eye irritation), some stand-alone methods have already been internationally adopted, such as those included in the Organization for Economic Co-operation and Development (OECD) Test Guidelines (TGs). These methods are accepted within regulatory frameworks and widely applied, for example, in the assessment of genotoxic potential or skin sensitization. However, for more complex toxicological endpoints (e.g., systemic effects after short or long-term repeated exposure), a single test might not be sufficient on its own to provide the necessary information. For this reason, the modern approach to applying NAMs is to use integrated testing strategies rather than a single alternative test. The integrated strategies combine various methodologies, including evidence-based and non-testing approaches, to create a comprehensive path from exposure to outcome [14, 15]. In this context, the new challenge of toxicology and in particular, regulatory toxicology is "to assemble individual NAMs into a comprehensive next-generation risk assessment (NGRA) strategy" [16], as a human-relevant, exposure-led, hypothesis-driven risk assessment designed to prevent harm. Furthermore, by integrating NAMs with advanced technologies and big data, RA becomes more precise, informative, and problem-oriented, capable of addressing gaps and uncertainties [11, 17-19]. A good example is the general NGRA workflow described by the EU Scientific Committee on Consumer Safety (SCCS) for the evaluation of cosmetic ingredients [20].

To this aim, the construct of the adverse outcome pathway (AOP) has been recognized as one of the fundamental tools to this transition. An AOP maps and describes a sequence of toxicodynamic events temporally and causally linked to different levels of biological organization (i.e., molecular, cellular, tissue, organ and organism), resulting from the exposure to a stressor (e.g., chemicals, physical stress, etc.) and causes a final adverse event in human health and/or the environment [21, 22]. Several biological, genetic, physical and chemical insults contributed to the onset of the disease; however, a deeper understanding of disease mechanisms and early and key events is an essential goal shared among toxicological and regulatory areas.

The sequence of events defined by the AOP begins with a molecular initiating event (MIE), which describes the interaction between a stressor and its molecular target (e.g., receptors, proteins, enzymes, DNA). This interaction triggers a sequential chain of intermediate events, called key events (KEs), which define certain measurable biological/physiological changes that can

Table 1
Classification and characteristics of new approach methodologies (NAMs)

Models	Model/Method	Main characteristics
In vitro	Continuous cell lines	Cultured cells from a single clone, derived from tumors, pluripotent cells, or cells taken from an organism and immortalized, grown under controlled conditions
	Microorganisms	Microbial cultures grown in predetermined culture medium under controlled laboratory conditions
	Primary cells	Cells taken directly from living tissue (e.g., biopsy material) and established for growth *in vitro*
	Spheroids	Spherical cellular units that are generally cultured as free-floating aggregates
	Organoids	Self-organized cells grown in 3D using scaffolds to mimic the extracellular matrix, typically derived from stem cells (pluripotent, fetal or adult). They form structural units that resemble the organ in structure, biology and function
	Reconstructed human tissues (e.g., epidermis)	Tissue model differentiates and stratifies similar to native human tissue (3D)
	Co-cultures	Different types of cells (2D and/or 3D) grown together under controlled conditions
	Organ-on-a-chip/micro-physiological systems	Multi-channel 3D microfluidic cell culture, integrated circuit (chip) that simulates the activities, mechanics and physiological response of an entire organ or an organ system
Ex vivo	Tissues or organs	"Outside of a living body", the living tissues or entire organs are directly taken from a living organism and studied in a limited time with no or minimal alterations
In chemico	Purified targets and biochemical assays	Enzymes, receptors, proteins for evaluation of reactivity, binding, changing in conformation after exposure to an agent; they are used to detect, quantify and/or study the binding or activity of biological molecules
	Isothermal titration calorimetry	A label-free quantification method used for the evaluation of a wide variety of biomolecular interactions
In silico and non-testing methods	Extensive literature search, systematic review and metanalysis	Useful tools for evaluating the status of research on a given topic and identifying data gaps and research needs
	Read across	Relevant information from one or more compound(s) is used to predict the properties of a target compound with similar chemical/ physical / toxicological properties (e.g., genotoxicity)
	Grouping	Evaluating more than one substance at the same time such as structural analogues and/or entire classes of chemicals
	(Q)SAR	Mathematical models providing a correlation between compounds chemical structure and a property or activity (qualitative or quantitative)
	TTCs	The establishment of a generic exposure level for a class of chemicals below which there would be no appreciable risk to human health.
	PBK models	Mathematical modelling technique for predicting the ADME of synthetic or natural chemical substances
	Weight of evidence	A process in which evidence is integrated to determine the relative support for possible answers to a scientific question.
	(Q)IVIVE	*In vitro* to *in vivo* extrapolation: the qualitative or quantitative transposition of experimental results or observations made *in vitro* to predict phenomena *in vivo*
	Data waiving	Avoiding performing test if: - not scientifically necessary - not technically feasible - relevant data already exist
	Data sharing	Existing studies involving or not vertebrate animals conducted can be shared for use to avoid unnecessary animal testing or reduce costs
High-content methods, others	HTS	The use of automated equipment to rapidly test thousands to millions of samples for biological activity at organism, cellular, pathway, or molecular level
	Advanced bioimaging and bioscanning	Methods that non-invasively visualize biological processes in real time
	Omics (genomics, transcriptomics, proteomics, or metabolomics, etc.)	Genomics can facilitate analysis of entire or component genome sequences of an organism. Transcriptomics and proteomics provide significant bodies of information on temporal and spatial expression of genes and gene products, respectively, whilst metabolomics captures data for a large pool of metabolites

ADME: absorption, distribution, metabolism and excretion; HTS: high-throughput screening; PBK: physiologically based kinetic; (Q)IVIVE: quantitative *in vitro* to *in vivo* extrapolation; (Q)SAR: quantitative structure-activity relationship; TTCs: thresholds of toxicological concern.

occur if the perturbation is sufficiently relevant in terms of power, duration, and frequency to exceed the threshold of adversity. These KEs are linked by a temporal, causal, empirical relationship and biological plausibility (key event relationship – KER). The resulting AOP framework provides a clear, evidence-based progression from the MIE to the observed apical event or adverse outcome (AO) (*Figure 1*) [23, 24].

While the primary goal of developing an AOP is to systematically identify and organize existing knowledge in a structured manner, this tool may also allow for identifying current knowledge gaps that, when addressed, may further enhance its predictivity. AOPs are also considered "knowledge bridge", reflecting their role in connecting mechanistic data with AOs. The following key characteristics underscore the stepwise development of AOPs and their utility in RA:

- stepwise organization of events: sequentially link alterations at the molecular level to broader biological changes at the organelle, tissue, and organism level;
- mechanistic integration: incorporate mechanistic information into a defined, causal and plausible construct of KEs leading to the AO, allowing the appropriate use of specific NAMs;
- relevance assessment: support the evaluation of the relevance of experimental toxicological and/or epidemiological data;
- uncertainty definition: define and identify the uncertainties related to the available data;
- data gap identification: identify any missing data points easily and immediately, aiding the refinement of pathways and methodologies;
- research and regulatory prioritization: address research needs and establish criteria for prioritizing regulatory/decision-making actions [25-27].

The implementation at the regulatory level of the AOPs is among the main objectives of various European and international bodies (i.e., ECHA, EMA, EEA, JRC-EURL ECVAM, OECD, WHO, FAO, US-EPA, FDA). Specific initiatives developed within these institutions have been launched to support the adoption and refinement of AOPs, among which:

- EU PARERE Network – Preliminary Assessment of Regulatory Relevance Network – a platform for assessing the regulatory relevance of new tools and approaches (https://shorturl.at/dswAR);
- PARC – Partnership for the Assessment of Risks from Chemicals – a collaborative EU initiative to improve RA methodologies and promote innovation in the chemicals RA sector (https://www.eu-parc.eu/);
- AOP-Wiki a collaborative online platform for organizing, sharing, and refining AOP-related data and information (https://aopwiki.org) [2, 7, 10, 11].

The AOP framework might be used as a starting point not only to organize the available data and information, but also to identify knowledge gaps, evaluate uncertainties, shape future research lines and regulatory and decision-making prioritization criteria.

While the scientific foundations of AOPs have also been recently reviewed in literature, as well as AOPs related to specific endpoints or chemicals [28], the present work provides a systematic and original analysis on how AOPs have been implemented within the EFSA regulatory framework. In reconstructing the NAM-based event flows, this paper identifies practical limitations in regulatory acceptance, and highlights opportunities for improvement, e.g., by strengthening toxicokinetic data integration, empirical support, and cross-AOP consistency. This regulatory-focused perspective may offer a novel viewpoint in RA and regulatory decision-making.

METHODOLOGY

This study, covering the period from 2010 (when the AOP concept was introduced) to July 2024, follows a systematic approach to collect, evaluate, and analyze existing data. The principles of the PROMETHEUS

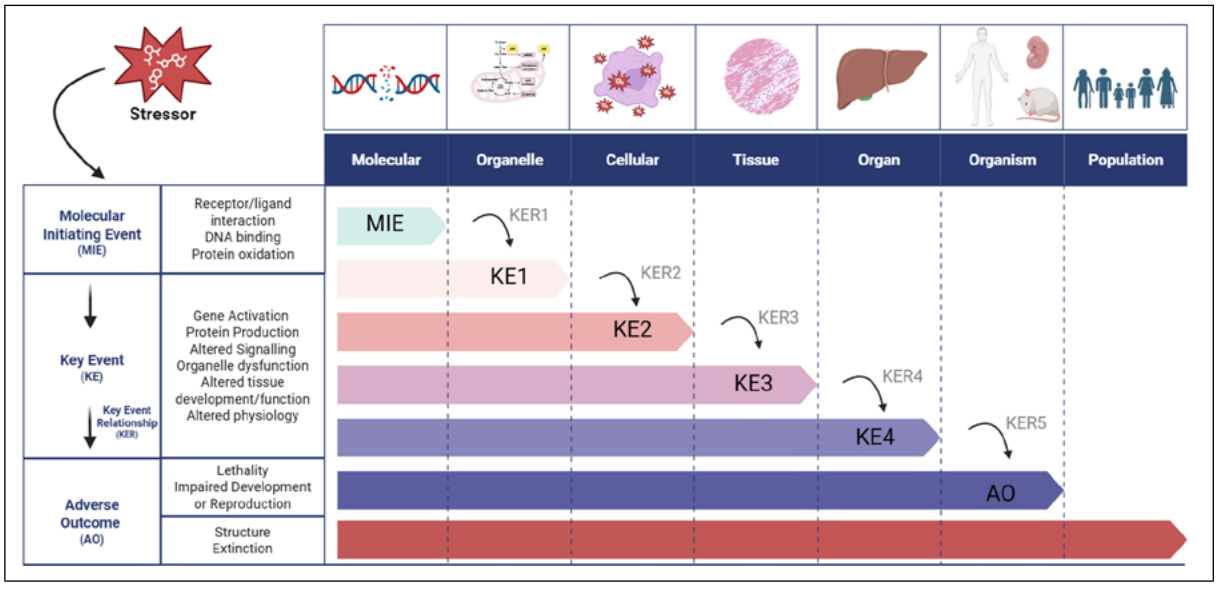

Figure 1
Summary of the adverse outcome pathway construct and levels of biological organization.

(Promoting methods for evidence use in scientific assessments) initiative, developed from EFSA, and PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) 2020 guidelines were used for this purpose [29-31]. In addition, a structured literature review using extensive literature search (ELS) methodologies was performed through three different databases, Web of Science, PubMed, and EFSA Search. The keywords were derived from the EFSA glossary and MeSH vocabulary (Medical Subject Headings). The generated search strings are reported in *Table S1 available online*.

The results were organized using EndNote Web and Rayyan software, enabling both manual and automated screening. Machine Learning (ML) tools were applied to streamline the organization of results, identify duplicates, and highlight relevant studies [32]. Studies meeting the inclusion criteria (summarized in *Table S2 available online*) underwent further analysis, while irrelevant studies were excluded.

To complement the literature review, the AOP-Wiki database was accessed to analyze and verify the status and applicability of the AOPs included in this study.

RESULTS

Figure 2 summarizes the results of the ELS, integrating systematic evidence collection with AOP evaluation to address the study objectives. Currently in the EFSA context, the use of AOPs/AOP networks is proposed for the assessment of toxicological properties linked to i) the development of Parkinson's disease (PD) and childhood leukemia (CHL) of some active substances used in plant protection products (PPPs) [33-36], ii) development of uterine adenocarcinoma by substances having endocrine disrupting properties [37, 38], iii) endocrine disrupting properties of perfluorooctanesulfonic acids (PFOS) (pollutants, excluded from this analysis) [39].

For this study, two AOPs were selected for in-depth analysis: (case 1) "mitochondrial dysfunction and neurotoxicity", relevant to PD, and (case 2) "Increased estradiol (E2) activating the ERα estrogen receptor", associated with endocrine disruption and uterine adenocarcinoma. These AOPs were chosen based on their direct relevance to PPP RA, robust scientific basis, and application within EFSA regulatory evaluations. While

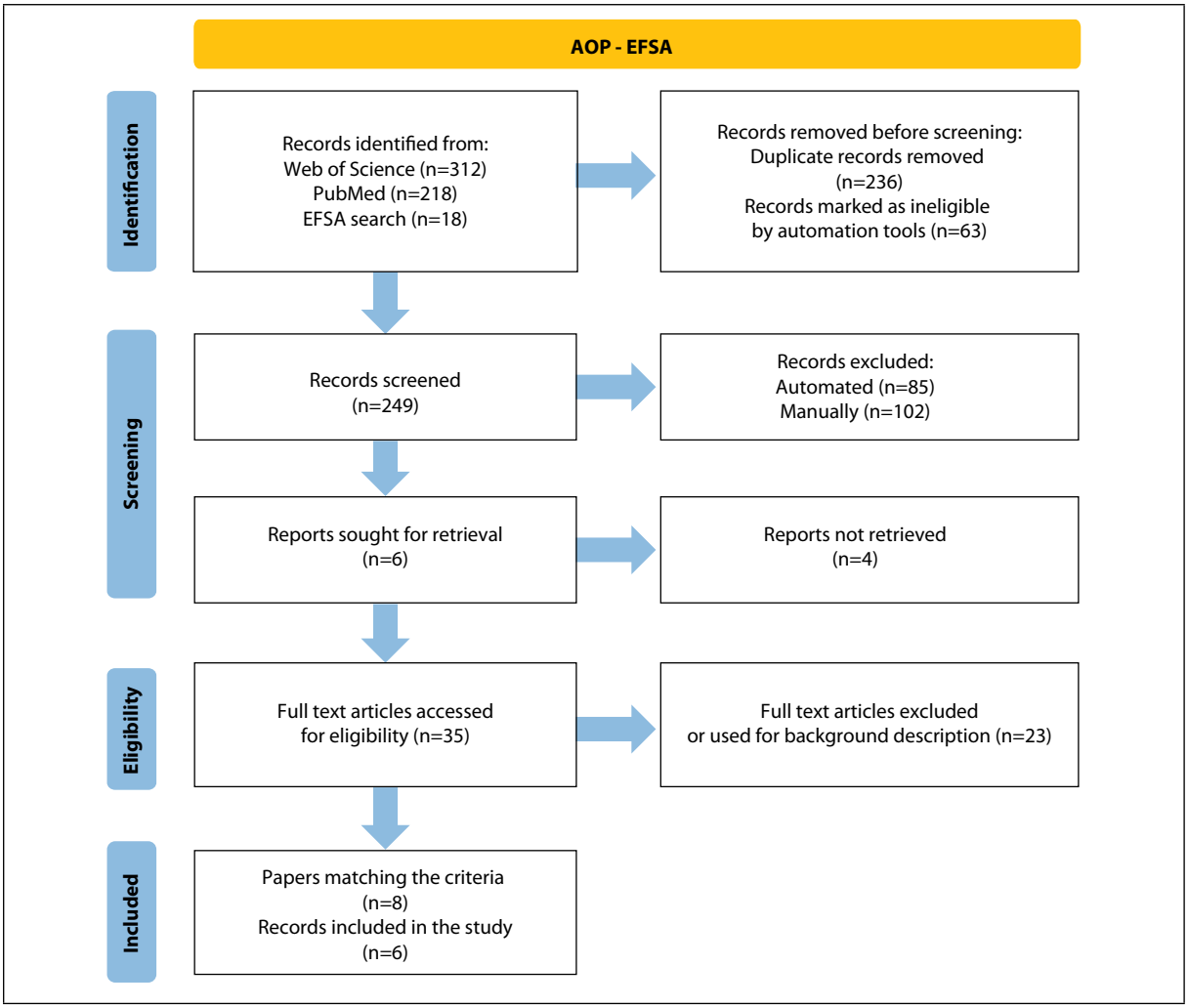

Figure 2
Preferred reporting items for systematic reviews and meta-analyses (PRISMA) flow diagram for the extensive literature search of adverse outcome pathways (AOPs) used in the European Food Safety Authority (EFSA) regulatory context.

additional AOPs were identified, including those linked to childhood leukemia and environmental pollutants (e.g., PFOS), they were excluded due to limited applicability to pesticide regulation. The selected AOPs represent distinct toxicological pathways, neurological and endocrine disruption, providing a focused yet comprehensive evaluation of the AOP framework in regulatory decision-making. The supporting publications and documents related to these AOPs were also analyzed [33-39].

In analyzing the AOPs, we considered evidence from both animal studies and NAMs, as identified through a systematic review. However, our analysis aimed to construct an AOP-based event flow using only NAMs whenever possible, critically evaluating their applicability and limitations. The resulting NAM batteries incorporate all available non-animal studies, except for the AO, where only limited alternatives currently exist.

Case 1: PPPs exposure and Parkinson's disease

In the preparatory stage, EFSA published the results of a literature review on epidemiological data linking exposure to active substances in PPPs to the occurrence of various health effects [33]. Subsequently, the EFSA Panel on Plant Protection Products and their Residues (PPR) was mandated to investigate exposure to PPPs as a risk factor for the development of PD and CHL. To define whether there was a biological plausibility between exposure to PPPs and these two diseases, the EFSA conducted a study, using the AOP construct as the most appropriate investigation tool. After an additional systematic review of the literature, the in-depth literature analysis by using the weight of evidence (WoE) approach was fundamental for the identification of the specific AOs and chemical stressors, as well as for the assessment of related MIEs, KEs and KERs [33-36].

Parkinson's disease – methodologies and AOP "mitochondrial dysfunction and neurotoxicity"

Epidemiological studies and ELS were indispensable tools for formulating the problem and the initial hypotheses. Using a top-down approach, the AO to be examined was identified. Studies on PD with genetic predisposition were excluded from the systematic review, in addition to those involving radiation and cytotoxic chemotherapeutic drugs. The studies related to mechanisms, mode of action (MoA) and KEs involved in PD pathogenesis and their organization levels were selected. Based on this groundwork, four AOPs for PD were formulated: three qualitative AOPs and a putative AOP. Two qualitative AOPs underwent structured evaluation based on OECD principles but are not fully validated, while the putative AOP remains at an early conceptual stage and requires further validation before integration into formal regulatory frameworks.

The AOP, having the inhibition of the mitochondrial complex I (Complex I – CI, NADH-ubiquinone oxidoreductase) of nigro-striatal neurons as MIE, and an AO characterized by Parkinsonian motor deficits, underwent OECD peer review, making it the most advanced among them in terms of regulatory application.

Different classes of pesticides (e.g., organochlorines, pyrethroids, carbamates, antifungals) or single active substances were analyzed in the ELS. Among these, three specific compounds were selected as prototypical neurotoxicants for assessing the MIE, KEs, and KERs: paraquat (a dipyridine insecticide), rotenone (a naturally derived insecticide/acaricide), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, a tetrahydropyridine compound). The three compounds are known to cause neurotoxicity and permanent PD symptoms.

In addition to one MIE, five KEs have been defined: three at the cellular level (Complex I inhibition, mitochondrial dysfunction and altered proteostasis) and two at the organ level (degeneration of dopaminergic neurons of the nigrostriatal pathway and neuronal inflammation). For each step, the NAMs used and/or usable for the assessment were identified, including *in vitro*, *ex vivo* tests, as well as the evaluation of existing data. The battery of tests that can be used for each event is summarized in *Table 2*.

The WoE methodological approach used in the AOP construction followed the modified Bradford-Hill criteria [28]. This approach evaluates: (1) biological plausibility, (2) empirical support in terms of linkage, (3) quantitative understanding of the relationship, (4) taxonomic applicability, life stage and gender-specific effects, (5) uncertainties and inconsistencies. Additional criteria used during this evaluation are i) analogy, i.e., a comparable association between the same outcome and a similar exposure or the same exposure and a similar outcome, and ii) coherence, in terms of concordance between the epidemiological data and the available experimental studies.

The relationships between the individual KER events were very complex and are described in detail by Terron *et al.*: eight KERs were analyzed using the WoE approach guided by the modified Bradford-Hill criteria [35].

The AOP delineated in the EFSA report (*Appendix A*) [32] was uploaded to the AOP-Wiki under the designation "mitochondrial dysfunction and neurotoxicity" (ID: 3). The evaluation process involved an initial peer review by the OECD Working Group of the National Coordinators of the Test Guidelines Programme (WNT) and the Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). Following these reviews, the AOP was endorsed. At this stage, the AOP remains open for consultation, citation, and refinement.

Case 2: AO uterine adenocarcinoma

In the second case study, the EFSA mandate to develop AOPs to be integrated into a network was examined. The AO under consideration was the development of uterine adenocarcinoma in mammals. The aim is the identification of active substances with MoA, characterizing them as endocrine-disrupting chemicals (EDCs) [37, 38].

The adopted methodology was an evidence-based approach, relying on non-testing methods with an initial mapping phase of scientific literature (e.g., ELS, literature mapping), by using a top-down approach. The

Table 2
New approach methodologies (NAMs) battery for the adverse outcome pathway (AOP) "mitochondrial dysfunction and neurotoxicity"

Event	Description	Proposed approach	Test
MIE	Binding of an inhibitor to NADH ubiquinone oxidoreductase (complex I)	*In silico* models	• Molecular docking simulations: based on mimicking the binding of chemicals to the pocket of NADH ubiquinone oxidoreductase
		In vitro/ex vivo	• Quantitative autoradiography
KE1	Inhibition of NADH ubiquinone oxidoreductase (complex I)	*In vitro* models: direct test (performed on homogenates of cells or tissues, and requires at least a partial purification of mitochondria or respiratory chain components)	• Forward electron transfer • Reverse electron transfer • Complex I activity dipstick assay
		Indirect test	• Oxygen consumption, • Intracellular ATP levels • Measurement of NADH/NAD+ ratio in mitochondria by imaging methods
KE2	Mitochondrial dysfunction	*In vitro* models: cells in culture isolated mitochondria	• Cellular oxygen consumption (ADP-to-O ratio) • Mitochondrial membrane potential • Mitochondrial PTP opening • mtDNA damage as a biomarker of mitochondrial dysfunction • ATP content assay
KE3	Impaired proteostasis	*In vitro* models: evaluation of UPS function	• General turnover assay by IHC or WB • Detection of a-synuclein aggregates by imaging, WB, MS or immuno-quantification • Proteasome reactivity assay immunocyto/histochemistry or western blotting
		Evaluation of ALP function	• Monitoring of autophagy-related molecules by mean of fluorescence-tags or quantification of lysosomes or autophagosomes
KE4	Degeneration of dopaminergic neurons of the nigrostriatal pathway	*In vitro* *	• Phenotypic histological marker measurement (TH, DAT, VMAT2) • Degenerating and/or degenerated neurons can be detected by the silver stains and the Fluoro-Jade stains • Cell counting
KE5	Neuroinflammation	*In vitro* **	• Quantification of cellular markers (e.g., pro- and anti-inflammatory cytokine expression, immunostimulatory proteins) • Quantification of released mediators
AO	Parkinsonian motor deficits	*In vitro* *In vivo* *Ex vivo*	• Determination of total contents of dopamine and its two metabolites HVA and DOPAC by HPLC-EC or HPLC-MS • Imaging by PET and SPECT • Detection of dopamine neuron terminals in the striatum • Behavioural tests • Regulatory studies (OECD 407, 408, 422, 424, 426, 443)

MIE: molecular initiating event; KE: key event; AO: adverse outcome; NADH: nicotinamide adenine dinucleotide hydrogen; ATP: adenosine triphosphate; ADP: adenosine diphosphate; PTP: permeability transition pore; UPS: ubiquitin–proteasome system; ALP: autophagy-lysosome pathway; IHC: immune-histochemistry; MS: mass spectrometry; TH: tyrosine hydroxylase; DAT: dopamine transporter; VMAT2: vesicular monoamine transporter type 2; HVA: homovanillic acid; DOPAC: dihydroxyphenylacetic; HPLC: high performance liquid chromatography; ED: electrochemical detector; PET: positron emission tomography; SPECT: single photon emission computed tomography.
*Some studies in *C. elegans*, *Drosophila*, zebrafish and *Lymnaea stagnalis* were also carried out; **some studies in zebrafish were also carried out.

WoE was used throughout the entire process, combined with expert judgment, in a structured and guided manner, i.e., Expert Knowledge Elicitation (EKE) [40]. After identifying the construct formulation questions and defining the scope of the evaluation, four AOPs were postulated with different MIEs and KEs. Through a hierarchical evaluation of the available data (human studies, *in vivo* and *in vitro*), literature mapping allowed the identification of the most plausible MIEs/KEs/KERs. Subsequently, ML techniques, including topic modelling, were employed to perform an automatic screening of the literature for the characterization and evaluation of the biological plausibility of KERs [32].

For the empirical support necessary for the WoE of KERs, two substances known to have estrogenic action, i.e., tamoxifen and estradiol, were selected. The strength of the KERs was assessed considering criteria of biological plausibility, empirical evidence, and essen-

tiality. The latter was evaluated by addressing whether blocking an upstream KE prevents downstream KEs or the AO. Finally, the developed AOPs were re-evaluated and integrated into a network.

According to a top-down approach, the common AO across the network is the development of uterine adenocarcinoma, with a key node, i.e., a meeting point between the various MIEs and/or KEs, identified as increased estradiol (E2) activating the ERα estrogen receptor. The identified MIEs were divided into uterine and extra-uterine molecular events.

Recently, the AOP 503 "Activation of uterine estrogen receptor-α leading to endometrial adenocarcinoma, via epigenetic modulation" has been formally submitted and publicly available on AOP Wiki, providing information on its applicability domain, KEs, and quantitative evidence. In AOP 503, the MIE is the activation of ERα in the uterus (KE 1065). The pathway then proceeds through four downstream KEs: (1) an epigenetic modification process (KE 2152), (2) altered expression of proliferation-related factors (KE 2153), (3) glandular epithelial hyperplasia in the endometrium (KE 772), and (4) genomic instability (KE 1896), which ultimately leads to type I endometrial (uterine) adenocarcinoma (AO 2154). A battery of tests with detailed MIE, KEs and AO was constructed and presented in *Table 3*.

Its life-stage applicability is the adult status; the taxonomic domain includes mammals, especially humans and mice. Its sex applicability is defined as female, consistent with the biology of the uterine endometrium. Empirical support remains rooted in the two agonists, which serve as prototypical stressors of ERα activation.

The evaluation of KERs is ongoing. One of the most important insights from the updated AOP is the role of epigenetic modulation (KE 2152): activation of ERα leads to changes in chromatin structure, such as histone acetylation or DNA hypomethylation, which then affect the expression of proliferation-driving genes (KE 2153). These altered expression patterns promote hyperplasia (KE 772), and over time, genomic instability (KE 1896) increases, setting the stage for malignant transformation into endometrioid adenocarcinoma.

DISCUSSION

General considerations

At a regulatory level, specific provisions govern the RA of certain substances, addressing unique regulatory needs. For complex adverse effects, such as those studied here, no single test – whether animal-based or NAM-based – is enough to fully describe toxicological properties and satisfy regulatory standards. Both the development of PD and estrogen-dependent uterine adenocarcinoma involve complex processes, with their MoA and pathogenesis not yet fully elucidated. Likewise, it remains critical to clarify and confirm whether exposure to certain substances poses a risk for the development of these pathologies. Keeping these complex questions in mind, the EFSA PPR Panel divided them into domains and considered the construction of AOPs as the most effective way for integrating information to better understand adverse events.

The goal of these constructs is to support regulatory applications by identifying those substances that share the same MIE and KE, leading to neurotoxic or endocrine-disrupting properties.

There are many approaches described in the literature for the development of an AOP: starting from the apical effect and, going backwards, building the path towards the MIE (top-down approach), or, on the contrary, starting from the MIE and building the path going towards

Table 3
NAMs battery for the AOP "ERα activation"

Event	Description	Level of biological organization	Proposed approach	Test
MIE	ERα activation	Molecular	*In vitro*	OECD 493 OECD 455
KE1	Epigenetic modulation	Cellular	*In silico* *In vitro* Omics	ToxCast Histone methylation assays DNA methylation assays miRNAs and LncRNA
KE2	Expression of factors ruling proliferation	Cellular	*in vitro* Omics	Luciferase Reporter Gene assay Protein expression (immunohistochemistry, western blot, immunoassay) transcriptome RNA sequencing
KE3	Increased proliferation (hyperplasia)	Tissue	*In vitro*	Cell proliferation assay
KE4	Genomic instability (accumulation of mutations)	Tissue	*In vitro/ex vivo*	FISH Flow cytometry OECD 476 OECD 490 OECD Guidance document 231
AO	Uterine adenocarcinoma (endometrioid adenocarcinoma Type I)	Organ	Human data *In vivo*	Human histological classification OECD 451

NAMs: new approach methodologies; AOP: adverse outcome pathway; MIE: molecular initiating event; KE: key event; AO: adverse outcome; ERα: estrogen receptor-alfa; FISH: fluorescence *in situ* hybridization.

the AO (bottom-up). Other types of approaches by analogy, case study or the middle-out approach can be considered similarly appropriate [27]. Although no single approach dominates, common steps are always involved:

1. hypothesis definition: establishing the purpose of the AOP;
2. KE and KER identification: researching and defining KEs, KERs, and tools (e.g., NAMs) for identifying and quantifying specific events;
3. biological applicability domains: defining "the boundaries" within which each KE operates. As an example, a biological event described by a KEs can be relevant only for one or a few species, gender or age (respectively, taxonomic, gender, or life stage applicability);
4. evaluation and verification: assessing assembled events and refining the AOP iteratively until sufficient confidence and predictability are achieved.

Before this assessment stage, an AOP is defined as putative, and its refinement and integration process are iterative. The confidence, predictability and regulatory fitness for an AOP must be evaluated. During all phases, ranging from development to evaluation of AOPs, what should not be underestimated is the identification and description of associated uncertainties [26].

A report is then prepared as described in the OECD guidelines and reviewed by the WNT and/or EAGMST groups to finalize the AOP endorsement (or rejection). It has been estimated that the average time to progress from the development of a single AOP to its inclusion on the AOP-Wiki, and completion of review and acceptance, exceeds three years [22, 25].

It should be emphasized that, by nature, AOPs are evidence-based constructs grounded in biological plausibility and empirical data. As such, they are living documents that evolve with technological advancements and regulatory requirements. Updates to AOPs happen as new evidence becomes available or when driven by regulatory or decision-making needs. However, as empirical evidence grows, so does the uncertainty surrounding the use of NAMs. In this context, considerable effort should be focused on assessing the relevance of both the NAMs used and the entire AOP, while also accounting for disease complexity from kinetic and dynamic viewpoints.

AOP "mitochondrial dysfunction and neurotoxicity"

In the first case study, the authors undertook the challenging task of capturing the onset of a complex disease (PD) by AOP. This AOP is included in the AOP-Wiki database and has undergone OECD evaluation and review, reaching "endorsed" status. As a result, it is open for regulatory consultation, further refinement, and application in toxicological RA.

Of the 429 AOPs currently present on the platform, only 29, approximately 7%, are in the approved state and only 10% are citable. The AOP inclusion in an internationally recognized database can encourage its use in research and chemical RA. However, the limited number of endorsed AOPs – partly due to the optional nature of the endorsement process – sheds light on the need for greater international collaboration and harmonization in chemical safety science. The applicability domains, supported by strong evidence, are adult (life stage), human and rat (taxonomy) and not sex dependent. According to all criteria, the strength of the link between each phase – MIE, KEs, KERs – and the overall AOP and between MIE and AO was evaluated as "strong" (in a scale foreseeing strong, moderate and weak). Similarly, the biological plausibility of this AOP is overall considered strong. The use of two known CI inhibitors, MPTP and rotenone, supports the WoE for the high relationship between the KEs. Experimental data with these two chemicals reveal significant concordance in dose-response relationships between MIE and AO and among KEs.

Based on the above considerations, this AOP has been considered within the overall neurotoxicity assessment, in compliance with (EC) Regulation n. 283/2013, since it is plausible that any compound that binds to the mitochondrial CI may eventually lead to Parkinsonian motor deficits.

Our analysis focuses on NAM-based tests applied within the AOP framework. This case study may represent a reference case for this aim, due to uncertainties linked to the extrapolation of data obtained with animal testing. Indeed, animal studies analyzed by EFSA PPR were carried out on knockout or mutational models. While these models may provide some information on familial PD pathogenesis, they fail to address mechanisms leading to primary idiopathic PD, which involves complex gene-environment interactions that are not fully replicated in rodents. Moreover, rodents are poorly representative of the extended human lifetime as well as of the human long-term exposure to the potential toxicants. Although rodents and humans share similar brain regions, significant differences exist in terms of size, structural organization, and complexity. Additionally, the kinetic behavior of neurotoxicants may vary significantly between species. Specifically, the prefrontal cortex, critical for higher cognitive functions in humans, is far less developed in rodents.

For these reasons, while the battery of NAMs presented in *Table 2* shows the potential for using simple and well-known tests, mainly *in vitro* or *in chemico*, several data gaps also emerge. Addressing these gaps requires targeted studies, particularly those leveraging NAMs. One example may be the establishment and implementation of mixed neurons/glial co-cultures to meet the data requirements for pesticide approval for neurotoxicity screening. These may play a key role in the development of *ad-hoc in vitro* batteries for neurotoxicity screening.

A promising scenario for advancing neurotoxicity assessment should be the use of human-derived cellular models, such as induced pluripotent stem cells (iPSCs) or brain organoids. These models allow the study of neurotoxicity in a species-specific context and support the development and validation of biomarker signatures for apical events, such as neuroinflammation, through high-content and omics methodologies. For any of the test systems selected, it should be underlined the importance of including a biokinetic study in any *in vitro* tests to understand the real cell exposure, avoiding to correlate any readout to the nominal tested

concentration(s), as also recommended by the OECD GIVIMP Document [41]. This also includes the characterization of the test system, particularly in terms of metabolic competence and transporter activity, crucial to carefully consider the biological relevance of these approaches, to enhance their translational value.

AO uterine adenocarcinoma

Recent updates to the AOP-Wiki confirm that AOP 503: activation of uterine estrogen receptor-α leading to endometrial adenocarcinoma, via epigenetic modulation is now formally deposited. Importantly, ERα activation (KE 1065) is not unique to this AOP but represents a shared MIE or early KE across several AOPs. For example, AOPs 445, 561, 565, and 609 all incorporate ERα activation as a mechanistic entry point for estrogen-mediated biological perturbations. This overlap underscores the centrality of this molecular event within endocrine-related AOP networks and highlights the necessity of evaluating uterine adenocarcinoma not through a single linear pathway, but within the broader context of interconnected AOPs converging on ERα-dependent signaling.

In AOP 503, ERα activation initiates downstream events including epigenetic modifications, altered expression of proliferation-related genes, endometrial epithelial hyperplasia, and genomic instability, ultimately leading to type I endometrial adenocarcinoma. However, because ERα activation also appears in multiple other AOPs, distinguishing tissue-specific downstream cascades (e.g., uterine *vs* mammary *vs* hypothalamic responses) remains a regulatory challenge. The presence of this shared node reinforces the need for harmonized criteria for evaluating ERα-driven mechanisms across AOPs, including standardized methodology for characterizing estrogen responsiveness, quantifying epigenetic alterations, and linking systemic and tissue-specific estradiol levels.

EFSA has identified data gaps that must be addressed before AOP 503 and its related network can be reliably used for regulatory screening of endocrine disruptors. These include the refinement of tissue-level estrogenic assays beyond receptor binding, improved quantitative correlations between circulating and uterine E2 concentrations, and NAMs specifically targeted to estrogen-dependent epigenetic mechanisms. Strengthening these areas will be essential for progressing AOP 503 toward OECD evaluation and potential regulatory adoption.

Limitations about the use of NAMs and AOP in regulatory framework

Introducing NAMs into regulatory frameworks is often hindered by specific legislative requirements. As an example, the CLP classification criteria for systemic toxicity and the corresponding information requirements under REACH refers to OECD TGs, largely based on traditional animal studies. NAM-based strategies especially for systemic toxicity end-point are not considered sufficiently valid and not validated, and are generally used for screening purposes by applicants, but not always included in the technical-scientific regulatory dossiers. It is therefore challenging to adapt NAMs to the current CLP system. Consequently, a new classification scheme is being developed, based on toxicodynamic and toxicokinetic properties obtained through NAM-based testing strategies. Under this scheme, chemicals would be ranked into three levels of concern: high, medium, and low [42].

Toxicological processes leading to an adverse outcome (AO) can be viewed as a continuum of events, starting from exposure to an external dose, progressing to the systemically available dose, and ultimately reaching the biologically effective dose at the target organ, tissue, cell, or receptor. This continuum may be described by MoA, which includes both toxicokinetic (TK) and toxicodynamic (TD), or by the AOP framework, which focuses solely on the TD dimension and is often considered "chemical agnostic". However, this distinction is important because a TK step, such as the metabolic bioactivation of a non-toxic chemical into a toxic metabolite, can itself constitute a KE. It is evident also from the case studies analyzed in this paper that among some limitations of the current AOP-based approach in pesticide RA, the TK information was not integrated into the AOP construct. The majority of KEs are anchored in a TD understanding, whereas critical determinants of internal dose are frequently absent. This limitation, together with the lack of information about *in vitro* biokinetics, restricts the extrapolation of *in vitro* NAM results (e.g., by a Quantitative *in vitro* to *in vivo* extrapolation – QIVIVE-modelling) to real exposure scenarios. Some improvements have been made, using the aggregate exposure pathway (AEP) framework or trying to quantify the KERs e.g., understanding *if* the magnitude of KE_1 is sufficient *then* to trigger KE_2 (the "if and then statement" or the biological plausibility of the relationship). Mathematical models enable the quantitative prediction of KERs using available biological data, facilitating the transition from an AOP to a qAOP [43]. While an AOP can be used to identify hazards, a qAOP can support risk evaluation, provided that an appropriate exposure scenario and complementary information on the chemical's biokinetic properties are available. Quantification is necessary for a more reliable prediction of chemical specific effects, including potency, which is a prerequisite for risk assessment [44, 45].

In addition, incorporating PBK models can help link external exposure to internal concentrations relevant to KEs [44]. Furthermore, the development of TK-TD models enables the quantification of temporal concordance and dose-response behavior along the pathway.

Furthermore, recent studies have emphasized the strong context-dependence of several neurotoxic outcomes, including Parkinsonian phenotypes, which are determined by factors such as age, developmental stage, co-exposures, and systemic inflammation [45]. It is evident that these aspects have not yet been captured by linear AOP structures. In order to enhance the regulatory applicability, specific methodological improvements can be pursued. The following four points are to be considered: (i) expanding the use of high-content and omics-based NAMs for KE quantification; (ii) de-

fining minimum performance criteria and reporting standards for NAMs included in regulatory batteries; (iii) promoting the development of qAOPs with defined quantitative KERs and exposure thresholds; and (iv) harmonizing cross-AOP analysis for shared biological nodes, such as ERα activation.

CONCLUSIONS

In conclusion, this study evaluated the application of AOPs and AOP networks by the EFSA, together with associated NAM batteries, through two case studies addressing pesticide-related Parkinson's disease and endocrine disruptor-induced uterine adenocarcinoma. The analysis of the complete AOPs and corresponding NAMs highlighted key strengths and limitations of the AOP-based approach and assessed their current level of integration within the regulatory framework. Looking to the future, the scientific and regulatory relevance of AOPs and qAOPs is expected to grow, supporting NGRA by providing reliable, human-relevant, and ethically sustainable tools for modern regulatory toxicology. This highlights the need for stronger international collaboration to streamline endorsement processes, ensure consistency in chemical safety assessments and accelerate the integration of AOPs, particularly their quantitative aspects, into both regulatory and non-regulatory frameworks.

Authors' contributions

Conceptualization and methodology, formal analysis, resources, data curation, writing – original draft preparation; writing – review and editing: TD, ET, EDC. All Authors have read and agreed to the published version of the manuscript.

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Conflict of interest statement

The Authors declare no conflict of interest.

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Indirect impact of COVID-19 pandemic on health and wellbeing: a narrative review

Teresa Valero-Gaspar[1], Cesar Garriga[2], Brigid Unim[3], Rodrigo Feteira-Santos[4], Luigi Palmieri[3], Asunción Díaz[5], Carmen Rodríguez-Blázquez[6] and Maria João Forjaz[7]

[1]Research Group "Food Behaviour, Nutrition and Gastrointestinal Health (CoNuSaGa)", Department of Nutrition and Food Science, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain
[2]National Centre for Epidemiology and CIBERESP, Carlos III Health Institute, Madrid, Spain
[3]Dipartimento Malattie Cardiovascolari, Endocrino-Metaboliche e Invecchiamento, Istituto Superiore di Sanità, Rome, Italy
[4]Instituto de Saúde Ambiental, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal
[5]National Centre for Epidemiology and CIBERINFEC, Carlos III Health Institute, Madrid, Spain
[6]National Centre for Epidemiology and CIBERNED, Carlos III Health Institute, Madrid, Spain
[7]National Centre for Epidemiology and RICAPPS, Carlos III Health Institute, Madrid, Spain

Abstract

Introduction. The COVID-19 pandemic had a profound impact on global health, notably affecting patients with non-COVID-19 conditions, who faced substantial disruptions in their treatment and care. The aim of this narrative review was to identify the main indicators used in literature to evaluate the indirect impact of COVID-19 on health and wellbeing.

Method. A literature search was conducted using PubMed from January 2021 to November 2022. The indicators were categorized into five main groups: burden of disease (BoD), life expectancy (LE), health-related quality of life (HRQoL), cost of illness and mental health status and were retrieved from 20 scientific articles.

Results. Disability-adjusted life years (DALYs) revealed substantial health losses; a decrease in LE was observed, with inequalities across population subgroups; HRQoL showed impairments in physical functioning, daily activities and emotional well-being; productivity losses were economically relevant and varied by context and elevated symptoms of anxiety and depression were reported.

Discussion. The compiled indicators may contribute to the development of sustainable pandemic mitigation policies.

Key words

- COVID-19
- life expectancy
- quality of life
- mental health
- illness cost

INTRODUCTION

The COVID-19 pandemic had a profound impact on global health, particularly for patients with non-COVID-19 conditions who experienced significant disruptions in their treatment and care. Hospitals postponed routine procedures for planned care [1-3], and public health systems issued guidelines to mitigate the risk of patients with cancer facing higher risks [4]. It was estimated that the disruption caused by the COVID-19 pandemic could result in an additional 18,000 cancer-related deaths in England (UK) [5]. A higher incidence of severe events in cancer patients have been reported based on a meta-analysis of 26 studies [6]. Furthermore, in June 2020, direct access to primary care was restricted, with general practitioners adopting telephone and video consultations in place of in-person visits [7]. Visits to urgent and emergency care services also decreased significantly following the onset of the pandemic, which likely resulted in patients postponing or forgoing essential medical attention [8]. Some patients required emergency care as their conditions worsened due to the lack of timely treatment [9, 10], while hospitalizations for chronic conditions may have risen as a consequence of the social-distancing measures implemented [11].

The increased pressure on health systems, caused by worsened health-status of patients who forewent timely treatment during the pandemic, has two potential implications. First, it might increase the costs to the national health systems due to increased need of medication [12, 13] and longer working hours for

Address for correpondence: Brigid Unim, Dipartimento Malattie Cardiovascolari, Endocrino-Metaboliche e Invecchiamento, Istituto Superiore di Sanità, Via Giano della Bella 34, 00162 Rome, Italy. E-mail: brigid.unim@iss.it.

health-care staff [14] (https://aspe.hhs.gov/reports/covid-19-health-care-workforce). Second, it may neglect the health of some groups who are more vulnerable than others (e.g., those with underlying comorbidities, children, homeless, pregnant women, migrants and people with disabilities) [15]. Early evidence suggested large differences between groups. For instance, for cancer deaths and other indirect deaths (i.e., drug- and alcohol-related deaths, suicides, fatal accidents, and all other causes), excess years of life lost (YLL) indirectly attributable to the pandemic ranged from 11,710 (95% CI: 2,694-20,725) in the least deprived quintile to 18,298 (95% CI: 10,754-25,810) in the most deprived quintile in England and Wales [16]. The impact of COVID-19 restrictions has been uneven across communities, with areas of higher deprivation and those with ethnic minorities suffering the worst of COVID-19 (https://ifs.org.uk/publications/are-some-ethnic-groups-more-vulnerable-covid-19-others).

Different types of indicators have been used to assess the indirect impact of the COVID-19 health crisis, such as burden of non-COVID-19 diseases [17] or life expectancy (LE) non-attributable to COVID-19 disease, among other indicators. Some examples of reviews published between 2021 and 2022 have described the impact of COVID-19 pandemic on quality of life [18], burden of disease [19] and mental health in the general population and vulnerable groups [20].

This study aimed to conduct a narrative review to identify and describe the key indicators employed in the research literature to assess the indirect impact of COVID-19 on health and wellbeing.

MATERIALS AND METHODS

Design

We conducted a narrative review to provide an overview of the most relevant indicators across various topics. The studies relevant to this review had heterogeneous designs, and many lacked the use of established data collection protocols and theoretical frameworks necessary for a comprehensive and quantitative synthesis. Our narrative review – supported by a predefined search strategy and selection criteria – offered a more flexible and feasible approach for identifying and analysing the indirect impact of COVID-19 on health and wellbeing.

Eligibility criteria

A literature search was conducted on PubMed, covering the period from January 2021 to November 2022. The selection criteria included studies published in peer-reviewed journals in the English language. Country reports and policy briefs were excluded, as well as grey literature such as conference proceedings, dissertations, abstracts, unpublished studies, and books.

Search strategy

The indicators were categorized into five main groups: burden of disease (BoD), life expectancy (LE), health-related quality of life (HRQoL), cost of illness and mental health status. The groups of indicators were chosen through a consensus between the authors and other experts in the field, who argued these were highly represented in the literature. The selection was based on previous guidelines [21-24].

Two reviewers applied the search strategy for each topic on November 30, 2022. Each search strategy included a combination of keywords with free text and Medical Subject Headings (MeSH) terms. Five collaborators from three European countries (Spain, Italy and Portugal) were allowed to modify search strategies to increase the chance of finding relevant articles *(Supplementary Material S1 available online)*.

Data extraction

Each collaborator was asked to select two to three publications featuring the same health indicator measuring an important health area indirectly affected by the COVID-19 pandemic. For each selected publication, the authors were instructed to extract and report specific information, including: indicator's name, what the indicator measured, its relevance – particularly in the context of COVID-19 – how it was calculated, and general comments on its application, strengths and limitations. The characteristics and implementation of each indicator were discussed in relation to its use in similar studies identified through the same search strategy. Collaborators also wrote an introductory paragraph about the relevance of their assigned topic. All the completed responses were collected and reviewed for inclusion in a summary table *(Supplementary Material S2 available online)*. This approach enables a focused synthesis of relevant evidence without the intention of quantifying the total number of retrieved publications. The included studies were selected collaboratively by the authors to ensure consistency and representativeness across the various indicators analysed.

RESULTS

The characteristics of the 20 studies included in the narrative review, and from which indicators were extracted, are reported in *Table 1*. The selection aimed at covering a range of geographic settings and study designs while maintaining a focus on clarity and relevance.

Burden of disease

The burden of disease (BoD) is an estimate of the impact of disease and injury on a population. It integrates the years of life lost due to living in poor health (non-fatal burden) with the years of life lost due to early death (fatal burden) [25]. The term "burden of disease" is commonly used to describe the total, cumulative impact of a specific disease or group of diseases on disability within a population. This encompasses not only the health effects but also the social consequences and the associated costs to society. The difference between optimal conditions, where everyone is free from disease and disability, and the cumulative current health status is described by the burden of disease. In the 1990s, the World Health Organization (WHO), together with Harvard University and the World Bank, developed a method for assessing the global burden of disease, based largely on statistical calculations of disability-adjusted life years (DALYs), which combine the time lost due to early mortality and the time spent living in poor health [26].

Table 1
Characteristics of the studies included in the narrative review

Author and year	Thematic area	Study design	Study country	Indicator/s extracted for this narrative review	Main results
Gravesteijn *et al.* (2021) [27]	BoD	Retrospective cohort study	The Netherlands	DALYs	The delay in surgical procedures led to a linear increase in DALYs per delay, with values ranging from 0.01 (shunt placement) to 0.23 (bypass surgery) DALYs/month.
Santomauro *et al.* (2021) [28]	BoD	Cross-sectional and longitudinal combination of studies	Worldwide	DALYs	Before the COVID-19 pandemic, major depressive disorder was responsible for 38.7 million DALYs globally, while anxiety disorders were responsible for 35.5 million DALYs. During the pandemic, major depressive disorder caused an additional 10.7 million DALYs, with 7.07 million among females and 3.62 million among males. Anxiety disorders caused an additional 9.05 million DALYs, with 6.11 million among females and 2.94 million among males.
Julien *et al.* (2022) [29]	BoD	Cross-sectional study	USA	DALYs	Drinking increases from 2020 to 2023 due to COVID-19 were projected to lead to 531,200 DALYs lost. This number was expected to rise significantly to 8,900,200 cumulative DALYs lost from 2020 through 2040.
Aburto *et al.* (2021) [30]	LE	Ecological study	England and Wales	LE at birth and lifespan inequality	The study found that LE at birth decreased during the first 47 weeks of 2020 in England and Wales. Women experienced a reduction of 0.9 years, while men saw a decrease of 1.2 years compared to 2019 levels.
Andrasfay and Goldman (2021) [31]	LE	Ecological study	USA	LE at birth and life expectancy at 65	This study presented estimated LE values under four projection scenarios. LE at birth would have been 78.61 y in 2020 had the COVID-19 pandemic not occurred, but all three mortality scenarios imply huge reductions in LE at birth for the USA in 2020. The medium scenario would bring about a decline of 1.13 y, whereas the higher and lower mortality scenarios project declines of 1.22 and 0.98 y, respectively. LE at age 65 y, which is estimated to have been 19.40 y in the absence of COVID-19, is projected to decline by 0.87 y under the medium scenario, 0.94 y under the higher mortality scenario, and 0.75 y under the lower mortality scenario. The disparities in LE declines were pronounced, with Black and Latino populations experiencing much larger reductions compared to Whites.
Ozyilmaz *et al.* (2022) [34]	LE	Ecological study	93 countries	LE at birth	Findings from social indicators show that LE negatively affects the number of deaths in the 25th and 50th quantiles. LE at birth and the share of the population over the age of 65 in the total population have a significant effect on the number of deaths. The population over the age of 65 has a statistically significant effect on the number of COVID-19 deaths in all quantile values.
Verveen *et al.* (2022) [48]	QoL	Prospective cohort study	The Netherlands	HRQoL-SF-36	Twelve months after illness onset, people with initial mild COVID-19 had health-related quality of life (HRQoL) within population norms, while those with moderate or severe/critical COVID-19 still had impaired HRQoL on more than half of the measured domains. Initial COVID-19 severity, migration background, number of comorbidities, and timing of public health measures all influenced long-term HRQoL outcomes.
O´Brien *et al.* (2022) [49]	QoL	Prospective longitudinal design	Ireland	HRQoL-SF-36	HRQoL measured by the SF-36 did not significantly improve in any domain over the 1-year follow-up. Scores remained lower than population norms in physical functioning, energy/vitality, role limitations due to physical problems, and general health. At 1 year, 31% of participants felt their general health was worse than a year prior.
Fernandes *et al.* (2021) [50]	QoL	Retrospective case-series study	Portugal	HRQoL-EQ-5D-5L	64.4% of COVID-19 critical illness survivors reported moderate to extreme problems in at least one dimension of the EQ-5D-5L questionnaire. The most affected areas were usual activities (51.1%), anxiety/depression (37.8%) and pain/discomfort (31.1%), followed by mobility (13.3%) and self-care (13.3%). The median self-rated health score (EQ-VAS) was 75.0 (on a scale from 0 to 100).

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Table 1
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Author and year	Thematic area	Study design	Study country	Indicator/s extracted for this narrative review	Main results
Kwon *et al.* (2022) [51]	QoL	Cross-sectional study	South Korea	HRQoL-EQ-5D	The study found that the overall EQ-5D index scores were significantly higher among individuals under quarantine during the COVID-19 pandemic (0.971) compared to those before the pandemic (0.964). Quarantine was associated with improved physical health dimensions, specifically showing significant improvements in "Pain/Discomfort" and "Mobility", but a significant deterioration in the "Depression/Anxiety" dimension.
Larsson *et al.* (2023) [52]	QoL	Prospective cohort study	Sweden	HRQoL-EQ-5D-5L and EQ-VAS	At both 4- and 12-month follow-ups, patients reported some problems in various dimensions, particularly in usual activity and pain/discomfort. There were no significant changes in any of the EQ-5D-5L dimensions between 4 and 12 months. Most of the participants reported lower EQ-VAS values than the general population at both follow-ups. For general health status, 28 (61%) participants at the first follow-up and 26 (57%) (p=0.414) at the second reported lower values than the general population.
Moens *et al.* (2022) [53]	QoL	Cross-sectional study	Belgium	HRQoL-EQ5D-3L and VAS score	89.58% of post-COVID-19 infected persons reported pain/discomfort, 82.45% indicated limitations when performing usual activities and only 13.16% indicated problems with self-care. The mean index score for normative population was significantly higher than the post-COVID-19 infected persons, with a mean difference of 0.31 (95% CI: 0.29 to 0.33, p<0.01). The mean score of chronic pain patients (PSPS-T2) was significantly lower than the score of COVID-19 infected persons, with a mean difference of -0.31 (95% CI: -0.29 to -0.33, p <0.01).
Barbieri *et al.* (2022) [54]	QoL	Cross-sectional study	Italy	HRQoL-KIDSCREEN-10	27% of parents' proxy reports indicated a low health-related quality of life (HRQoL) for their children. For children and adolescents aged 11-19 years: 33% self-reported a low HRQoL while 31% of parents reported a low HRQoL for their children in this age group. Children aged 7-10 years were significantly less affected by low HRQoL compared to children aged 11-19 years, according to proxy reports. Female adolescents showed significantly higher frequencies of low HRQoL than corresponding proxy reports. The self-reported low HRQoL of 33% in South Tyrol was similar to the 35% reported in Germany during the third wave of the COPSY Germany survey in September-October 2021 [55].
Ravens-Sieberer *et al.* (2022) [55]	QoL	Cross-sectional study	Germany	HRQoL-KIDSCREEN-10	The percentage of children and adolescents reporting low HRQoL increased substantially from 15.3% before the pandemic to 40.2% during the pandemic. Stratified by gender, a higher proportion of girls reported low HRQoL than their male peers before and during the pandemic. Younger children (aged 11-13) showed a greater decline in HRQoL compared to older adolescents (aged 14-17), rose from 7.7% to 41.3% in 11-to13-year-old children and from 17.1% to 39.3% in 14- to 17-year-olds (p<0.001).
Van Ballegooijen *et al.* (2021) [57]	Cost of illness	Cross-sectional study	Belgium and The Netherlands	Productivity losses (iMTA Productivity Cost Questionnaire-iPCQ-)	5.1% of respondents in Belgium and 4.4% in the Netherlands reported losing their job due to the pandemic. About 39-40% of respondents in both countries were somewhat to extremely worried about losing their profession. Weekly work hours decreased from 34.4 to 26.9 in Belgium and from 30.6 to 26.6 in the Netherlands during COVID-19. 35.7% of Belgian respondents experienced absenteeism compared to 18.7% in the Netherlands. By contrary, 29.5% of Belgian respondents and 33.7% of Dutch respondents experienced presenteeism. The mean value of lost production per person per week, including absenteeism and presenteeism, was €161.39 for Belgium and €82.69 for the Netherlands.

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Table 1
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Author and year	Thematic area	Study design	Study country	Indicator/s extracted for this narrative review	Main results
de Paiva *et al*. (2022) [58]	Cost of illness	Cross-sectional study	Brazil	Rate of absenteeism per year and total cost of absenteeism per year	The mean sickness absenteeism rate was 3.25%. There was a significant increase during the pandemic period, with the rate rising to 5.10% compared to 2.97% in the pre-pandemic period. The total cost of sickness absence was R$8,158,117.20, with a mean daily cost of R$3,525.55. During the pandemic, the mean daily cost increased to R$7,380.38, which is 2.49 times greater than the pre-pandemic period (R$2,960.12).
Faramarzi *et al*. (2021) [59]	Cost of illness	Cross-sectional study	Iran	Lost productivity cost ($US) due to absenteeism	The total cost of absenteeism due to COVID-19 among hospital personnel was estimated to be nearly $1.3 million. The average cost per patient was $671.4, with a median of $649. The mean cost of absenteeism was higher among males ($688.7) compared to females ($659.9). Patients aged over 50 years had the highest mean cost ($872.6), while those under 30 had the lowest ($597.7). Physicians had the highest mean cost per patient ($827.5), while other staff had the lowest ($603.7). Permanent employees had a higher mean cost ($756.2) compared to non-permanent employees ($604). The total number of missed workdays was 32,209, with an average of 16.44 days per patient.
Davis *et al*. (2022) [60]	Mental health status	Cross-sectional study	Canada	GAD-7	82% of the home dialysis patients surveyed reported symptoms of anxiety and depression either "not at all" or "for several days" on the GAD-7 scale. The results indicate that most symptoms of anxiety were experienced "some days" or "never" in more than 80% of the respondents.
Buneviciene *et al*. (2021) [62]	Mental health status	Cross-sectional study	Lithuania	GAD-7	Both pre-existing medical conditions and poor perceived health status were associated with an increased risk of moderate to severe anxiety symptoms, as measured by the GAD-7 questionnaire. After adjusting for demographic and behavioural factors, pre-existing medical conditions were linked to a significantly higher risk for moderate to severe anxiety symptoms (GAD-7 score ≥10). When both pre-existing conditions and perceived health status were considered together, pre-existing conditions were associated with a 1.5-fold increased risk (OR 1.526, p=0.016), while poor perceived health status was associated with a 4.6-fold increased risk (OR 4.556, p<0.001) for moderate to severe anxiety symptoms.
Ho-Fung *et al*. (2022) [64]	Mental health status	Cross-sectional study	China (Hong Kong SAR and Shanghai), Norway, Sweden, Switzerland, Taiwan and USA	GAD-7	25.7% of pregnant women in the study had moderate to severe generalized anxiety symptoms, as measured by GAD-7 (score ≥10). The total median GAD-7 score was 6.0 (IQR: 3.0-10.0). Women with sick family members had higher GAD-7 scores (median 7.0 [4.0-12.00]) compared to those without (median 6.0 [3.0-9.0]), and this difference was statistically significant (p=0.003). Risk factors for higher GAD-7 scores (anxiety) included having a sick family member (aOR 2.218, 95% CI [1.376, 3.573], p=0.001) and experiencing a substantially stressful life event (aOR 2.427, 95% CI [1.471, 4.005], p=0.001). Younger age (18-30) was protective against anxiety.

BoD: burden of disease; DALYs: disability adjusted life-years; EQ-VAS: EuroQoL Visual Analogue Scale; EQ-5D-3L or 5L: EuroQoL 5-Dimension 3 or 5-level questionnaire; GAD-7: generalized anxiety disorder-7; HRQoL: health related quality of life; LE: life expectancy; QoL: quality of life; SF: short form.

A study of elective surgical procedures in a Dutch hospital estimated the health impact of postponing these procedures [27]. Survival data were used to inform the model, which incorporated years lived with disability (YLD) and years of life lost (YLL) due to premature death, resulting in the calculation of DALYs. DALYs were used to assess the outcome of delaying surgery. The expected health outcomes with surgery at two weeks were compared with the expected health outcomes at 52 weeks to determine the health lost per 50 weeks. This measure of health loss provided an indication of urgency, which was later converted to health lost per month of delay. This was used to rank the surgical procedures, with a high DALYs/month indicating urgent surgery. The quality of data for this type of studies could be limited because the surgical procedures being evaluated are often part of standard clinical practice. Therefore, data may be biased (e.g., selection bias in the survival analysis of patients without treatment because patients opt for palliative care) or unavailable. The results from the indicator calculation provided valuable information for the development of a

decision model to support the prioritisation of surgical care in times of limited surgical capacity, such as the COVID-19 pandemic.

It was also observed that DALYs were used to quantify the impact of the COVID-19 pandemic on the prevalence and burden of major depressive disorder and anxiety disorders worldwide. The data revealed an increase in depressive and anxiety disorders in 2020 due to the pandemic [28]. Another study used DALYs to project alcohol-associated liver disease (ALD) from 2020 to 2040 in the USA and concluded that a short-term increase in alcohol consumption during the COVID-19 pandemic could substantially increase long-term ALD-related morbidity and mortality [29].

Life expectancy

The study of life expectancy (LE) in the context of the COVID-19 crisis allowed researchers to compare the cumulative effect of the pandemic with mortality rates from previous years and current trends across different countries. This comparison is feasible because LE is standardized and routinely monitored, allowing for the tracking of changes and differences in mortality [30]. This enables an analysis of the impact of the COVID-19 pandemic on survival rates while adjusting for the age distribution of the underlying populations [31]. LE at birth is defined as the average LE of a newborn baby, assuming no change in current mortality rates. Gains in LE at birth can be attributed to several factors, including improved standards of living, improved lifestyles and education, and better access to quality health services. This indicator is usually presented as a total and by sex, and is measured in years [32]. Another related measure is LE at 65: the average number of years a person of that age can be expected to live, assuming that age-specific mortality remains constant. Estimates of LE can vary by fractions of a year depending on the calculation used, which may vary slightly between countries [33].

In light of the COVID-19 pandemic, a cohort study analysed all-cause mortality for England and Wales calculating LE (average mortality) and the variation in length of life between individuals in a population (lifespan inequality) [30]. Estimating the number of deaths caused by the COVID-19 pandemic was crucial to understanding the impact of the disease. The authors assessed the impact of the COVID-19 pandemic on LE and lifespan inequality in 2020 by using baseline mortality data reflecting deaths in the absence of COVID-19 and applied fitted models to estimate excess deaths. LE at birth for women and men in 2020 was 82.6 and 78.7 years, respectively, with 0.9 and 1.2 YLL compared to 2019. Lifespan inequality decreased due to the rise in mortality among older age groups. Furthermore, LE was used to illustrate the impact of the Coronavirus pandemic on the Black and Latino populations in the United States [31]. Estimates were calculated for LE at birth and at age 65 for 2020, with results stratified by race and ethnicity. The study found that LE at birth in the US reached its lowest level since 2003, alongside a 0.87-year reduction in LE at age 65. The decline in LE at birth was 2.10 years for Black populations, 3.05 years for Latino populations, and 0.68 years for White populations. Another study analysed confirmed cases and determinants of COVID-19 fatalities in 93 countries [34]. The study projected a mortality model that incorporated social indicators, including LE at birth, sourced from the World Bank Open Data (https://data.worldbank.org/).

LE at birth was strongly associated with the number of COVID-19 deaths in countries with a low number of cases (25th quantile). The study estimated that a 1% increase in LE corresponded to a 10.82% reduction in COVID-19 deaths. Conversely, a 1% increase in the population aged 65 and older was associated with an increase in the number of deaths. This effect is attributed to their inclusion in the group with a higher-risk for adverse outcomes related to COVID-19.

Health-related quality of life

COVID-19 can lead to varying outcomes, including persistent symptoms that impact the daily lives of those infected [35-38]. Furthermore, measures implemented to control the virus' spread caused disruptions to individuals' daily activities on multiple levels [18, 39]. Given this, assessing quality of life (QoL) and its association with the long-term health consequences of COVID-19 is essential [40, 41]. The long-term effects of COVID-19 infection or pandemic countermeasures can lead to physical and mental health deterioration, as well as impairments in health-related quality of life (HRQoL) [18, 39]. Various measurement tools have been used to evaluate HRQoL [42], many of which are widely validated across different languages. These instruments assess multiple dimensions of HRQoL, including physical, mental, social and emotional functioning [43]. Tools commonly used to evaluate the impact of COVID-19 infection or pandemic-related contexts on HRQoL include the Medical Outcomes Study Short Form 36-item health survey (SF-36) [44], the EQ-5D [45, 46] (https://euroqol.org/euroqol/) and the KIDSCREEN-10 index [47].

The SF-36 was used to assess how contextual factors, such as public health control measures, affected patients' recovery [48] and their ability to return to pre-infection levels of function [49]. Verveen *et al.* [48], in a cohort study conducted in the Netherlands, monitored individuals with a confirmed SARS-CoV-2 infection at 1 and 12 months post-diagnosis. The study aimed to assess the impact of infection severity on HRQoL in both the short and long term. One month after a COVID-19 diagnosis, HRQoL was significantly below the population average in all SF-36 domains among individuals with mild COVID-19, except for general health and body pain. However, after 12 months, the HRQoL levels were within population standards. The study by O'Brien *et al.* [49] assessed COVID-19 patients at 10 weeks, 6 months, and 1 year after hospital discharge to monitor which patients or groups experienced greater impairment in HRQoL. The authors found no change in SF-36 scores across any domain during the study period. The scores remained lower than population standards in the domains of physical functioning, energy/vitality, role limitations due to physical problems and general health.

The EQ-5D-5L was employed to evaluate the impact of a SARS-CoV-2 infection on HRQoL among patients aged 18 years or older in Portugal, between the 30th and 90th day after hospital discharge [50]. The authors found that 29 patients (64.4%) reported moderate to extreme problems in at least one dimension of the EQ-5D-5L questionnaire. The most affected dimension was usual activities (51.1%), followed by anxiety/depression (37.8%) and pain/discomfort (31.1%). Kwon *et al.* [51] used the instrument to evaluate the impact of quarantine on HRQoL among individuals aged 19 years and older in South Korea. A comparison was made between EQ-5D scores collected after the onset of the pandemic and data systematically collected before the pandemic, starting in 2008. The overall EQ-5D index scores were significantly higher in the group under quarantine during the COVID-19 pandemic (0.971±0.064) compared to the pre-pandemic scores (0.964±0.079, Diff. 0.007±0.101, p=0.043). The EQ-5D was also employed to evaluate the burden of illness and its impact on health and work-related circumstances among patients who had been treated for SARS-CoV-2 infection in Sweden at both four- and 12-month intervals following their discharge from intensive care [52]. The findings revealed no improvements between the first EQ-5D score and the second follow-up in any of its domains: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Finally, a Belgian study evaluated the HRQoL of individuals who had recovered from COVID-19 and were active on social media platforms between June and August 2021. HRQoL was measured using the EQ-5D-3L tool. The authors found that low scores were associated with difficulties in performing activities and pain/discomfort across the EQ-5D-3L dimensions [53].

The KIDSCREEN-10, an instrument for assessing QoL in children, was used to evaluate the HRQoL of children in Italy during the second year of the pandemic. The objective was to identify strategies for safeguarding children's mental health. The study found that 33% of children and adolescents aged 11 to 19 years self-reported a low HRQoL, while parents reported a low HRQoL for 31% of their children in this age group [54]. Furthermore, the KIDSCREEN-10 was employed in a study utilising a representative sample of children and adolescents aged 7-17 years in Germany. The study measured the HRQoL of the participants before and after the onset of the pandemic. Up to 15.3% of children and adolescents reported low HRQoL prior to the pandemic (n=146; based on weighted data from the BELLA study), compared to 40.2% during the pandemic (n=418; based on weighted self-reported data from the COPSY study) [55]. The identification of children and adolescents' needs enables the dissemination of information regarding their mental health to policymakers, paediatric healthcare professionals and parents. This can be considered a strength of the tool, as it reports findings from both the children themselves and their parents' perspectives.

Assessing the population's HRQoL requires the use of valid instruments, such as the SF-36, EQ-5D, or KIDSCREEN-10 index, and the collection of primary data. This process can be resource-intensive and expensive. Typically, these instruments must be administered after the exposure in question, such as a COVID-19 infection or the pandemic context, to capture relevant HRQoL outcomes. Consequently, the size of the studied sample or population may vary depending on the available resources and the specific research question. The resource-intensive nature of HRQoL assessments also leads to varying timeframes for data collection, which can impair the reliability of result comparisons between different populations. Consequently, the available data may not always be sufficient for disaggregating results to the extent necessary for comparing HRQoL across all population subgroups. This limitation can hinder the ability to draw comprehensive and equitable conclusions about the impact of events such as COVID-19 on different population subgroups. When data collection relies on self-administered methods, certain population subgroups may be less likely to provide or complete the questionnaires, thus limiting the ability to compare HRQoL across groups. Additionally, some HRQoL scales may not be suitable for all populations. For instance, the SF-36 is challenging to apply to individuals with severe mental disorders. Similarly, the KIDSCREEN-10 provides a single global HRQoL score, which can result in a loss of detailed information when compared to longer versions of the KIDSCREEN, such as KIDSCREEN-27 or KIDSCREEN-52 [56].

Cost of illness

The impact of the novel coronavirus on the ability to resume work and perform at a normal capacity after infection has been demonstrated [57, 58]. The term "productivity loss" can be defined in terms of two distinct categories: presenteeism, which refers to the inability to work at one's normal capacity, and absenteeism, which encompasses the inability to work at all [57].

A study conducted in Belgium and the Netherlands evaluated the impact of the pandemic on stress levels, QoL, medical resource utilisation and productivity losses in the general population during the initial eight weeks of the coronavirus lockdown. The indicator of productivity losses related to COVID-19, described in terms of absenteeism and presenteeism, was recorded using the Productivity Cost Questionnaire (iPCQ). The authors calculated the mean value of lost production among respondents in paid professions on a per-person and per-week basis. The number of hours lost due to the impact of the pandemic on remunerated work was calculated by multiplying the total number of hours lost by the average hourly income in a specific country, adjusted for age; all costs were presented as weekly costs in euros [57]. A similar study was conducted to estimate the lost productivity cost of absenteeism due to COVID-19 among hospital staff in Iran. The monetary value for a working day for absent employees was multiplied by the number of missed workdays to estimate the absenteeism cost expressed in US dollars [59]. Another study estimated the cost of absenteeism among healthcare workers in Brazil from 2014 to 2020. The cost of absenteeism was calculated similarly to previous studies; however, it excluded periods when workers

were receiving sickness benefit, as salaries were not paid during these times. The authors also calculated the rate of absenteeism per year by dividing the number of days absent in a year by the number of days that could have been worked. This was based on the weekly number of hours worked and the worker's job title, providing a clearer understanding of absenteeism relative to the total potential workdays in a given year [58].

Mental health status

The COVID-19 pandemic caused significant consequences on global mental health including fear of acquiring and spreading infection to family members, loneliness, anxiety, depression and suicide. These effects were likely driven by national lockdowns implemented to contain the virus spread, leading to isolation and family separation. Additionally, the proliferation of misinformation and disinformation on social media, low health literacy, scarcity of basic needs, financial losses and increasing fear and vulnerability due to the uncertainty of disease progression may also be contributing factors [60, 61]. Therefore, public health emergencies, i.e., the COVID-19 pandemic, can increase the risk of new-onset mental health complications or exacerbate pre-existing mental disorders in the general population and in vulnerable individuals with pre-existing conditions. Addressing the risks for mental health complications during health emergencies is crucial to ensure the optimal allocation of health resources and to mitigate the adverse consequences of the disease [62].

In a study conducted in Lithuania, it was observed that pre-existing medical conditions (e.g., cardiovascular, pulmonary, obesity, diabetes, mental disorders) were associated with an increased risk of mental health complications during the recent pandemic. The correlation between self-perceived health status and the likelihood of developing mental health issues during the pandemic was also highlighted [62]. The study specifically assessed depressive symptom severity and anxiety symptom severity, measured by the Generalized Anxiety Disorder-7 (GAD-7) score [63]. The GAD-7 score was also employed to assess the prevalence of perinatal anxiety, alongside depression and acute stress reaction, among pregnant women in Sweden. This was part of a cross-sectional study that also explored the association of these symptoms with mental health outcomes. According to the study, 121 participants (25.7%) exhibited moderate to severe generalized anxiety symptoms, as indicated by a GAD-7 score of 10 or higher [64]. In a single-centre study of home dialysis patients in Canada, the GAD-7 score indicator was employed to describe levels of anxiety and quality of life during the SARS-CoV-2 pandemic. In this case, 80% of respondents reported experiencing symptoms of anxiety and depression "some days" or "never"[60].

The use of validated generic scales, such as GAD-7, is an effective approach for the acquisition of indicators defining the impact of the COVID-19 pandemic on mental health. These scales are simple to use and provide standardized self-reported measures of core mental health disorder symptoms. However, they rely on self-reports and evaluate only probable diagnoses, which should be confirmed by other means, such as psychiatric interviews. Moreover, they may not be appropriate for all population groups [63, 65, 66].

DISCUSSION

The impact of COVID-19 on global health conditions or health status has been assessed through various indicators, including DALYs, LE at birth or at 65 years old, HRQoL, cost of illness and mental health status. Some indicators, such as HRQoL and mental health outcomes, were evaluated using standardized scales obtained from different questionnaires [44, 46, 47, 63, 66].

According to the revised studies, DALYs revealed substantial health losses associated with delays in elective surgeries, increases in mental health disorders and alcohol-related liver disease. LE declined globally in 2020, with disproportionate reductions among Black and Latino populations in the United States. HRQoL scales (SF-36 and EQ-5D) showed impairments in physical functioning, daily activities and emotional well-being among COVID-19 patients, with children and adolescents also reporting significant deterioration, as shown by the KIDSCREEN-10. Productivity losses, quantified through absenteeism and presenteeism, were economically relevant and varied by context. Finally, mental health impacts were pervasive, with elevated symptoms of anxiety and depression reported in both the general population and high-risk groups, as identified using instruments such as the GAD-7. These indicators, widely used in scientific literature, have also been implemented in European policy documents and decision-making tools [23, 67-69].

Furthermore, these indicators not only were useful for assessing the effects of the COVID-19 pandemic, but they have also been widely applied to chronic and communicable diseases to capture indirect health impacts and guide public health decision-making. Overall, the indicators showed a widespread effect of the COVID-19 pandemic on health, economic and psychosocial dimensions across different populations and contexts.

Burden of disease

Burden of disease metrics, such as DALYs, facilitate monitoring the direct impact of COVID-19 infection on population health. They also offer opportunities to assess the indirect impact of the pandemic that has occurred due to national lockdowns and restrictions to vital healthcare services [70]. DALYs have also been used to assess the impact of other national public health programs and interventions around the world [71-73]. Their models translate distributed units of intervention into DALYs averted, allowing cost per DALY calculations and comparisons across programs and settings [74]. Such studies typically adopt cost-effectiveness calculations and cohort simulations to estimate the number of DALYs averted. This includes a wide range of health areas: mental health, infectious disease control, immunization or primary care coverage.

Life expectancy

The impact of the pandemic resulted in a reduction in life expectancy (LE) between 2020 and 2021 [75].

However, the precise age-specific mortality rates for any given birth cohort cannot be known in advance. As the impact of the pandemic in terms of mortality is decreasing, the actual life spans are likely to be higher than the LE estimates based on the average mortality rates in 2020. The studies included in this review highlight the diverse impacts of COVID-19 on LE and lifespan, emphasizing the inequalities that have emerged as a result of the pandemic [30, 31, 34]. To better inform national policies for future health crises, it is essential to stratify data by factors such as socioeconomic status, education level and ethnicity. Harmonizing these contextual variables is key to enhancing surveillance systems. Furthermore, in the studies reviewed, LE was calculated based on all-cause mortality, allowing the indicator to capture both the direct and indirect effects of COVID-19. Studies that identify specific causes of death could help distinguish between the direct and indirect impacts of a pandemic. However, during the first wave of a pandemic, when health systems are overwhelmed, such studies may not be feasible. In this context, the excess mortality indicator will continue to play a critical role in future health crises.

In addition, LE improvements are commonly used to assess the impact of national public health programmes and broader health policies around the world. This indicator is used in a way comparable to its application in assessing the effectiveness of lockdown measures during the COVID-19 pandemic. Numerous national public health programmes have demonstrated significant impacts on LE. A modelling study projected the effect of raising tobacco prices (by 5%, 10%, or 20%) on smoking behaviour, COPD burden, and LE in Italy (alongside England and Sweden). Using a multi-state simulation model and Italian demographic and smoking data, the study estimated that, under a 20% price increase, LE gains in Italy for a 20-year-old ranged from 0.25 to 0.45 years over 40 years, driven by reduced smoking and COPD incidence [76]. In the United States, a national analysis found that 44% of the 3.3-year increase in LE between 1990 and 2015 was due to public health improvements, including tobacco control and environmental health [77]. In England, the Health Inequalities Strategy (1997-2010) targeted deprived areas and successfully narrowed gaps in LE, reversing previous trends of widening disparities [78]. Modelling based on UK Biobank data suggests that middle-aged adults who adopt optimal dietary patterns could gain up to 10 additional years of life, depending on baseline habits [79]. Similarly, a European microsimulation study estimated that if all adults met physical activity guidelines, LE would increase by approximately 3 months, while also reducing chronic disease risk [80]. Lastly, global data from the Institute for Health Metrics and Evaluation (https://www.healthdata.org/news-events/newsroom/news-releases/life-expectancy-increased-world-addressed-major-killers) show that LE increased by 6.2 years worldwide between 1990 and 2021 [81], largely due to public health interventions that reduced infectious and chronic disease mortality.

Health-related quality of life

Several well-designed studies have demonstrated improvements in health-related quality of life (HRQoL) associated with public health interventions. For example, a US-based community lifestyle program adapted from the Diabetes Prevention Program (Group Lifestyle Balance Program) significantly improved EQ-5D-VAS scores (+7.4 at 6 months, +6.7 at 12 months) and modestly increased EQ-5D index values among adults with prediabetes or metabolic syndrome, demonstrating the utility of EQ-5D in evaluating community-based diabetes prevention efforts [82]. In China, a community-based public health service targeting middle-aged and older adults with chronic conditions constructed an SF-36-based HRQoL scale from CHARLS variables and found that this service demonstrated a significant association with an increased overall SF-36 score (β=3.539, p<0.001) supporting the use of SF-36 to assess programmatic interventions in chronic disease management [83]. A Brazilian study comparing elderly participants in a community physical activity program with sedentary peers and found higher scores in SF-36 domains of functioning capacity and general health perceptions for program participants [84]. Although fewer studies directly link KIDSCREEN-10 to specific public health programs [85], its use in population-based surveys supports its potential to evaluate child and adolescent health initiatives. Together, these studies illustrate the value of EQ-5D and SF-36 in measuring QoL gains from public health interventions at sub-national levels [86-88].

Cost of illness

Studies have used cost-of-illness measures – including sickness absence days, production loss, and presenteeism – to evaluate workplace or community-level public health interventions. For example, a cluster-randomised controlled trial in Sweden implemented a work-directed problem-solving intervention within occupational health services for employees with common mental disorders. It led to almost 15 fewer registered sickness absence days over one year compared to usual care – a reduction that was cost-beneficial from a societal perspective, despite increased short-term productivity loss recorded by employers [89, 90]. A follow-up within primary care showed similar reductions in self-reported sick leave and improved mental health and return-to-work, again demonstrating effectiveness using real-world registry data [91]. Additionally, a systematic review of workplace nutrition and physical activity programs demonstrated that multi-component interventions (including organizational, environmental, and individual-level elements) consistently reduced absenteeism and improved work performance and productivity [92].

In terms of the indirect impact of communicable diseases measured with cost of illness indicators there are studies about influenza vaccination [93, 94]. The systematic review of workplace influenza interventions across multiple countries concluded that both pharmacological and non-pharmaceutical strategies, including paid sick leave, significantly reduced absenteeism and

were consistently cost-saving from the employer perspective [94].

Mental health status

Mental health screening tools such as the GAD-7 and GHQ-12 (General Health Questionnaire) have been widely used in cross-sectional and cohort studies to document the psychological impact of communicable disease outbreaks [95, 96]. However, despite their frequent use in epidemiological monitoring, these mental health instruments are rarely embedded in formal evaluations of non-pharmaceutical public health interventions – such as mass testing, lockdown mandates, or vaccination rollouts. For example, a small mobile telephone survey measured anxiety using GAD-7 during the early phase of COVID-19 in Wuhan and Shanghai in China but did not tie changes in mental health to any specific intervention [97]. More broadly, most public health policy evaluations focus on clinical and epidemiological outcomes – such as infection rates, hospitalizations, and mortality – while mental health metrics are often included only in secondary analyses or smaller sub-studies. This trend was evident in longitudinal UK studies that used the GHQ-12 to track mental health changes before and after national lockdowns, though without linking those changes directly to specific policies or intervention efficacy [98].

Strengths and limitations

We have emphasized the identification of methodological issues and the characteristics of the indicators used across the literature, rather than the findings of individual studies. While these indicators provided valuable insights, limitations included resource-intensive data collection, reliance on self-reports and potential sampling biases. Furthermore, the main limitation of rating scales is the subjectivity in the allocation of scores and the ordered level of items (representing a sorted classification rather than true numerical values) adopted for the majority of them. Nevertheless, rating scales are easy to apply in a multitude of contexts and settings, necessitating no supplementary resources or expenditures. They encompass numerous aspects of health status, providing a comprehensive overview of physical, mental and social wellbeing. These characteristics have facilitated their use as indicators during the pandemic, enabling comparison with previous years in research studies.

CONCLUSIONS

The present review offers a rapid vision on the indirect impact provoked by the COVID-19 crisis, presenting a comprehensive synthesis of health indicators identified through extensive research, evaluating the indirect effects on health status and wellbeing for both COVID-19 and non-COVID-19 patients.

Although the pandemic has ended, understanding the key indicators used to evaluate health status and wellbeing remains essential. Lessons learned during the pandemic have underscored the critical importance of preparing robust, inclusive and resilient health systems capable of adapting to challenges and changes across various levels. Such systems should ensure the continuity of medical consultations in both primary and specialized care, which were affected not only by the excessive burden on healthcare workers but also by the impact on patients in long-term care facilities with main focus on mental health services of long term care recipients and workers [99].

The indicators identified in this review could serve as valuable tools for assessing the impact of future pandemics. To maximize their utility, it is essential to standardize their calculation, facilitating comparisons over time and across different populations, settings and countries. During global health emergencies, having a harmonized, equity-sensitive set of indicators, clearly disseminated through policy documents and decision-making tools and categorized by the primary areas affected, is crucial. Such a framework would enable public health institutions to monitor disease elimination strategies, implement early prevention measures and sustain robust health systems, all with the overarching goal of preserving the physical and mental health of populations.

Despite the extensive body of evidence reviewed, significant gaps persist – especially concerning the pandemic's impact on specific occupational groups such as healthcare workers, educators, and frontline service providers. Urgent research is needed to evaluate the long-term physical and mental health effects on these populations and to guide the development of occupational health policies that prioritize their safety and psychological well-being. These indicators should be systematically integrated into both research frameworks and policy planning to ensure better preparedness and equitable responses in future public health emergencies.

This review summarizes the evidence on the indirect impact of COVID-19 on health and wellbeing, aiming to guide the development of priorities and mitigation strategies to support recovery. The compiled indicators can contribute to shaping sustainable pandemic response policies.

Disclaimer

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Authors' contributions

TVG drafted the manuscript. TVG and CG developed the search strategy. CG, TVG, CRB, AD and MJF contributed to the development of the selection criteria and data extraction criteria. CG, TVG, BU, RFS and LP retrieved papers, indicators and their characteristics. All Authors read, provided feedback and approved the final manuscript.

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Data availability

The data underlying this article are available in the article and in its *Supplementary material available online*.

Conflict of interest statement

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The hidden pain of bullying: somatic symptoms and physical health consequences

Ramona Di Stefano[1], Rodolfo Rossi[2], Valentina Baldini[3], Adriano Ranieri[4], Francesca Pacitti[4] and Alessandro Rossi[4]

[1]*Dipartimento Integrato di Salute Mentale, Neuropsichiatria dell'Infanzia, dell'Adolescenza e delle Dipendenze Patologiche (DSM-DP), Azienda Sanitaria Locale Roma 5, Colleferro, Rome, Italy*
[2]*Dipartimento di Medicina dei Sistemi, Università degli Studi di Roma "Tor Vergata", Rome, Italy*
[3]*Dipartimento di Scienze Biomediche e Neuromotorie (DIBINEM), Alma Mater Studiorum, Università di Bologna, Bologna, Italy*
[4]*Dipartimento di Scienze Cliniche Applicate e Biotecnologiche (DISCAB), Università degli Studi dell'Aquila, L'Aquila, Italy*

Abstract

Background. Bullying is a pervasive public health issue among children and adolescents, associated not only with psychological distress but also with the development of physical health complaints.
The present study aims to explore the relationship between bullying victimization and somatic symptoms in youth, with particular attention to the Italian context, and to identify potential mechanisms and intervention strategies.
Materials and Methods. A narrative review of the literature was conducted to examine the association between bullying victimization and somatic symptoms in children and adolescents. Relevant studies were identified through a non-systematic search of major electronic databases, including PubMed, Scopus, and Google Scholar. Search terms included combinations of keywords such as bullying, cyberbullying, somatic symptoms, psychosomatic complaints, adolescents, and school-based intervention. Additional articles were identified by manually screening the reference lists of selected papers.
Results. Multiple studies indicate a consistent association between bullying victimization and increased prevalence of somatic symptoms among children and adolescents. In Italy, national programs have shown some success in reducing bullying, yet the problem remains significant. High affective empathy and limited emotional competence may increase vulnerability to psychosomatic outcomes.
Conclusions. Somatic symptoms in youth may be important indicators of bullying-related stress. Early identification, multidisciplinary care, and school-based prevention programs are essential.

Key words
- bullying
- somatic symptoms
- adolescents
- cyberbullying
- emotional regulation
- public health

INTRODUCTION

Bullying is defined as a persistent threatening and aggressive physical behavior or verbal abuse by an individual or a group, directed toward other people, especially those who are younger, smaller, weaker, or in some other situation of relative disadvantage [1].

Bullying is characterized by a power imbalance between the perpetrator and the victim, which may arise from disparities in physical strength, social status, or other contextual factors. It manifests in multiple forms, including direct physical aggression (such as hitting, kicking, or pushing), verbal aggression (encompassing taunting, name-calling, and threats), relational aggression (involving behaviors intended to harm social relationships or reputations, such as rumor-spreading and social exclusion), and cyberbullying, which occurs through digital platforms and communication technologies [2, 3].

METHODS

A non-systematic but comprehensive search was conducted using electronic databases including PubMed, Scopus, and Google Scholar. The search terms included combinations of bullying, cyberbullying, somatic symptoms, psychosomatic complaints, adolescents, and school-based intervention. Studies were selected if

Address for correspondence: Rodolfo Rossi, Dipartimento di Medicina dei Sistemi, Università degli Studi di Roma "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy. E-mail: rudy86.rossi@gmail.com.

they addressed any form of bullying (physical, verbal, relational, cyber, or identity-based), involved child or adolescent populations, and explored the occurrence, mechanisms, or outcomes of somatic symptoms. Both cross-sectional and longitudinal studies were included, as well as meta-analyses, narrative reviews, and relevant reports from organizations. Particular attention was given to literature focusing on the Italian context and interventions implemented within Italy. The included studies were synthesized qualitatively to highlight epidemiological trends, psychological and biological mechanisms, and implications for clinical practice and public health strategies. Due to the narrative nature of this review, no formal quality assessment of studies or statistical meta-analysis was performed.

RESULTS

Types of bullying

Bullying can be broadly categorized into four types, each representing unique modes of aggression (*Table 1*):

- *physical bullying* involves direct acts of aggression such as hitting, kicking, or pushing. Although it is less commonly reported than verbal or relational forms of bullying, it tends to be more visible and easily recognized. Physical bullying is more prevalent among younger children, particularly those in elementary school, and its occurrence generally declines with age. It is typically more common among boys than girls, both as perpetrators and as victims [4, 5];
- *verbal bullying* consists in using words to harm, such as name-calling or insults. Verbal bullying is one of the most common forms of bullying across all age groups. It typically begins in early childhood and remains prevalent throughout adolescence. This form of bullying is frequently perpetrated by, and directed toward, both boys and girls [4-6];
- *relational bullying* involves behaviors intended to harm an individual's social relationships or reputation, such as spreading rumors, social exclusion, or manipulating friendships. This form of bullying is highly prevalent, particularly during adolescence. Its occurrence tends to increase with age and is most commonly observed in middle and high school. While relational bullying is more frequently associated with girls, boys also engage in such behaviors [7, 8];
- *cyberbullying* refers to bullying behaviors that occur through digital platforms such as social media, text messaging, or online forums. It has become increasingly common, particularly among adolescents and teenagers. While relatively rare among younger children, its prevalence rises significantly during middle school and peaks in high school. Both boys and girls engage in cyberbullying; however, girls are slightly more likely to be victims of relational forms of cyberbullying [9, 10];
- *sexual and identity-based bullying* involves harassment or victimization based on characteristics such as sexual orientation, gender identity, race, ethnicity, religion, or disability. This form of bullying disproportionately affects individuals from marginalized groups. It becomes more prevalent during adolescence, a period marked by intensified identity formation and social categorization. Victims are often LGBTIQ+ youth, students with disabilities, and members of racial or ethnic minority groups [11, 12].

While all types can co-occur, studies have shown that verbal and relational bullying are more commonly reported than physical aggression [8, 13, 14].

Epidemiology

Bullying is a global issue, with prevalence rates varying by region, country, and study methodology [15-18]. According to a 2019 United Nations Educational, Scientific and Cultural Organization (UNESCO) report, nearly one-third of children worldwide reported experiencing bullying in the past month, with a smaller proportion experiencing it on six or more days within that period [15]. Regional differences are evident, with reported rates ranging from 22.8% in Central America to 48.2% in sub-Saharan Africa. Studies across multiple countries reveal similar variability. For example, a study involving 23 countries found that traditional bullying prevalence ranged from 8% to 50%, with an average of 27%. Another analysis of 28 countries reported prevalence rates ranging from 6.3% among girls in Sweden to 41.4% among boys in Lithuania. In the United States, approximately 29% of students report being victims of school-based bullying. In Norway, prevalence rates range between 8.3% and 12.5%, depending on the study [15, 17]. These variations highlight the complexity of accurately measuring bullying prevalence due to differences in definitions and methodologies.

According to the World Health Organization (WHO), boys are generally more likely than girls to engage in both traditional and cyberbullying behaviors. Although the prevalence of victimization from traditional bullying is relatively similar between boys and girls, girls are more frequently targeted through cyberbullying. While the overall rates of bullying perpetration have declined since 2014, the proportion of adolescents experiencing

Table 1
Types of bullying

Bullying types	Common age range	Prevalence trend
Physical	Elementary school	Declines with age
Verbal	All ages	Stable across age
Relational	Middle to high school	Increases with age
Cyberbullying	Middle to high school	Increases with technology access
Identity-based	Adolescence	Increases in diverse environments

bullying has remained largely unchanged. Additionally, over one in ten adolescents reported being cyberbullied during the same period, with girls (14%) more likely than boys (12%) to report such experiences. Although girls are less likely to cyberbully others, they are disproportionately affected as victims of cyberbullying. The prevalence of bullying perpetration peaks at age 15 for boys and age 13 for girls. Younger adolescents, in general, are particularly vulnerable to victimization. Moreover, boys are significantly more likely than girls to engage in physical fighting, with 15% of boys and 5% of girls reporting involvement (https://www.who.int/europe/initiatives/health-behaviour-in-school-aged-children-(hbsc)-study/highlights).

It is important to highlight that the interpretation of what constitutes bullying varies according to culture and age group. These differences may account for part of the large variation in prevalence rates reported across studies (*Table 2*).

Age and cultural differences in the perception of bullying

The interpretation of what constitutes bullying is deeply embedded within cultural norms and values and therefore varies considerably across societies.

Developmental factors play a role, as younger children are less likely to identify or report indirect or relational forms of aggression such as exclusion or rumor-spreading, while older adolescents become more sensitive to these subtler dynamics [19].

In bullying research, many studies rely on questionnaire-based responses, which limits the findings to children who are old enough to read, comprehend, and complete such instruments proficiently. Nevertheless, some investigations have explored much younger age groups, including children as young as four years old [20, 21]. It is important to recognize that the way younger children conceptualize bullying evolves with age. Early on, their understanding does not typically emphasize elements such as power imbalance, repetition, or intent to harm, which are more commonly identified by older children. Instead, younger children tend to describe bullying in terms of the negative impact on the victim or behaviors that deviate from what is perceived as normal, often regardless of whether the actions are repeated or unprovoked [22]. According to this broader interpretation, a child may be labeled a bully irrespective of intent.

An Italian study published in 2002 compared definitions of bullying provided by teachers and students aged 8 to 14 years. The findings revealed that older students were more likely to classify a wider range of behaviors as bullying, including social or gender-based exclusion and verbal bullying, compared to teachers. Educators, on the other hand, tended to overlook these relational or verbal forms, focusing instead on more overt behaviors such as physical aggression, even when their descriptions aligned more closely with fighting than with bullying [23].

However, cultural context remains a decisive factor in shaping both recognition and reporting. For instance, research conducted in Scandinavian countries demonstrates that behaviors like social exclusion or rumor dissemination are consistently categorized as bullying and systematically monitored in school settings, reflecting a strong societal emphasis on equity and inclusion [24, 25].

Recent research underscores additional complexity: while racial and ethnic minority youth report comparable or even higher levels of victimization behaviors than their majority peers, they are less likely to label those experiences as "bullying" when asked via definition-based measures [26, 27].

In sub-Saharan African contexts, such as Ghana or Uganda, bullying is often equated with physical violence, while verbal or relational aggression is less likely to be recognized, reflecting broader socialization patterns where direct confrontation is more salient [28].

Cyberbullying adds another layer of cultural variation, since its salience is tied to digital penetration and attitudes toward online interaction. In East Asian countries such as South Korea and Japan, cyberbullying is a predominant concern, often linked to cultural patterns of group conformity and social reputation [29].

Ethnic and cultural harassment further illustrates how minority stress frameworks apply to bullying. In

Table 2
Reported bullying prevalence

Macro-area	Range of reported prevalence	Examples from reviewed studies
Europe [15]	25%	Sweden (6.3% girls), Lithuania (41.4% boys), Norway (8.3-12.5%)
North America [15, 17]	29.0-31.7%	About 19.5% of high school students reported being bullied on school property in the past 12 months, and 15.7% reported being cyberbullied during the same period
Central America [15]	22.8%	Sexual bullying is prominent. Psychological/relational bullying 7.5% as the highest regional median
South America [15, 16]	30-40%	Brazil 20% cyberbullying among 9-17 years of age internet users
Middle East [15, 18]	41.1-45.1%	Physical and sexual bullying are prominent
Asia [15]	7-40%	7% in Tajikistan is the global low; 29% in Philippines
North Africa [15]	42.7%	Physical and sexual bullying are prominent
Sub-Saharan Africa [15, 18]	48.2%	Highest reported regional physical-bullying prevalence. Less frequent psychological/relational bullying
Caribbean [15, 16]	30-40%	Limited data

multicultural school settings in the United States, discriminatory harassment based on race or immigrant status is explicitly recognized as bullying, with institutional mechanisms for redress. Yet minority and immigrant youth remain disproportionately vulnerable to bias-based bullying, which is consistently associated with more severe mental health and academic consequences than non-bias bullying, including depression, suicidal ideation, and school disengagement [30, 31].

These findings highlight how cultural, ethnic, and age-related differences shape not only prevalence estimates but also the reactions of victims, peers, and educators. They emphasize the importance of developing culturally responsive measurement tools and interventions that are sensitive to diverse populations.

Bullying in Italy

The 2023 National Institute of Statistics (Istituto Nazionale di Statistica, ISTAT) survey "children and youth: behaviors, attitudes, and future plans" involved over 39,000 young people aged 11 to 19 across Italy, focusing on bullying and cyberbullying. About 68.5% reported experiencing at least one offensive or violent episode, and 21% were victims of repeated bullying. The most common forms included verbal abuse, social exclusion, and online attacks, with a higher impact on minority groups such as foreign nationals, LGBTIQ+ youth, and neurodivergent individuals. Repeated bullying affected 20% of youth in Southern Italy, slightly less than in other regions where rates ranged from 21% to 22.1% [32, 33].

A study using data from 28 countries in Europe and North America in 1997-98 found that the proportion of students being bullied in Italy varied. The prevalence was lower than in some other countries, such as Lithuania, where 38.2% of girls and 41.4% of boys reported being bullied [34]. More recent data from the UNESCO report "Behind the numbers" indicates that Italy has seen a significant decrease in the prevalence of bullying. Italy was one of six countries that has seen a decrease in the prevalence of bullying, physical fights and physical attacks [15]. Italy has invested significantly in research and evaluation of anti-bullying interventions and programs. Evaluations of the school-based programs, "No Trap!" (beginning in 2008) and *kiusaamista vastaan* (KiVA), (beginning in 2013) have demonstrated their effectiveness in reducing school violence and bullying in Italian schools. Italy is also one of two countries that report teacher training on online safety and the prevention and reporting of cyberbullying [35-37].

Bullying remains a significant public health concern in Italy, despite ongoing efforts to curb its prevalence. Research has consistently demonstrated a direct association between bullying victimisation and the development of somatic complaints among Italian children, such as headaches, stomachaches, and sleep disturbances, indicating that the impact of bullying extends beyond psychological harm to physical well-being [38]. Moreover, cyberbullying has emerged as a parallel and equally concerning phenomenon. Italian studies have shown that cybervictimisation is significantly correlated with both psychological distress and physical symptoms, reinforcing the complex and multidimensional effects of online harassment [39]. Although national intervention programs and school-based strategies have contributed to some reductions in bullying rates, the persistence of both traditional and cyberbullying suggests that more comprehensive and sustained efforts are needed. These should include educational campaigns, psychological support services, and continuous monitoring, all of which must be tailored to address the evolving dynamics of youth aggression and its health-related consequences in the Italian context.

DEFINITION AND CLINICAL SIGNIFICANCE OF SOMATIC SYMPTOMS

Somatic symptoms refer to physical complaints, such as headaches, fatigue, dizziness, abdominal pain, and sleep disturbances, that are not fully explained by organic medical conditions. According to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5), somatic symptom disorder (SSD) involves the presence of these physical symptoms in conjunction with excessive thoughts, feelings, or behaviors related to health concerns, resulting in functional impairment [40]. The prevalence of recurrent somatic complaints in adolescents is considerable, ranging from 2% to 10%, with up to 50% of youths reporting isolated somatic symptoms [41]. These symptoms are associated with increased health service use, school absenteeism, and long-term risk of psychiatric comorbidities [42, 43].

BULLYING AS A LONGITUDINAL PREDICTOR OF SOMATIC SYMPTOMS

Emerging evidence identifies bullying as a significant psychosocial risk factor for the development of somatic symptoms. Bullying victimization is consistently associated with a higher prevalence of somatic complaints in adolescents.

Children and adolescents identified as bully-victims, victims, or bullies exhibited a significantly elevated risk of experiencing psychosomatic problems, including headaches, compared to their uninvolved peers [38, 44, 45].

Various forms of bullying victimization, including physical aggression, verbal abuse, and social exclusion, collectively accounted for approximately 6% of the variance in subjective well-being (SWB) among children, with variations observed across regions, age groups, gender, geographic settings, and socio-economic backgrounds. This proportion is likely to be higher among subgroups directly exposed to repeated victimization. More frequent bullying experiences were consistently associated with lower levels of SWB. Notably, while these forms of victimization had a significant negative impact, the persistence of relatively stable SWB levels suggests that homeostatic mechanisms may play a mediating role. Furthermore, diminished SWB has been linked to increased physical complaints, highlighting the potential psychosomatic pathway through which bullying affects children's health [46].

In a study by Espejo-Siles *et al.*, experiences of bullying victimization were significantly associated with increased somatic symptoms at the time of assessment and persisted one year later [47].

A prospective study by Fekkes *et al.* provided evidence that children exposed to bullying developed a greater number of somatic symptoms within a six-month period, supporting a unidirectional causal relationship in which bullying precedes and contributes to the onset of physical health complaints [48].

Hunter and colleagues found that both victims and perpetrators of bullying were nearly twice as likely to report sleep difficulties compared to uninvolved peers [49]. A meta-analysis by van Geel *et al.* involving over 360,000 children and adolescents, confirmed that peer victimization is linked to significantly more sleep problems, particularly among younger children [50].

The biological plausibility of bullying-induced somatic symptoms centers on chronic stress and its impact on neuroendocrine and immune function. Sustained exposure to psychosocial stressors like bullying is thought to dysregulate the hypothalamic-pituitary-adrenal (HPA) axis, resulting in elevated cortisol levels and inflammatory responses that may manifest as physical symptoms. A study found that bullied children had higher cortisol reactivity to stress compared to nonbullied peers, suggesting heightened HPA axis activity. Conversely, other studies have observed blunted cortisol responses in maltreated or bullied children, indicating potential HPA axis dysregulation [51, 52]. Chronic activation of the HPA axis can lead to sustained cortisol secretion, which, over time, may suppress immune function and increase inflammation. Elevated levels of pro-inflammatory cytokines, such as IL-1β and TNF-α, have been associated with stress-related conditions and can contribute to physical symptoms like fatigue, sleep disturbances, and pain [53-55].

Affective empathy, or the capacity to emotionally share others' experiences, has also been implicated. Espejo-Siles *et al.* found that higher levels of social and emotional competencies were associated with lower levels of somatic symptoms one year later. In contrast, elevated affective empathy was linked to greater somatic symptom reporting both concurrently and longitudinally [47]. This association may be mediated by poor emotional regulation, as supported by MacDonald and Price, who showed that college students with high affective empathy reported more internalizing symptoms. In contrast, cognitive empathy was not significantly linked to somatic symptoms, suggesting a differential role of empathy subtypes [56].

This aligns with prior findings where emotional intelligence and social skills were inversely related to various problem behaviors, including internalizing disorders and psychosomatic complaints [57, 58]. Supporting this, studies have shown that adolescents with alexithymia are more likely to report increased somatic symptoms [59]. Thus, enhancing emotional competence may serve both preventative and therapeutic roles in adolescent health.

PUTATIVE STRATEGIES AND FUTURE DIRECTIONS

Given the established link between bullying victimization and the emergence of somatic symptoms in youth, future clinical and research efforts must emphasize early identification, multidisciplinary intervention, and targeted prevention.

Clinicians should be trained to recognize somatic complaints, particularly recurrent symptoms such as headaches, fatigue, or abdominal pain, as potential red flags for underlying psychosocial distress, including peer victimization.

Routine use of structured psychosocial screening tools in pediatric and adolescent settings may aid in the timely detection of bullying and associated psychological sequelae. Psychotherapeutic approaches, especially cognitive-behavioral therapy (CBT), have shown promise in addressing somatization and underlying emotional dysregulation. Integrating emotional skills training, stress-reduction techniques, such as mindfulness-based interventions, and trauma-informed care into clinical practice could help mitigate both psychological and physiological manifestations of chronic stress in bullying victims. Family-centered approaches and school-based mental health services should be prioritized, particularly in moderate to severe cases, to address environmental contributors and promote systemic resilience.

From a public health standpoint, school-wide anti-bullying programs that incorporate peer mediation, social-emotional learning (SEL), and empathy development may reduce victimization rates and buffer against somatic symptom onset.

Tailored interventions may be needed for students with high affective empathy, who appear especially vulnerable to internalizing distress. Early identification and support for bullied students and those with emotional dysregulation could reduce long-term somatic and psychological sequelae.

CONCLUSIONS

The relationship between bullying victimization and somatic symptoms highlights the profound impact of psychosocial stress on both mental and physical health in adolescents. Bullying not only fosters emotional distress but also contributes to chronic physiological changes, which can manifest as physical complaints like headaches, fatigue, and sleep disturbances. These somatic symptoms may serve as critical, though not exclusive, indicators of underlying psychological distress, particularly for those who have experienced persistent peer victimization. They should be interpreted as part of a wider psychosocial assessment rather than as standalone markers. Clinicians should be vigilant in recognizing these symptoms, as they provide an opportunity for early intervention and support for both the psychological and physical well-being of bullied youth. Furthermore, the implementation of comprehensive school-based programs and trauma-informed care, coupled with family and community involvement, is essential for addressing the root causes of bullying and mitigating its harmful effects on adolescents' overall health. By fostering environments that promote emotional resilience, social-emotional learning, and peer support, we can reduce the prevalence of bullying and its associated health consequences, ultimately supporting healthier and more adaptive developmental trajectories for youth.

Authors' contributions

All Authors contributed equally to the conception, design, drafting, and critical revision of the manuscript. All Authors approved the final version and agree to be accountable for all aspects of the work.

Conflict of interest statement

The Authors declare that there are no conflicts of interest related to the conduct or findings of this study. No financial or personal relationships with other people or organizations have inappropriately influenced this work.

Artificial intelligence use statement

Artificial intelligence (AI) tools were used solely to assist with language editing and proofreading of this manuscript. No AI tools were used for data analysis, content generation, interpretation of results, or decision-making.

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Determinants of vaccine hesitancy and interventions aimed at contrasting this issue in Europe: an overview of systematic reviews

Giuseppina Lo Moro[1], Maria Ferrara[2], Elisa Langiano[2], Alessandro Durbano[1], Sonia Pinto[1], Alex Pivi[1], Caterina Grisafi Schittone[1], Elena Cerino[1], Carola Linot[1], Maurizio Esposito[2], Elisabetta De Vito[2] and Roberta Siliquini[1]

[1]*Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università di Torino, Turin, Italy*
[2]*Dipartimento di Scienze Umane, Sociali e della Salute, Università degli Studi di Cassino e del Lazio Meridionale, Cassino, Italy*

Abstract

Introduction. Vaccine hesitancy remains a major public health challenge in Europe, with declining confidence in vaccine safety and efficacy despite high overall acceptance. Evidence on its determinants and effective interventions is fragmented, underscoring the need for a comprehensive synthesis. This overview of systematic reviews aimed to describe the most recent determinants of vaccine acceptance, barriers to vaccination, and effective interventions to reduce hesitancy or increase uptake in Europe.

Methods. The search was conducted in PubMed, Embase, Epistemonikos. Reviews were eligible if they included European data.

Results. Of 3,363 papers, 88 reviews were included, classified into children, adolescents, and parents (n=29); pregnant women (n=11); healthcare workers (HCWs) (n=12); and the general population (n=36) reviews. Determinants of vaccine acceptance included high education, socioeconomic status, gender, trust in government/health authorities, and HCW recommendations, with associations that varied across populations, vaccines, and contexts. Emerging determinants such as health engagement and social media were reported. Main barriers included fear of side effects, safety concerns, preference for natural immunity, and logistical challenges. Interventions included educational campaigns, reminders, organizational changes, school-based programs, and digital tools. Active reminders, school-based programs, and organizational changes showed the most consistent effectiveness.

Conclusions. Vaccine hesitancy is multifaceted, requiring tailored, evidence-based strategies. Future research should prioritize rigorous study designs and underexamined factors.

Key words

- vaccination hesitancy
- Europe
- vaccine acceptance

INTRODUCTION

Vaccine hesitancy has been a recurring challenge since the advent of vaccinations [1]. Recognizing the global significance of this issue, the World Health Organization (WHO) identified vaccine hesitancy as one of the top ten threats to global health in 2019 [2]. In Europe, vaccine hesitancy has increased over the past two decades [3]. In particular, the most recent State of Vaccine Confidence in the European Union (EU) analyzed over 25,000 questionnaires across the 27 EU member states, highlighting fluctuations in public perceptions of vaccine importance, safety, and efficacy [4]. While overall confidence in vaccines remains high, perceptions of their safety and efficacy have diminished in most countries post-2020 [4]. As vaccination remains a cornerstone of public health, addressing vaccine hesitancy is essential to achieving high coverage rates, ensuring community protection, and preventing the resurgence of vaccine-preventable diseases. Despite the growing body of literature on vaccine hesitancy, evidence in the European context remains fragmented across different vaccines, population groups, and analytical approaches. While numerous primary studies and systematic reviews have explored specific determinants, barriers, or interventions, a comprehensive synthesis integrating these dimensions across populations and settings is still lacking.

Address for correspondence: Giuseppina Lo Moro, Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università di Torino, Via Santena 5 bis, 10126 Turin, Italy. E-mail: giuseppina.lomoro@unito.it.

Within this context, the Addressing Vaccine Hesitancy in Europe (VAX-TRUST) study was funded by the European Union's Horizon 2020 research and innovation programme and was initiated in 2021. VAX-TRUST investigated vaccine hesitancy as a complex transnational, yet region- and context-specific phenomenon in Europe. With a specific focus on childhood vaccines, the study particularly emphasized interactions between healthcare workers (HCWs) and parents during vaccination visits, identifying these encounters as critical moments influencing vaccine hesitancy [5]. One of VAX-TRUST's objectives was to support HCWs in addressing hesitancy through tailored interventions [6]. To inform these interventions, a systematic review described existing strategies and interventions for HCWs [7], while complementary research, including the present work, focused on mapping the key determinants of vaccine hesitancy in Europe and identifying the strategies used to address it. Specifically, it intended to explore the factors influencing vaccine hesitancy, highlighting the differences in these determinants across various population groups that may attend vaccination centers, to better guide both research and patient interactions. Therefore, this overview of systematic reviews aimed to provide a comprehensive description of the most recent determinants of vaccine acceptance, barriers that hinder vaccinations, and potential effective interventions to reduce vaccine hesitancy or increase vaccine uptake in Europe.

MATERIALS AND METHODS

A systematic search was conducted across multiple databases, including PubMed, Embase, and Epistemonikos, to retrieve the most recent systematic reviews on the topic. The search was performed on March 22, 2022, and included only studies published from 2017 onward. The search strategy was structured using the population intervention comparison outcome (PICO) framework. The population included the European population, with a particular focus on the general population, pregnant women, parents, children and adolescents, as well as HCWs and healthcare students. The interventions/exposures analyzed were determinants of vaccine acceptance, barriers hindering vaccinations, and interventions designed to reduce hesitancy or increase vaccine uptake. Reviews that included studies with any control groups, as well as those without control groups, and any outcomes related to vaccine hesitancy and vaccine uptake were considered eligible. The search terms are available in the *Supplementary material available online* ("*List of search terms*"). The protocol was registered on the International prospective register of systematic reviews (PROSPERO) (CRD42022379111). This article is a revised and expanded version of a conference abstract presented at the 17th World Congress on Public Health (Rome, Italy, 2023) [8].

Eligible reviews had to meet the following criteria: systematic review or meta-analysis with the primary aim that includes exploring determinants of vaccine hesitancy and/or interventions aimed at reducing vaccine hesitancy or increasing vaccine uptake; published in peer-reviewed journals; focused on European populations. Studies were excluded if they: examined only non-European populations; were preprints; focused solely on specific subpopulations (e.g., individuals with diseases, minorities); addressed only vaccine efficacy, side effects, or vaccination coverage without discussing determinants, barriers, or interventions; analyzed pipeline vaccines, timely administration of the hepatitis B (HBV) vaccine birth dose, or were scoping reviews, narrative reviews, overviews, primary studies, editorials, letters, or conference papers. Exclusively qualitative systematic reviews were excluded because this overview aimed to compare determinants, barriers, and intervention types based on evidence allowing assessment of their recurrence and consistency across studies and populations.

The authors independently screened the titles and abstracts using the web application Rayyan [9]. Each article was evaluated by at least two authors. As training, 5% of the articles were independently screened by all authors, and disagreements were resolved through consensus before proceeding with the remaining papers. Then, full texts of potentially eligible studies were assessed against the inclusion and exclusion criteria by at least two authors, with decisions tracked in Excel. Reasons for exclusion were documented. Throughout the process, reviewers were blinded to each other's decisions, and disagreements were resolved through consensus.

Data were extracted into pre-designed Excel spreadsheets. The extracted information included: author and year of publication; vaccinations studied; target populations; search dates and databases; number, design, and years of included studies; continents and participants of included studies; determinants of vaccine acceptance and barriers hindering vaccinations in Europe; interventions targeting vaccine hesitancy or increasing vaccine uptake in Europe (with data on the results of interventions, if available). For each systematic review, at least one author extracted the data, and another author verified it. Discrepancies were resolved by discussion. When systematic reviews provided Europe-specific analyses or summaries, these were directly extracted and synthesized. When Europe-specific results were not reported, all primary studies included in the review were screened to identify those conducted in European countries, based on study setting and population. Data and conclusions from these European primary studies were then extracted and synthesized to generate a Europe-focused summary.

The risk of bias of the included systematic reviews was assessed using the "A MeaSurement Tool to Assess systematic Reviews 2" (AMSTAR 2) [10]. At least one author conducted the assessment, and another author verified it. Any disagreements were addressed through consensus-based discussions.

The narrative synthesis was organized by macro-categories of populations, summarizing how frequently data on determinants of vaccine acceptance, barriers hindering vaccinations, and interventions to address vaccine hesitancy or increase vaccine uptake were cited in the systematic reviews. For determinants and barriers, the synthesis followed the structure of the Vaccine Hesitancy Determinants Matrix, which categorizes fac-

tors influencing the decision to accept, delay, or reject vaccines into three domains: contextual influences, individual and group influences, and vaccine/vaccination-specific influences [11]. For interventions, the information on efficacy/effectiveness was summarized as "significant" if the systematic review reported significant improvements in any outcome, and "non-significant/conflicting" if no significant results were reported or if the studies included in the review showed mixed or conflicting findings.

RESULTS

Selection process

Out of 3,363 papers, 88 were selected. *Figure S1* (*Supplementary material available online*) shows the PRISMA flow diagram [12]. The included systematic reviews were classified, according to available European data, as primarily concerning children, adolescents, and parents (CAP) (n=29, 33% of the reviews); pregnant women (PW) (n=11, 12.5%), HCWs (n=12, 13.6%), and general population (GP) (n=36, 40.9%).

Characteristics of the systematic reviews

Included reviews were published between 2017 and 2022 considering all the subpopulations, except for PW (2018-2022). The year with the highest number of published articles was 2020 for CAP (n=8, 27.6% of CAP reviews) and 2021 for all the other categories (PW: n=5, 45.5%; HCWs: n=5, 41.7%; GP: n=12, 33.3%).

Search dates had no lower date limit in 24.1% of CAP reviews (n=7), 27.3% of PW reviews (n=3), 100% of HCWs reviews (n=12), and 16.7% of GP reviews (n=6). The most used lower limit in CAP reviews was 2006 (n=4, 13.8%), mostly related to the human papillomavirus (HPV) vaccination introduction. Considering the other populations, the most frequent range was around 2020-2021, due to the COVID-19 pandemic focus (PW: n=3, 27.3%; HCWs: n=3, 25.0%; GP: n=9, 25.0%).

Overall, the reviews used an average of 5 databases to conduct the search (databases mean: CAP 6; PW 4; HCWs 5; GP 5). The most used databases were: PubMed/MEDLINE (n=28, 96.6%), Embase (n=15, 51.7%), and Scopus (n=14, 48.3%) for CAP; PubMed/MEDLINE (n=11, 100.0%), Embase (n=5, 45.5%), and Web of Science (n=5, 54.5%) for PW; PubMed/MEDLINE (n=12, 100.0%), Embase (n=8, 66.7%), Web of Science (n=5, 50.0%) and CINAHL (n=5, 50.0%) for HCWs; PubMed/MEDLINE (n=36, 100.0%), CINAHL (n=16, 44.4%), and Embase (n=15, 41.7%) for GP.

The most frequent target populations of the CAP reviews were adolescents and young adults (n=10, 34.5%), parents of children (n=9, 31.0%), parents of children and adolescents (n=8, 27.6%). All PW reviews were on pregnant women. Few HCW reviews focused on a specific subgroup: 1 on midwives (8.3%), 1 on nurses (8.3%), and 1 on obstetrics/gynecologists (8.3%). Similarly, few GP reviews focused on some subgroups: 7 on elderly individuals or risk groups that included elderly (19.4%), 1 on women (2.8%), and 1 on university students (2.8%).

The most frequent target vaccinations of the CAP reviews were HPV (n=13, 44.8%), pediatric vaccines with no specific restriction (n=8, 27.6%), vaccines in adolescents (n=2, 10.3%), and COVID-19 (n=2, 10.3%). Considering PW reviews, the most frequent vaccinations were against COVID-19 (n=4, 36.4%) and flu (n=3, 27.3%). In HCW reviews, the most frequent vaccinations were against COVID-19 (n=3, 25.0%) and vaccinations with no specific restriction (n=3, 25.0%). Lastly, in GP reviews, the most frequent vaccinations were vaccines with no restrictions (n=13, 36.1%), COVID-19 (n=11, 30.6%), and flu (n=7, 19.4%).

Included studies within the reviews ranged between 6 and 103 for CAP (mean: 30 studies), 9 and 120 for PW (mean: 28), 6 and 96 for HCWs (mean: 27), and 5 and 470 for GP (mean: 45). Among these, studies conducted only in Europe per review ranged between 1 and 103 for CAP (mean: 11), 1 and 25 for PW (mean: 6), 1 and 82 for HCWs (mean: 14); 1 and 176 for GP (mean: 13). Only 5 reviews were exclusively based on European studies (3 CAP; 1 PW; 1 HCWs; 0 GP). Overall, the continent with the highest number of papers was America, with the following mean number of papers: 15 (CAP), 13 (PW), 10 (HCWs), and 19 (GP).

Details about the abovementioned characteristics are presented in the *Supplementary material available online* (*Table S1*).

European data

Considering the included European studies, most reviews (n=52) were focused only on determinants and/or barriers (CAP: n=14, 48.3%; PW: n=9, 81.8%, HCW: n=8, 66.7%; GP: n=21, 58.3%). Fewer reviews (n=27) were mostly focused on interventions (CAP: n=12, 41.4%; PW: n=2, 18.2%, HCW: n=2, 16.7%; GP: n=11, 30.6%). In some cases (n=9), the reviews included both papers on determinants/barriers and interventions due to their main aims (CAP: n=3, 10.3%; HCW: n=2, 16.7%; GP: n=4, 11.1%).

Studies on determinants/barriers included in the reviews were all observational studies (including quantitative, qualitative, and mixed methods designs). Considering the 27 reviews with European studies only on interventions, the percentage of randomized controlled trial (RCT) varied from 0% (PW: 0/3 primary articles), to 33.3% (CAP: 13/39 primary articles), 64.3% (GP: 27/42 primary articles), and 100.0% (HCW: 10/10 primary articles).

The publication year of the European primary papers included in the reviews ranged between 1980 and 2021 (CAP: 1980-2021; PW: 2010-2021; HCW: 1998-2021; GP: 1997-2022). The sample size ranged between 5 to 8,020,000 individuals (CAP: 5-1,204,588; PW: 14-247,316; HCW: 10-50,351; GP: 20-8,020,000) (*Supplementary material available online*, *Table S1*).

Determinants of vaccine acceptance

Figure 1 shows the determinants of vaccine acceptance that have been most frequently reported across multiple identified macro-categories. Considering contextual influences, the most frequently mentioned determinants of vaccine acceptance were higher educational attain-

	CAP review (n=29)	PW review (n=11)	HCW review (n=12)	GP review (n=36)
	Type of vaccine (n)	Type of vaccine (n)	Type of vaccine (n)	Type of vaccine (n)
CONTEXTUAL INFLUENCES				
Mothers'/Parents' higher education	HPV (3) Pediatric vaccines (2)	COVID-19 (4)	–	–
Higher socio-economic situation	HPV (2) Vaccines (1)	–	–	COVID-19 (4) Flu (2) HPV (1) Meningoc. (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1) Vaccines (1)
Higher educational level	–	–	COVID-19 (1)*	COVID-19 (4) HPV (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1) Vaccines (1)
Male gender	–	–	COVID-19 (1)*	COVID-19 (6) Flu (2) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Being in favor of mandatory vaccinations	–	Pediatric vaccines (1)	Vaccines (1)*	–
INDIVIDUAL AND GROUP INFLUENCES				
Personal vaccination history (i.e., the participant or the parent has already been vaccinated for other vaccinations)	HPV (2)	Pregnancy vaccines (1) COVID-19 (4) Flu (1)	COVID-19 (3)* Flu (1)** Vaccines (1)**	COVID-19 (2) Flu (2) HPV (1) Vaccines (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Perceiving VPDs as severe	–	Pregnancy vaccines (1)	COVID-19 (1)* Vaccines (1)*	COVID-19 (6) Flu (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Awareness/knowledge regarding the specific vaccine or disease of focus	HPV (1) Vaccines (1)	Pregnancy vaccines (1)	Flu (1)* Flu (1)** Vaccine s (1)**	Flu (1) Meningoc. (1) Vaccines (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Trust in government policies and health authorities	COVID-19 (1) HPV (1)	–	Vaccines (1)**	COVID-19 (4) Flu (1) Vaccines (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Perceiving vaccines as important/ useful	COVID-19 (1) HPV (2) Pediatric vaccines (2)	Pregnancy vaccines (1) Pediatric vaccines (1)	Vaccines (1)**	COVID-19 (2) Flu (1) Meningoc. (1)
Perceiving the risk of contracting VPDs	–	–	COVID-19 (1)* Vaccines (1)* Flu (1)**	COVID-19 (2) Flu (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Having a chronic disease	–	Flu (1)	–	COVID-19 3) Flu (3) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Having received advice to get vaccinated (by other figures than physicians)	HPV (1)	–	–	Flu (1)
Older age	–	–	COVID-19 (1)* Vaccines (1)**	COVID-19 (6) HPV (1) Meningoc. (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Younger age	–	–	HBV, measles, rubella, varicella, and flu (1)*	COVID-19 (1) Meningoc. (1)
VACCINE – AND VACCINATION – SPECIFIC ISSUES				
Healthcare professional recommendation	HPV (2) Pediatric vaccines (1)	Pregnancy vaccines (1)	–	COVID-19 (1) Flu (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)

50%	40-49%	30-39%	20-29%	10-19%	1-9%	n.m.

Figure 1
Determinants of vaccine acceptance.

The figure shows the number of reviews that mention a determinant per type of vaccine. If the type is "vaccines", it means the review refers to vaccines without any restrictions. The color of the cells represents the percentage of reviews that mention a determinant relative to the total number of reviews in each column category (i.e., CAP: children, adolescents, and parents; GP: general population; HCWs: healthcare workers; PW: pregnant women). The darker the color, the higher the percentage of reviews that mention the determinant.
HPV: human papillomavirus; VPDs: vaccine-preventable diseases; Meningoc.: meningococcal disease; MERS: Middle East Respiratory Syndrome; n.m.: not mentioned; SARS: Severe Acute Respiratory Syndrome; *HCW: considered as vaccine (review: n=8); **HCW: considered as providers (review: n=4).

ment (of the parents or of the individual) and higher socioeconomic status, which represent structural determinants, as well as male gender, whose association with vaccine acceptance likely reflects context-specific social and cultural factors. A positive attitude towards mandatory vaccination policies was also mentioned both for pregnant women and HCWs.

Other contextual determinants were mentioned less frequently. Indeed, CAP reviews also mentioned being exposed to media and social media that promote vaccination (1 review on HPV) and having multiple options to access the vaccination (1 review on vaccines in general). Considering GP reviews, feeling close to radical parties or not feeling close to any party were also reported (1 review on COVID-19), as well as living in an urbanized area (1 review on vaccines in general) and being white (4 reviews on COVID-19, 2 on flu, and 1 on Severe Acute Respiratory Syndrome, Influenza A/H1N1, Middle East Respiratory Syndrome, Ebola Virus Disease, and COVID-19). Two GP reviews on COVID-19 and meningococcal vaccinations reported female gender as a positive determinant, in contrast with a higher number of abovementioned studies.

With regard to individual and group influences, the most frequently cited determinants across most macro-categories (HCW, GP and at least one between CAP and PW) were history of vaccination, perceiving vaccine-preventable diseases (VPDs) as severe, knowledge on VPDs or vaccines, trust in government and health authorities, perceiving vaccines as important and/or useful. For HCW and GP reviews, the perceived risk of contracting VPDs and age (with conflicting data) represented determinants that appeared in multiple reviews. Having received advice to get vaccinated (by other figures than physicians) and having a chronic disease were cited for GP and CAP/PW (*Figure 1*).

As for determinants specific for one macro-category, CAP reviews mentioned the perception of HPV vaccine benefits by parents (2 reviews on HPV), declaring willingness to protect own child (1 review on HPV and 1 on vaccines in general), parental perception about the importance of future partner protection (1 review on HPV), sexual habit (1 review on HPV), peer encouragement (1 review on HPV and 1 on vaccines in general), influence of grandparents (1 review on HPV), and child's older age (1 review on HPV). PW reviews highlighted in 4 reviews on COVID-19: maternal age higher than 30 years, proximity of childbirth/being in the third trimester, living with someone older than 65 years, having had COVID-19. The perception of responsibility and being physician or midwife were reported for both HCWs as vaccinees (1 review on vaccines in general and 2 reviews on COVID-19, respectively) and HCWs as providers (2 reviews on vaccines in general); while less years of work experience (1 review on COVID-19 and 1 review on HBV, measles, rubella, varicella and influenza) and working in intensive care units (1 review on HBV, measles, rubella, varicella and influenza) were reported for HCW as vaccinees. GP reviews mentioned health engagement (1 review on COVID-19) and having given up smoking (1 review on flu), perceiving vaccines as effective (4 reviews on COVID-19 and 1 on flu), considering side effects less risky than VPD (1 review on COVID-19 and 1 on flu), social norms/social pressure (2 reviews on COVID-19, 2 on flu, 1 on meningococcus, 1 on Severe Acute Respiratory Syndrome, Influenza A/H1N1, Middle East Respiratory Syndrome, Ebola Virus Disease, and COVID-19), perceived poor health (2 reviews on flu), being married (1 review on COVID-19 and 1 on flu), being a HCW (3 reviews on COVID-19), living with other people/having a large household (1 review on flu and 1 on vaccines in general).

Last, considering vaccine and vaccination-specific issue, the recommendation by HCWs was frequently mentioned for all categories (except HCW reviews) (*Figure 1*). CAP reviews specifically reported health insurance coverage (2 reviews on HPV) and PW reviews the presence of an obstetrician following the pregnancy (4 reviews on COVID-19). HCW reviews that considered HCW as providers reported 1 review on vaccine in general that described as determinant: general positive attitude towards vaccination, not having false beliefs about vaccines (reported also in another review on flu), having seen VPDs, patients' clinical status, willingness to vaccinate own child, routine recommendation of vaccinations, working alone, and being ready to discuss sexuality with patients for the HPV vaccine. For HCW as vaccinee, 1 review on HBV, measles, rubella, varicella and influenza reported receiving the proposal by an occupational health physician and a general practitioner or from the vaccination service as determinant.

The *Supplementary material available online* (*Table S1* and *Table S2*) shows further details considering each review.

Barriers hindering vaccinations

Figure 2 shows the barriers hindering vaccinations that have been most frequently reported across multiple identified macro-categories. Most of such barriers were the opposite of abovementioned determinants of vaccine acceptance, i.e., belonging to an ethnic minority, perceiving VPDs as not severe, lack of knowledge, distrust of information received by government and health authorities, and perceiving vaccines as not important/useful. Similarly, the role of HCWs was also highlighted by barriers such as lack of recommendation by HCWs (*Figure 2*) and receiving recommendations against the vaccination (also by healthcare professionals) (3 CAP reviews, 2 on HPV and 1 on pediatric vaccines). Although they were reported in only one macro-category, other variables that were quite the opposite of the aforementioned determinants were noted as barriers: having a low education level (4 PW reviews, 3 on COVID-19 and 1 on flu) or parents' low education level (4 CAP reviews, 1 on COVID-19, 2 on HPV and 1 on pediatric vaccines), female gender (6 GP reviews, 4 on COVID-19 and 2 on flu), socioeconomic barriers such as low income and living in poor areas (9 GP reviews, 5 on COVID-19, 2 on flu, 1 on Severe Acute Respiratory Syndrome, Influenza A/H1N1, Middle East Respiratory Syndrome, Ebola Virus Disease, and COVID-19, and 1 on vaccines in general), being against mandatory vaccination policies (2 CAP reviews on COVID-19) and

	CAP review (n=29)	PW review (n=11)	HCW review (n=12)	GP review (n=36)
	Type of vaccine (n)	Type of vaccine (n)	Type of vaccine (n)	Type of vaccine (n)
CONTEXTUAL INFLUENCES				
Religious beliefs	HPV (2) MMR (1)	–	–	COVID-19 (1)
Being immigrant/ethnic minority	HPV (1) Adolescent vaccines (1)	–	–	COVID-19 (5) Flu (1)
INDIVIDUAL AND GROUP INFLUENCES				
Perceiving VPDs as not severe/poor risk perception	HPV (1)	Flu (1)	Flu (1)**	COVID-19 (2) Meningoc. (1) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1) Vaccines (1)
Previous negative experience with vaccinations	HPV (1)	–	–	Flu (1)
Lack of knowledge or lack of information	HPV (6) MMR (1) Pediatric vaccines (2) Adolescent vaccines (1)	Flu (1) Pediatric vaccines (1)	Flu (1)** Measles, mumps, and rubella (1)** Pediatric vaccines (1)** Vaccines (1)**	COVID-19 (5) Flu (2) Meningoc.(1)SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Distrust of information received by government and health authorities	HPV (1) MMR (1)	Pediatric vaccines (1)	Vaccines (1)*	COVID-19 (5) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1)
Fear of side effects	HPV (5) MMR (1) Pediatric vaccines (2) Adolescent vaccines (1)	Flu (1) Pediatric vaccines (1)	–	–
Perceiving vaccines as not important/useful	COVID-19 (1) HPV (4) Pediatric vaccines (2) Vaccines (1)	Flu (1)	–	–
Perceiving vaccines as unsafe/harmful	COVID-19 (1) HPV (4) Pediatric vaccines (2) Vaccines (1)	Pregnancy vaccines (1) Flu (1)	COVID-19 (2)* Vaccines (1)* Flu (1)** Measles, mumps, and rubella (1)** Vaccines (1)**	–
Thinking that children are too young/preferring to wait when children are older	HPV (2) MMR (1)	Pediatric vaccines (1)	–	–
Preference for natural immunity	MMR (1)	Flu (1) Pediatric vaccines (1)	–	–
Perceiving vaccines as not effective	HPV (1)	–	COVID-19 (2)* Vaccines (1)* Flu (1)** Measles, mumps, and rubella (1)** Vaccines (1)**	COVID-19 (6) Flu (2) SARS, Flu A/H1N1, MERS, Ebola, and COVID-19 (1) Vaccines (1)
Too busy schedule	HPV (1)	–	–	Meningoc. (1)
Unemployment	–	COVID-19 (3)	–	COVID-19 (1)
VACCINE – AND VACCINATION – SPECIFIC ISSUES				
Physical barriers to vaccination (e.g., economic reasons such as costs; access to clinic etc)	HPV (5) COVID-19 (1) Adolescent vaccines (1)	–	–	COVID-19 (1) Flu (1) Meningoc. (1) Vaccines (1)
Lack of recommendation by healthcare providers	–	Flu (1)	–	Flu (1)

50%	40-49%	30-39%	20-29%	10-19%	1-9%	n.m.

Figure 2
Barriers hindering vaccinations.

The figure shows the number of reviews that mention a barrier per type of vaccine. If the type is "vaccines", it means the review refers to vaccines without any restrictions. The color of the cells represents the percentage of reviews that mention a barrier relative to the total number of reviews in each column category (i.e., CAP: children, adolescents, and parents; GP: general population; HCWs: healthcare workers; PW: pregnant women). The darker the color, the higher the percentage of reviews that mention the barrier.
HPV: human papillomavirus; VPDs: vaccine-preventable diseases; Meningoc.: meningococcal disease; MERS: Middle East Respiratory Syndrome; n.m.: not mentioned; SARS: Severe Acute Respiratory Syndrome; *HCW: considered as vaccine (review: n=8); **HCW: considered as providers (review: n=4).

general negative attitude towards vaccinations (1 PW review on flu), not living in an urbanized area (2 CAP reviews, 1 on HPV and 1 on vaccines in adolescents), parents' age less than 30 years (1 CAP review on COVID-19), being not married (2 GP reviews, 1 on flu and 1 on vaccines in general), living alone (2 CAP reviews on flu), unhealthy behaviors such as alcohol consumption (1 CAP review on flu), perceived good health (4 GP reviews, 1 on COVID-19, 2 on flu and 1 on Severe Acute Respiratory Syndrome, Influenza A/H1N1, Middle East Respiratory Syndrome, Ebola Virus Disease, and COVID-19). Instead, some variables described as determinants in some reviews were reported as barriers in other: working as HCW (3 PW reviews on COVID-19) complications from other diseases or poor health (1 GP review on COVID-19), and age, both young age (4 GP reviews, 3 on COVID-19 and 1 on flu) and older age (1 HCW review on vaccines in general, with HCWs as providers).

However, additional variables were reported.

As for contextual influences, religious beliefs were reported both in CAP and GP reviews (*Figure 2*). Moreover, relying on information found in the web/social media (3 reviews on COVID-19 and 1 review on measles, mumps, and rubella, MMR) and disagreement between experts on the safety of vaccines (1 review on pediatric vaccines) were described in CAP reviews. Not having a regular source of care was reported in 1 GP review on flu.

Considering individual and group influences, previous negative experience with vaccinations, fear of side effects, perceiving vaccines as unsafe/harmful, thinking that children are too young/preferring to wait when children are older, preference for natural immunity, perceiving vaccines as not effective, too busy schedule, and unemployment were reported across multiple macro-categories (*Figure 2*). In addition, other variables were reported in only one macro-area: having never experienced VPD (2 GP reviews on flu), false beliefs about vaccines (6 GP reviews, 4 on COVID-19 and 2 on flu), parental concerns about children sexual activity (1 CAP review on HPV), fear of harming the fetus (2 PW reviews, 1 on pregnancy vaccinations and 1 on flu), perceiving that vaccines are too many (1 CAP review on HPV), anxiety status (3 PW reviews on COVID-19), having had previous pregnancies (4 PW reviews, 2 on COVID-19 and 2 on flu) and being pregnant (3 PW reviews on COVID-19), and living with children (1 GP review on vaccines in general). Regarding HCW as providers 1 review on vaccines in general highlighted practicing alternative medicine as a barrier. When considering HCWs as vaccinees, another general review reported the perception that vaccination should be an autonomous decision.

Lastly, physical barriers to vaccination (e.g., costs) were considered as additional variables related to vaccine and vaccination-specific issue reported both in CAP and GP reviews (*Figure 2*). Focusing on variables presented only in one macro-area, there were: fear of injection (2 CAP reviews on HPV) and administrative errors (1 CAP review on pediatric vaccines). Specific barriers were related to HCW as providers: being uncomfortable administering more injections during the same consultation (1 review on pediatric vaccines), lack of guidelines/clear official recommendations (3 reviews: 1 pediatric vaccines, 1 vaccines in general, 1 flu; 1 review on flu also considering HCW as vaccinees), lack of training (3 reviews: 1 flu, 1 pediatric vaccines, and 1 vaccines in general), having a role less involved in vaccination (1 review on pediatric vaccines), and workload and organizational issues (3 reviews: 1 pediatric vaccines, 1 vaccines in general, 1 flu).

The *Supplementary material available online* (*Table S1* and *Table S2*) shows further details considering each review.

Interventions

A total of 15 CAP reviews considered interventions aimed at improving vaccinations intentions or rates. Most of them were on HPV (n=7) or about pediatric or adolescents' vaccines in general (n=6). Only one review specifically focused on pertussis and one on MMR. Considering PW, 2 reviews reported interventions, one on pertussis and one on flu and pertussis. As for GP reviews (n=15), most works were about vaccines with no particular restrictions (n=10), 3 on flu, 1 on COVID-19, and 1 on HPV. Last, 4 HCW reviews containing interventions were about HBV, measles, rubella, varicella and flu (n=1), flu (n=1), respiratory diseases (n=1), and vaccines in general (n=1).

Seven areas of interventions have been identified. *Figure 3* shows the interventions that have been most frequently reported across multiple macro-categories.

The most discussed intervention across all reviews involved the provision of educational materials and campaigns targeting populations (17 reviews). The distribution of printed materials, such as leaflets, and social media educational campaigns were the most frequently mentioned interventions, with conflicting results for both (*Figure 3*). Additionally, one CAP review on MMR reported positive results for in-person educational campaigns, while a CAP review on HPV highlighted the effectiveness of television and magazine communication campaigns. One GP review (vaccines in general) showed non-significant results for mailed educational programs.

A total of 12 reviews mentioned interventions based on active invitations and reminders for the population. Reminders via phone, text, or email were the most cited across all macro-categories and consistently yielded significant results (*Figure 3*). Similarly, nurses' calls to parents showed positive results in a CAP review on adolescent vaccines. Letters and postcards were effective in 4 GP reviews (2 on vaccines in general, 2 on flu), while reminder postcards for motivating the elderly did not show significant results (1 GP review on vaccines in general). Active invitations with significant results included invitation letters in one CAP review on HPV and telephone appointments in 2 GP reviews (1 on flu and 1 on vaccines in general).

Twelve reviews discussed organizational changes. The most frequently mentioned interventions across macro-categories were increased involvement of general practitioners (e.g., in patient management or com-

Intervention	CAP review (n=15)	PW review (n=2)	HCW review (n=4)	GP review (n=15)
	Type of vaccine (n)	Type of vaccine (n)	Type of vaccine (n)	Type of vaccine (n)
Financial incentives for general practice	Pediatric vaccines (1)+	Flu/pertussis (1)-	–	–
Printed materials (e.g., leaflets)	HPV (1)+ Pediatric vaccines (1)-	Flu/pertussis (1)-	–	Vaccines (7)+ COVID-19 (1)+ Flu (1)+
Social media education campaign	Pediatric and adolescent vaccines (1)+ HPV (2)+-	Flu/pertussis (1)-	–	Vaccines (1)+
Reminder call/text/email for the population	MMR (1)+ Pediatric and adolescent vaccines (1)+ Pediatric vaccines (1)+ HPV (1)+	–	–	Flu (4)+
GP patients management/GP communications	MMR (1)+	Flu/pertussis (1)-	–	Vaccines (1)+
Community pharmacy programme	–	Flu/pertussis (1)-	–	Flu (1)-
Opt-out approach	–	–	Vaccines (1)- Respiratory diseases (1)-	Vaccines (1)-
Communication tools and engagement of HCWs	–	Pertussis (1)+	.	Vaccines (1)+
Training sessions and materials for HCW	–	–	Respiratory diseases (1)+	Flu (1)+
Reminders and feedback for HCW (e.g., periodic messages and coverage updates)	Pediatric vaccines (1)+	–	Respiratory diseases (1)+	–

50%	40-49%	30-39%	20-29%	10-19%	1-9%	n.m.

Figure 3
Interventions to reduce vaccine hesitancy and/or increase vaccine uptake.

The figure shows the number of reviews that mention an intervention per type of vaccine. If the type is "vaccines", it means the review refers to vaccines without any restrictions. The color of the cells represents the percentage of reviews that mention an intervention relative to the total number of reviews in each column category (i.e., CAP: children, adolescents, and parents; GP: general population; HCWs: healthcare workers; PW: pregnant women). The darker the color, the higher the percentage of reviews that mention the intervention. When a plus sign (+) is present, it indicates a significant improvement in outcomes according to the review; a minus sign (-) means no significant results or conflicting findings.
HPV: human papillomavirus; MMR: measles, mumps, and rubella; n.m.: not mentioned.

munication), community pharmacy programs, and opt-out approaches (*Figure 3*). However, except for general practitioner involvement, most interventions did not show significant improvements. Other reviews reported effective interventions that included promoting and administering vaccines for newborn parents in maternity wards (1 CAP review on pertussis), midwives providing vaccines (1 PW review on flu and pertussis), offering free vaccines at the workplace (1 HCW review on flu), providing free vaccinations (3 GP reviews on vaccines in general and flu), and facilitating easier access to vaccinations (1 GP review on HPV).

Seven reviews (only CAP reviews) mentioned school-based interventions, primarily focusing on school-based educational programs (7 reviews, with 4 on HPV and 3 on pediatric and adolescent vaccines). Additionally, 2 reviews (1 on HPV and 1 on pediatric vaccines) involved school nurses, and 1 review on pediatric vaccines described vaccine administration in schools. All these interventions were reported as effective.

Six reviews addressed digital interventions. Three CAP reviews showed positive results with computer- and web-based tailored interventions (2 on HPV) and mobile apps for vaccination promotion and reminders (1 on pediatric vaccines). However, three other reviews reported non-significant results for websites using storytelling (1 CAP review on pediatric vaccines), web-based interventions with virtual assistants (1 GP review on vaccines in general), and digital game-based interactive simulations (1 GP review on vaccines in general).

Six reviews reported interventions specific for HCWs. Communication tools and engagement of HCWs, training sessions and materials for HCWs, and reminder and feedback (e.g., periodic messages and coverage updates) for HCWs were mentioned across multiple macro-categories (*Figure 3*), all showing significant positive results. Programs that involved the training of leaders (2 HCW reviews, 1 on flu and 1 on respiratory diseases) and the implementation of policies emphasizing staff vaccinations (1 HCW review on flu) reported as well good results. One HCW review on respiratory disease vaccines revealed that reminders based on social norms were not effective. Additionally, 1 HCW review reported successful multifaceted interventions based on education, active promotion, and easy access to influenza vaccination.

Five reviews described financial incentives. Financial incentives for general practice were reported across multiple macro-categories (*Figure 3*), showing conflicting results. Financial incentives for the population were addressed in three CAP reviews: 2 reviews (1 on HPV

and 1 on adolescent vaccines) showed positive results and 1 review on HPV reported non-significant results.

The *Supplementary material available online* (*Table S1* and *Table S2*) shows further details considering each review.

Risk of bias

Details of the AMSTAR 2 evaluation for each review are reported in *Supplementary material available online* (*Table S3*). Overall, the most frequent issues were common across the reviews with the different targets populations: lack of a clear and complete explanation about the reasons of the selection of study designs (CAP: n=26; PW: n=5; HCW: n=11; GP: n=34); lack of a complete list of excluded studies with reasons (CAP: n=26; PW: n=10; HCW: n=10; GP: n=30); lack of reporting about funding of the included studies (CAP: n=21; PW: n=9; HCW: n=11; GP: n=23); lack of an interpretation of risk of bias assessment in the discussion (CAP: n=21; PW: n=7; HCW: n=8; GP: n=27).

DISCUSSION

The aim of this overview was to describe potential determinants of vaccine hesitancy that should be considered in both research and clinical practice in Europe and explore potential interventions.

First, the distribution of reviews showed that CAP and GP received the most attention, likely because they are the largest population groups. Their importance in public health vaccination strategies also makes them a key focus. The publication peak in 2020-2021 reflected the possible dual impact of the COVID-19 pandemic: it increased research specifically on COVID-19 vaccines while also intensifying the broader public debate around vaccinations [13]. Among specific vaccines, those against HPV, COVID-19, and influenza received the most attention. This emphasis highlighted their significance in public health priorities. While COVID-19 naturally gained substantial attention due to the timing of the overview, the focus on HPV and influenza vaccines may stem from the fact that their coverage rates in Europe [14] remain well below the established targets [15, 16], necessitating an in-depth understanding of underlying reasons and possible strategies.

While observational studies dominated research on determinants and barriers, the limited inclusion of RCTs in intervention-focused reviews highlighted a significant evidence gap, restricting the possibility to plan effective strategies to enhance vaccine uptake. The HCW category exhibited the highest percentage of RCTs, likely due to the relative ease of conducting such studies among healthcare professionals. However, the scarcity of RCTs in vaccine research may also reflect broader challenges, including complex regulatory and ethical systems, patient recruitment difficulties, insufficient funding, and limited access to skilled staff and infrastructure [17, 18]. Addressing these barriers is essential to enable more robust trials and advance evidence-based approaches.

Considering determinants and barriers, one key finding is the consistent influence of contextual determinants, particularly education level and socioeconomic status on vaccine acceptance. These factors appeared across various macro-categories, suggesting their broad utility as predictors of vaccination behavior. A high level of education, whether that of the individual or their parents, emerged as a significant determinant in most reviews. Socioeconomic status also reflected the ongoing disparities in vaccine uptake. This finding highlighted the ongoing need for more targeted interventions to overcome these barriers. Interestingly, Sacre *et al*. suggested that key mechanisms linking socioeconomic status to vaccine uptake include knowledge (access to or understanding of information) and confidence (in vaccines or decision-making) [19]. Social and political contexts are also commonly cited as influencing vaccine acceptance. For instance, for certain populations, such as HCWs and PW, support for mandatory vaccination emerged as a positive determinant. This support may reflect a broader dynamic, where individuals are more willing to accept stricter measures for themselves and others when they perceive the promoted behaviors, such as vaccination, as highly effective in achieving health benefits [20], highlighting the importance of clear and evidence-based communication from policymakers to build trust and demonstrate the impact of such measures.

Exposure to media, particularly social media, promoting vaccination has emerged as a determinant of vaccine acceptance, especially for the HPV vaccine. This highlighted the dual role of media as both a potential barrier (e.g., misinformation) and a facilitator in promoting vaccine uptake. The growing influence of social media in shaping public opinion highlights the need for deeper exploration of its impact on vaccine hesitancy. While social media provides opportunities for wider engagement and diverse viewpoints, it also demands literacy skills to help users to understand the complexities of digital information and discern credible sources effectively [21].

This overview also described how determinants and barriers can differ across populations. For example, parents' decisions to vaccinate (especially regarding HPV) were influenced by perceptions of the vaccine's benefits, peer encouragement, and the influence of grandparents. These factors may be key in shaping vaccine acceptance within family dynamics. Pregnant women showed unique vaccine hesitancy or acceptance patterns influenced by maternal age, proximity to childbirth, and household demographics. This highlights the importance of tailoring strategies to address the specific social and familial contexts that shape individual decision-making. Interestingly, while HCWs may have greater knowledge of vaccine-preventable diseases and vaccines due to their profession, their vaccine uptake can still be influenced by personal experiences, attitudes toward health behaviors, and perceived risks. This suggests that while HCWs are likely to be better informed about vaccines, addressing individual health perceptions, social influences, and emotional barriers is important to improve uptake. HCWs as vaccine providers face additional challenges, such as the perceived responsibility to recommend vaccines and the organizational constraints related to workload, lack of clear guidelines, and insufficient training. Furthermore,

HCWs' personal beliefs and practices, such as alternative medicine preferences, were found to be barriers, suggesting that providers' attitudes may affect their vaccine recommendations and practices, which in turn influence vaccine uptake among the general population. Thus, it is essential to develop targeted interventions that address both personal attitudes and professional responsibilities to strengthen HCWs' roles as vaccine promoters.

An underexplored factor was health engagement, which was found to be a relevant determinant in some GP reviews. Individuals with higher health engagement or those who gave up smoking appeared more likely to accept vaccines, in line with broader behavioral models that link health literacy and proactive health behaviors. This is consistent with findings from Nudelman *et al*. [22], who reported that higher engagement in healthy lifestyle behaviors prior to the pandemic correlated significantly with greater adherence to coronavirus protective measures. Similarly, the framework of Multiple Health Behavior Change [23] suggests that health behaviors are interrelated, with perceived health status mediating their impact on overall health outcomes. However, the relationship between health engagement and vaccine hesitancy remains relatively unexplored in the existing literature and warrants further investigation. Unhealthy behaviors, such as alcohol consumption, noted as barriers in some studies, further highlighted the complexity of these relationships. Havigerová [24] found that health behaviors, including nutrition, substance use, and physical activity, may be influenced by multiple factors like attitudes and economic status, rather than a single latent factor. Understanding how these behaviors interact and influence vaccine hesitancy could provide essential information.

Interestingly, some variables were reported as both determinants and barriers depending on the study. For example, being an HCW was identified as both a positive determinant in some populations and a barrier in others. This contradictory finding could reflect the complexity of HCWs' attitudes toward vaccination, where professional responsibilities might promote a sense of duty to vaccinate, while personal experiences, such as burnout or lack of institutional support, may contribute to vaccine hesitancy. These mixed results highlight the need for nuanced strategies to address vaccine hesitancy in healthcare professionals. Further contrasting variables include age, which was a determinant in some reviews (particularly among younger populations) but a barrier in others. Similarly, complications from other diseases or poor health were identified as barriers in some studies, particularly regarding COVID-19 vaccines for GP reviews. These contrasting results indicate the complexity of vaccine hesitancy, whereby the same variables may act as determinants or barriers depending on contextual, population-specific, and vaccine-related factors, underscoring the need for tailored approaches that consider the specific circumstances of different groups.

Considering interventions, one of the most common strategies identified across multiple macro-categories was the provision of educational materials and campaigns. Although widely implemented, the results were inconsistent. Printed materials and social media campaigns were often mentioned, but their effectiveness varied across reviews. For instance, while in-person educational campaigns for MMR and television-based campaigns for HPV were deemed successful in some CAP reviews, other types of campaigns, such as mailed educational materials in GP reviews, showed no significant impact. This variability aligns with findings from Zhao *et al*. [25], who highlight that health communication campaigns often have modest effects on behavior change. The heterogeneous effectiveness of educational interventions may partly reflect differences in delivery modalities. Passive approaches, such as mailed materials, were more often associated with no effects, whereas more interactive strategies, including in-person campaigns, appeared more likely to be effective. This suggests that information provision alone may be insufficient to address vaccine hesitancy. Despite this, even small impacts can lead to substantial public health benefits at the population level. Furthermore, the effectiveness of campaigns is highly context-dependent, with stronger results reported in areas like road safety or tobacco control compared to sexual health or mammography [25]. These findings suggest that the impact of educational campaigns targeting vaccinations warrants further exploration, particularly in relation to specific contexts and delivery modalities. When it comes to social media campaigns, the challenges in evaluating their effectiveness become even more pronounced. In social media campaigns, evaluating effectiveness is more complex, with many studies unable to determine whether objectives were met [26]. Engagement has become a central metric for success; however, not all engagement is beneficial, as negative engagement, such as misinformation, remains a challenge [26]. This is in line with the earlier observation that media, especially social media, can act as both a facilitator and a barrier in promoting vaccine uptake, suggesting that improving digital literacy and critical engagement with online content could be essential components in improving the effectiveness of social media-based interventions.

Active reminders, including phone calls, text messages, and emails, emerged as one of the most consistently effective interventions across all groups. These findings showed the importance of proactive communication and the role of personalized, direct engagement with individuals. Overall, the consistent effectiveness of active reminders across multiple populations and settings highlights their potential as scalable, low-cost interventions that can be readily integrated into routine vaccination programs. However, while reminders were generally effective, reminder postcards for elderly individuals in GP reviews did not show significant results, indicating that targeted approaches based on the population's specific needs are crucial. Given these insights, it would be worth to compare different reminder methods in more robust studies, as seen in other prevention areas [27] and emerging research on vaccination interventions [28].

Organizational changes emerged as a common theme in the interventions, particularly across the GP and

HCW categories. Among these, the increased involvement of general practitioners in patient management and communication was one of the most frequently cited interventions, consistently showing positive results. While organizational changes can be beneficial, their success may depend on how they are integrated into existing healthcare infrastructures and whether they address the specific needs of the population being targeted. For example, offering free vaccinations in maternity wards and workplaces showed promising results in some reviews, highlighting the importance of making vaccines easily accessible within familiar settings. However, other organizational changes, such as community pharmacy programs and opt-out approaches, did not always lead to significant improvements. Interestingly, opt-out approaches generally increased participation in other contexts, e.g., in colorectal cancer screening [29] and HIV testing [30]. However, challenges arise when communication is unclear, as shown by resistance to opt-out flu vaccination policies in the English National Health Service [31]. Therefore, an effective implementation may require clear communication and engagement with the target population. Overall, approaches building on existing healthcare structures, such as greater involvement of general practitioners, may appear more broadly transferable, whereas setting-specific strategies (e.g., workplace- or maternity ward-based vaccination and opt-out policies) may be more context-sensitive and dependent on local organizational and communication conditions. Accordingly, organizational interventions should be interpreted in light of their implementation context, with transferable strategies being more suitable for large-scale programs and context-sensitive approaches requiring local adaptation.

School-based interventions were found to be consistently effective in CAP reviews. This reinforces the value of using schools as settings for vaccination campaigns, particularly in reaching adolescents and young children. As noted in previous research, schools provide one of the most accessible avenues for implementing health promotion and preventive measures for school-aged children [32], emphasizing their important role not only in vaccination initiatives but also in advancing comprehensive public health efforts for younger populations.

The effectiveness of digital interventions was more variable. While some CAP reviews found positive results with web-based tailored interventions and mobile apps, other digital strategies, such as storytelling websites or game-based simulations in GP reviews, showed no significant impact. These mixed results suggest that while digital tools hold promise, their success may depend on various factors that require further examination. Jakob *et al*. [33] highlighted that adherence to tools such as mobile apps may depend on factors like personalization, ease of use, and addressing barriers such as low technical competence and privacy concerns, which should be addressed in the development of such interventions. These findings further indicate that usability, personalization, and users' digital literacy should be clearly reported and systematically evaluated in future studies, as they are likely key determinants of the effectiveness of digital vaccination interventions.

In terms of interventions specific to HCWs, communication tools, training, and feedback mechanisms consistently yielded positive results. Training healthcare leaders and implementing policies emphasizing staff vaccinations were also shown to be effective. These findings highlight the importance of engaging HCWs in vaccination promotion and ensuring they are well-equipped with the necessary information and resources. Complementing these results, our other work within the VAX-TRUST project, a systematic review aimed at identifying interventions targeted at HCWs, with few categories of interventions showing consistently positive findings. Nevertheless, it highlighted that newer strategies, such as apps, gaming, and simulations, could offer promising avenues to support HCWs [7]. Thus, while digital interventions could represent an innovative approach, the mixed results in both our works suggest that the characteristics of successful interventions need further exploration.

Last, financial incentives demonstrated mixed results. While some CAP reviews, particularly on HPV and adolescent vaccines, showed positive impacts of financial incentives, others reported non-significant results. Similarly, financial incentives aimed at general practices showed conflicting outcomes. According to Miranda *et al*. [34], it is worth noting that many studies on incentives fail to report details such as the rationale, magnitude, and design of incentive strategies. Addressing these aspects could help refine financial incentives and improve their effectiveness in promoting healthy behaviors.

This work had several limitations that should be acknowledged. The overview was constrained by the specific needs of the VAX-TRUST project, posing time and geographical restrictions. Search windows varied across the included systematic reviews, which may have affected comparability of findings. This is particularly relevant given that vaccine hesitancy is a complex and dynamic phenomenon that evolves over time and across contexts [11]. Determinants and barriers identified in this overview are based predominantly on observational evidence; therefore, causal relationships cannot be inferred and residual confounding cannot be excluded. Determinants and barriers were reported as framed in the included systematic reviews, with some reviews describing factors associated with vaccine acceptance and others reporting opposite characteristics as barriers. This overview cannot determine whether these reflect symmetric or distinct underlying mechanisms. The reliance on systematic reviews as the primary unit of analysis may have introduced biases related to the quality and scope of the included reviews. A further limitation relates to the extraction of Europe-specific evidence from global systematic reviews. When European results were not explicitly reported, primary studies conducted in European countries were identified and synthesized based on their reported setting. While this approach enabled the inclusion of relevant European evidence, it depends on the quality of reporting in the original reviews and primary studies and may have introduced selection bias or incompletely

captured contextual heterogeneity across Europe. The quality of the included reviews posed challenges. Common issues across the reviews included insufficient explanation of the reasons for selecting study designs, incomplete reporting of excluded studies with reasons, lack of transparency regarding funding sources, and inadequate interpretation of the risk of bias assessments. Therefore, while the overview provides valuable insights, it must be interpreted with these quality concerns in mind. In addition, the exclusion of exclusively qualitative systematic reviews may have resulted in the loss of rich contextual and experiential insights into vaccine hesitancy Last, the binary classification of intervention effectiveness as "significant" *versus* "non-significant or conflicting" was adopted to enable synthesis across highly heterogeneous systematic reviews; however, this necessary simplification may not fully capture variations in effect size, outcome relevance, and contextual factors, and the findings should therefore be interpreted with caution.

CONCLUSIONS

This overview highlights vaccine hesitancy as a multifaceted, dynamic, and context-dependent phenomenon, characterized by the coexistence of determinants and barriers that may vary across populations, vaccines, and settings, underscoring the need for tailored, evidence-based strategies.

Key gaps, including the limited use of RCTs, hinder the development of effective interventions. Among the most novel and underexplored determinants, health engagement is reported as a relevant factor, reflecting the need for a holistic approach that integrates broader health behaviors. Similarly, social media's dual role as both a facilitator and a barrier emerged with its growing influence, requiring deeper investigation and targeted strategies to improve its impact. Our findings suggest prioritizing interventions with more consistent effectiveness and higher scalability, such as active reminders, school-based programs, and organizational changes embedded within existing healthcare structures. Strengthening the role of primary care providers and reducing structural and logistical barriers appear particularly suitable for large-scale implementation, whereas more context-sensitive strategies require careful local adaptation and stakeholder engagement. In contrast, digital interventions and financial incentives warrant further evaluation to clarify the conditions under which they are effective. Overall, future research should prioritize rigorous study designs and implementation-focused studies to support evidence-informed vaccination policies across Europe.

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Conflicts of interest statement

The Authors declare no conflicts of interest.

Authors' contributions

Conceptualization: GLM, MF, EL, ME, EDV, RS; methodology: GLM, MF, EL, ME, EDV, RS; investigation: GLM, AD, SP, AP, CGS, EC, CL; data curation: GLM, AD, SP, AP, CGS, EC, CL; writing – original draft preparation: GLM, AD, SP, AP, CGS, EC, CL; writing – review and editing, visualization: GLM; supervision: MF, EL, ME, EDV, RS; project administration: GLM, MF. All Authors have read and agreed to the published version of the manuscript.

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Building AI-ready health systems: the AULSS6 (Padua, Italy) local health authority artificial intelligence implementation strategy

Carlo Giordani[1*], Marcello Di Pumpo[1*], Fabio Senarigo[1], Chiara Zambon[1], Maddalena Valli[2], Luca Gino Sbrogiò[1], Nadia Milano[1], Giulia Gusella[1], Tullio Zampieri[1], Francesco Allegro[1], Chiara Bovo[1], Laura Biasi[1], Elena Debora Toffanello[1], Pietro Viena[1], Angela Vinelli[1], Susanna Visonà[1], Michela Barbiero[1] and Paolo Fortuna[1]

[1]*Azienda ULSS6 Euganea, Regione del Veneto, Padua, Italy*
[2]*Grimaldi Alliance, Padua, Italy*
[*]*These Authors equally contributed to the paper and share first authorship*

Abstract

Objectives. To present the strategy developed by the Local Health Authority AULSS6, Padua Province, Italy, for implementing artificial intelligence (AI) in healthcare systems.

Methods. Between December 2024 and December 2025, a series of structured meetings of a multidisciplinary Steering Committee were conducted. Ideas, priorities and initiatives were systematically collected, categorized, and refined through consensus.

Results. The strategy produced several outcomes: an AI ethics and readiness checklist ensuring compliance with the EU AI Act, GDPR, (EU General Data Protection Regulation) and cybersecurity requirements; multi-level education programs for staff; systematic technology evaluation guidelines and HTA (Health Technology Assessment) evaluation studies planning; active participation in projects on AI for public health and clinical outcomes prediction and resource optimization; communication strategies designed to enhance transparency and trust. Collectively, these initiatives enabled AULSS6 to build institutional capacity for responsible AI adoption at the local level.

Conclusions. AULSS6 shows that local health authorities can lead responsible AI implementation in healthcare. Its multidimensional approach close to the population offers a replicable, scalable model for health systems across Italy and Europe, advancing digital transformation through a bottom-up strategy close to the communities served.

Key words

- artificial intelligence
- implementation strategy
- local health authority
- governance
- ethics
- health service research

INTRODUCTION

Artificial intelligence (AI) is revolutionizing healthcare, bringing forward a transformative era across diagnostics, therapeutics, prevention and organization. Recent scientific literature illustrates how AI tools are enhancing clinical accuracy, optimizing workflows, and reducing administrative load [1]. AI-powered algorithms are now demonstrating high accuracy in multiple medical tasks. A 2023 umbrella review reported that AI systems for cancer imaging achieve strong diagnostic metrics. In breast imaging specifically, deep-learning tools improve detection rates, reduce false positives, and lighten radiologist workloads [2]. Public health and primary care also benefit greatly from this revolution [3, 4]. While much of AI's success centers on single-use-case applications (e.g., image analysis or chatbot assistants), the bigger challenge lies in embedding AI within population health systems, those that manage entire community health, chronic diseases, and service networks. Addressing this requires robust population-level strategies: comprehensive data infrastructures, interoperability, equitable algorithm design, governance frameworks, stakeholder engagement, and outcome-driven evaluation. Population health implementation must ensure AI tools are interoperable across primary, secondary, and social care platforms, calibrated for diverse socioeconomic, age, and ethnic groups, aligned with policy standards and voted dedicated to real-world application [5].

The current landscape of advanced healthcare technology is characterized by a significant gap between high-level scientific findings and standardized practical

Address for correspondence: Marcello Di Pumpo, Azienda ULSS6 Euganea, Via Enrico degli Scrovegni 12, 35131 Padova. E-mail: marcello.dipumpo@aulss6.veneto.it.

implementation. While literature demonstrates numerous cutting-edge experiences, these are typically isolated, existing as single-institution pilot studies or research trials with limited, non-diverse datasets. This restriction may compromise their generalizability and robustness when applied to new settings, a phenomenon supported by scientific evidence indicating potential performance drop-offs during external validation [6].

Comprehensive frameworks, spanning from ethics regulation (EU and Italian regulations) to implementation science (RE-AIM) [7] have been extensively developed to provide a structured, repeatable blueprint for responsible scaling. However, the practical implementation of these frameworks at the healthcare system level remains scarce. This scarcity is principally due to systemic barriers, the main ones including organizational and cultural inertia within risk-averse healthcare systems, the high cost and complexity of achieving interoperability across fragmented IT infrastructures (EHRs), and the challenge of establishing sustainable standardized reimbursement models, which prevents hospitals from establishing a clear, sustainable return on investment (ROI) [8, 9].

It is in this context that the Azienda ULSS 6 Euganea (AULSS6) Local Health Authority of the Padua Province has launched a comprehensive strategy to integrate AI within its healthcare and administrative systems according to the following dimensions: Governance, Ethical and Regulatory Assessment, Education, Technology Evaluation, Healthcare Organization and Public Health, Research, and Communication.

METHODS

The aim of this study was to perform a narrative account of an institutional initiative for AI multilevel implementation strategy at AULSS6. A series of structured meetings of a multidisciplinary Steering Committee were conducted between December 2024 and December 2025. Ideas, priorities and initiatives were systematically collected, categorized, and refined through consensus. The output of the analysis was descriptively reported.

RESULTS

With regards to Governance, the first move was the establishment, in November 2024 [10] of a multidisciplinary AI Steering Committee mandated with governing AI implementation in AULSS6, meeting monthly, composed by: Director General, Administrative Director, Innovation and Organizational Development Director, Information and Communication Technology Director, Data Protection Officer, Department of Hospital Management Director, Department of Prevention Director, Department of Primary and Community Care Director, General Affairs Director, Anti-corruption Director, Clinical Engineering Director and AI scientific expert.

Considering how The EU AI Act [11] classifies healthcare AI as "high-risk", mandating rigorous compliance with risk management, data quality, transparency, human oversight, and cybersecurity standards is necessary. The Steering Committee's dedicated staff and the Data Protection Officer has provided an organizational AI Ethics and Readiness Checklist. Main principles that are required to be upheld are: GDPR compliance i.e., ensuring all data processing activities, particularly the use of sensitive patient data for AI training, strictly adhere to the principles of data minimization, purpose limitation, and lawful basis for processing, alongside robust data subject rights management; data quality, i.e., guaranteeing that training, validation, and testing datasets are representative, accurate, and free from bias to prevent discriminatory outcomes; transparency and explainability i.e., requiring clear documentation of how AI models function and the rationale behind their outputs, implementing proactive strategies to identify and mitigate algorithmic bias and enhance explainability (e.g., SHAP or LIME methodologies) to end-users and patients; i.e., human oversight, ensuring that autonomous decision-making remains subject to appropriate human intervention and validation; cybersecurity resilience, i.e., protecting sensitive health data and the AI infrastructure from attacks; human-in-the-loop (HITL) approach: mandating clearly defined protocols for human review, validation, and override of AI outputs, particularly for critical healthcare tasks, transforming the clinician's role into one of augmented decision-maker.

This last principle is highly emphasized by the AULSS6 strategy. Under this model, every decision is AI-supported, but the AI is never intended as a substitute for humans. The final decision-makers are always healthcare professionals, and their decision is considered a healthcare act with related responsibility. Professionals, however, never act alone, but always inside a framework of clear policies and regulations provided by the governance. Their daily duties are, in fact, included within a comprehensive care process, never resulting in automated or fragmented services but always upholding the importance of each healthcare-related act (e.g., diagnostic, therapeutic, etc.). AI implementation within complex clinical environments poses significant practical and organizational challenges that extend far beyond technical integration. A primary concern may be alert, where multiple AI warnings could cause clinicians to ignore truly critical alerts, fatigue and the risk of de-skilling, with over-reliance on accurate predictions (automation bias) potentially eroding fundamental professional capabilities and judgment. Moreover, the introduction of AI may complicate the handling of accountability and liability among the different actors of the process, namely technology providers, management, and end-users. Therefore, upholding the HITL principle requires not just technical integration but a fundamental coordinated rethinking of clinical workflows, training, and risk management procedures.

In the domain of education, the Steering Committee developed a comprehensive training strategy to build awareness, skills, a shared vision and ethical culture around AI in healthcare. This included the creation of multiple targeted courses and dissemination events: a foundational course on AI and LLMs (large language models) for healthcare and administrative profession-

als (hosted in collaboration with the Italian Society for Artificial Intelligence in Medicine (Società Italiana Intelligenza Artificiale in Medicina, SIIAM), a specialized program focused on ethics and regulatory frameworks tailored for the Scientific Committee, a regional-level Congress dedicated to AI implementation for hospital management, and also dissemination of free institutional learning materials. To enhance practical understanding and foster innovation, site visits to leading AI development centers were organized, offering hands-on exposure to real-world applications. This education providers will also help guide the AI implementation process at various level of the AULSS6 organization. Additionally, the results of scientific research were presented in dedicated sessions of scientific international conferences to broaden knowledge dissemination and stimulate interdisciplinary dialogue across the healthcare community.

In the area of technology evaluation, AULSS6 is working on producing a dedicated checklist to enable a thorough assessment of AI-based technologies to be adopted, ensuring their compliance with current regulatory standards and the production of appropriate documentation, e.g., FRIA (fundamental rights impact assessment), which systematically evaluates the potential impact of the AI system on users' and patients' fundamental rights (e.g., non-discrimination, privacy) [11]. AULSS6 developed and disseminated clear guidelines to support systematic technology evaluation across the organization. Additionally, AULSS6 is scanning internal promising technologies as candidates for Health Technology Assessment (HTA) pilot studies, applying structured dedicated frameworks [12]. This involves applying structured, dedicated frameworks to assess the clinical effectiveness, cost-effectiveness, organizational impact, and ethical implications of a technology for future widespread adoption. These efforts aim to promote evidence-based decision-making, enhance the value of technological investments, and ensure that innovation aligns with clinical needs and regulatory requirements.

Regarding public health and healthcare service organization, multiple tools are present in literature addressing problems like: staffing optimization i.e., utilizing predictive modelling to forecast patient load and allocate clinical and administrative staff efficiently; hospital length-of-stay control i.e., employing algorithms to identify patients at risk of prolonged stays, allowing for targeted intervention and care path modification; emergency room overcrowding prevention i.e., implementing real-time predictive models to forecast ER bottlenecks and manage patient flow proactively [1, 13, 14]. The same is true for public health interventions: early disease detection i.e., leveraging AI for rapid analysis of complex data (e.g., genomic, imaging, or surveillance data) to identify early signs of disease, such as infectious disease outbreaks or chronic conditions [15]; cancer screening programs i.e., applying AI to enhance the efficiency and accuracy of image analysis in screening programs, improving detection rates and reducing false positives [4]. These solutions are being thoroughly evaluated at AULSS6 and selected for potential future implementation, always ensuring an ethical approach and upholding quality and safety issues.

With regards to scientific research, AULSS6 actively participated in funded research calls, engaging in diverse set of projects addressing critical areas of innovation, from infectious disease outbreak early detection i.e., utilizing advanced algorithms for the early identification and prediction of infectious disease spread patterns, to clinical outcomes prediction, i.e., developing sophisticated algorithms for early prediction of critical outcomes in complex environments like Intensive Care Units (ICUs), enabling timely interventions. To support and streamline these efforts, AULSS6 established a standardized submission process to efficiently receive and evaluate research proposals. This efficient, integrated approach ensures the effective receipt, rigorous ethical and scientific evaluation, and enhanced potential for successful financing and execution of research proposals. This integrated approach not only fosters scientific collaboration with academic and industry partners but also accelerates the translation of research into practical real-world solutions that enhance patient outcomes and strengthen our healthcare system's preparedness and response capabilities.

Recognizing that the successful adoption of AI depends not only on technical and regulatory readiness but also on public understanding and healthcare professionals' engagement, AULSS6 dedicated specific resources to strategic communication. Targeted internal and external communication is being gradually established, in order to accompany the rollout of AI initiatives, addressing the specific concerns and information needs of different stakeholder groups. The dual goals of transparency and trust are maintained: the communication efforts are designed to ensure full transparency regarding the function, limitations, and impact of deployed AI systems, while actively working to enhance trust. Three core recipient groups are identified: healthcare and administrative professionals, by clarifying the AI's role as a supportive tool rather than a replacement, focusing on clinical practice augmentation rather than substitution; patients, by providing accessible information on how their data is used and how AI impacts their care decisions; institutional stakeholders, by demonstrating compliance, ethical diligence, and the strategic value of the AI investment. This integrated communication effort is crucial for fostering a culture of responsible innovation and ensuring participatory governance, which are essential for the long-term, ethical, and successful integration of AI into healthcare delivery.

CONCLUSIONS

What makes the AULSS6 initiative particularly notable is that the project emerges from a local health authority in direct contact with the community level and stakeholders and directly responsible for delivering care to about one million residents of the Veneto region. By designing a comprehensive, compliant, and ethically grounded strategy for AI adoption, AULSS6 is demonstrating that innovation in AI can and must happen also at the local level, close to the citizens it ultimately

serves. As such, AULSS6 further aims at providing a replicable and scalable model for other regional health systems across Italy and Europe, offering a powerful signal: the digital transformation of healthcare can be advanced with a bottom-up strategy complementing national and regional authorities' guidance.

Ethical approval

This study did not involve human participants, patient data, or experimental interventions. It was based on a descriptive analysis of institutional strategy and internal organizational processes. Therefore, ethical approval was not required.

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Conflict of interest statement

The Authors declare no competing interests.

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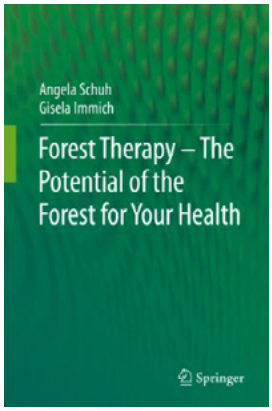
Book Reviews, Notes and Comments

Edited by
Federica Napolitani Cheyne



FOREST THERAPY
The potential of the forest for your health
Angela Schuh, Gisela Immich
Springer Berlin, Heidelberg; 2022
130 p.
ISBN 978-3-662-64279-5
32,65 €

Who would have thought that forests, or rather outdoor life, preferably in unspoiled ecosystems, could have demonstrable and "scientifically" proven effects on our health conditions? For some time now, green, especially urban green, has become an important, almost priority-level, issue for public health in metropolitan areas in various parts of the world [1].

It is well known that when hospitalized patients are exposed to the green color, but also to blue, (with windows overlooking green areas), such a visual immersion exerts an accelerating effect on recovery performances and times [2, 3]. With regard to pediatric emergency facilities it has been reported that, irrespective of country, community or genetic background, striking similarities in children's spontaneous preferences regarding hospital care seem to consistently emerge [4].

This book is written by two basic-research authors who have been carrying out for some time intensive research into the therapeutic effects of forest exposure and climate conditions at Ludwig Maximilian University in Munich, promoting concepts related to the preventive use of forest exposure and forest therapy itself.

Obviously, air temperature, humidity, rainfall, wind, oxygenation, and air "cleanliness" in general are reviewed in the text. Gas exchange with the atmosphere is also originally reviewed as a critical parameter. It is an actual novelty that "quietness" is also discussed, touching on the delicate and increasingly topical subject of the so-called "soundscape", to which the human senses are evolutionarily attuned, in addition to sight and the analogous "odourscape" (here we refer to VOCs, Volatile Organic Compounds, which have been extensively studied) [5].

The effects of relief from thermal stress, understood as protection of the particularly looming thermoregulatory and cardiovascular systems, are one of the most original phenomena taken into consideration.

Similarly, the potential health effects of phytoncides, thanks also to studies by Russian authors published in the second half of the 1950s, represent only an apparent return to the role, now once again considered pivotal, of terpenes emitted, for example, by common trees of the *Pinaceae* family, which are almost inevitable elements in a variety of ecosystems even in urban areas of our Mediterranean country. In particular, in a "One Health" Third Millennium perspective, an agent such as alpha-pinene has long been the subject of characterization and depicted functional role.

In addition to cognitive effects, memory and attention performance, or psychiatric and/or cardiovascular syndromes, other pathologies (from autism to ADHD to individual pain thresholds) are all parameters influenced by such a type of immersive practice. The entire Chapter 5, a major "bone" of this small volume, focuses on operational activities, providing a sort of guideline on "Which forest is suitable for forest therapy" (title of a successful paragraph).

The text highlights the technical and training requirements for "forest health trainers" and "forest therapists," emerging professionals who are clearly considered essential in the authors' scientific and clinical ecosystem. They also usefully focus on the role of mindfulness as an integrative therapeutic approach to forest immersion practices. While the literature on forest therapy is growing rather rapidly, few volumes dwell on these aspects of novel job offers.

In sum, the present one is a rather original, at times provocative, text, which may arouse surprise, if not a certain degree of aversion, in some sectors of contemporary clinical practice. The volume is particularly recommended for young people starting a career in the biomedical world who may be interested in approaches that are unusual within the current educational curriculum but promising given the accumulating evidence of their beneficial effects on psychological well-being.

Gemma Calamandrei, Stella Falsini
and Enrico Alleva
Istituto Superiore di Sanità, Rome, Italy
gemma.calamadrei@iss.it

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GUT MICROBIOME AND ENVIRONMENTAL TOXICANTS
Impact on Human Health
Gupta G, Hussain S, Dua K, Gilhotra R, Dhanasekaran M (Eds).
Boca Raton: CRC Press; 2025. 294 p.
ISBN 9781032787343

Gut Microbiome and Environmental Toxicants: Impact on Human Health is a timely and comprehensive volume that addresses one of the most rapidly evolving areas in biomedical and environmental health research: the role of the gut microbiome as a key mediator of human responses to environmental toxicants. By integrating perspectives from toxicology, microbiology, immunology, and clinical sciences, the editors provide a multidimensional framework for understanding how environmental exposures translate into health outcomes through microbiome-dependent mechanisms.

The book is organized into thematic chapters that examine a wide spectrum of environmental toxicants and endocrine-disrupting compounds, including heavy metals, pesticides, bisphenol A, phthalates, organic pollutants, and emerging contaminants such as microplastics. Across these chapters, a central concept consistently emerges: the gut microbiome functions as a dynamic and metabolically active interface between environmental exposures and host physiology, significantly influencing susceptibility, toxicity, and disease progression.

A major strength of the volume lies in its detailed exploration of *mechanistic pathways*. Several chapters focus on the microbiome's ability to metabolize xenobiotics through enzymatic processes such as reduction, hydrolysis, and deconjugation. These microbial transformations can result in either detoxification or bioactivation of environmental chemicals, thereby altering their toxicity, bioavailability, and systemic distribution. This perspective challenges traditional toxicological models by positioning the microbiome as an additional metabolic "organ" that must be considered in risk assessment.

The book provides an in-depth discussion of key *molecular signaling pathways* involved in microbiome-toxicant interactions. Notably, the aryl hydrocarbon receptor (AhR) is highlighted as a critical signaling node through which microbial metabolites and environmental pollutants converge to regulate immune homeostasis, epithelial barrier integrity, and inflammatory responses. Dysregulated AhR signaling, driven by toxicant-induced alterations in microbial metabolism of tryptophan, is linked to chronic intestinal inflammation and systemic immune dysfunction.

Equally important is the examination of *Toll-like receptor (TLR) signaling*, particularly TLR4, in mediating inflammation associated with toxicant-induced dysbiosis. Increased intestinal permeability resulting from microbiome disruption facilitates the translocation of lipopolysaccharides (LPS), activating TLR-dependent pathways and triggering downstream signaling through NF-κB. The sustained activation of NF-κB is shown to promote chronic low-grade inflammation, a hallmark of metabolic disorders, cardiovascular disease, and immune-mediated conditions.

The volume also gives considerable attention to the role of the gut microbiome in maintaining *intestinal barrier integrity*. Several chapters describe how environmental toxicants reduce the production of short-chain fatty acids (SCFAs), particularly butyrate, leading to impaired tight junction protein expression and increased gut permeability. This "leaky gut" phenomenon amplifies systemic exposure to toxicants and microbial products, further exacerbating inflammation and oxidative stress.

Of particular interest are chapters addressing *neurotoxicity and the gut-brain axis*. The editors effectively synthesize evidence linking microbiome alterations caused by heavy metals and persistent organic pollutants to changes in neurotransmitter synthesis, neuroinflammation, and hypothalamic-pituitary-adrenal (HPA) axis dysregulation. These mechanisms provide a compelling biological basis for associations between environmental exposure and neurodevelopmental, cognitive, and neurodegenerative disorders.

Beyond mechanistic insights, the book explores *clinical and translational implications*, emphasizing the gut microbiome as a promising therapeutic target. Strategies such as dietary modulation, prebiotics probiotics and personalized microbiome-based interventions are discussed as potential tools to mitigate toxicant-induced health effects. This translational focus enhances the practical relevance of the volume for clinicians and public health researchers.

Overall, *Gut Microbiome and Environmental Toxicants: Impact on Human Health* stands out for its scientific depth, interdisciplinary scope, and forward-looking perspective. It successfully bridges experimental research and clinical relevance, making it a valuable reference for researchers, clinicians, toxicologists, and graduate students. The book underscores the necessity of incorporating microbiome science into environmental health risk assessment and opens new avenues for preventive and therapeutic strategies aimed at reducing the burden of environmentally driven diseases.

From a toxicological perspective, the volume highlights individual contaminants in depth, yet *real-world exposures are typically chronic and involve complex mixtures of chemicals*. The combined and potentially synergistic effects of multiple toxicants on the gut microbiome remain insufficiently characterized. This represents a critical gap, particularly for environmental health risk assessment, where mixture toxicity is the norm rather than the exception.

Looking forward, the book implicitly points toward several promising *future research directions*. Longitudinal human cohort studies integrating microbiome profiling with exposomics, metabolomics, and clinical phenotyping will be essential to establish causal relationships between environmental toxicants, microbiome alterations, and disease outcomes. Advances in systems biology, including multi-omics integration and machine learning approaches, may help disentangle the complex networks linking microbial metabolism, host signaling pathways (such as AhR, TLR, and NF-κB), and health effects.

Furthermore, future work should prioritize the development of *standardized methodologies* for microbiome analysis and toxicant exposure assessment, enabling more robust comparisons across studies. From a translational standpoint, controlled clinical trials are needed to validate microbiome-targeted interventions – such as probiotics, prebiotics, and dietary strategies – as viable tools to mitigate toxicant-induced health effects.

In conclusion, while the volume effectively consolidates current knowledge and highlights key mechanistic pathways, it also underscores the need for integrative, human-centered research approaches. Addressing these limitations will be crucial for translating microbiome science into actionable strategies for environmental health protection and personalized medicine.

Renata Alleva
Università di Bologna, Bologna, Italy
rena.alleva@gmail.com

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Publications from International Organizations on Public Health

Edited by
Annarita Barbaro

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO)

World food and agriculture – Statistical yearbook 2025. Rome: FAO; 2025. 418 p. ISBN 978-92-5-140174-3. The Statistical Yearbook 2025 offers a synthesis of the major factors at play in the global food and agricultural landscape. Statistics are presented in four thematic chapters, covering the economic importance of agricultural activities, inputs, outputs and factors of production, their implications for food security and nutrition and their impacts on the environment. The digital version of the yearbook presents the data in an interactive way, which facilitates the visualization of figures and tables and their reuse. The Yearbook is meant to constitute a primary tool for policymakers, researchers and analysts, as well as the public interested in the past, present and future path of food and agriculture.

Callan A, Sabirovic M, Caceres H, Abreu D, Tenenbaum N. **Progressive pathway for emergency preparedness self-assessment user guide 2025**. Rome: FAO; 2025. 52 p. ISBN 978-92-5-140377-8. The Progressive Pathway for Emergency Preparedness (PPEP) self-assessment user guide is a comprehensive guide that helps national authorities evaluate and improve their emergency preparedness for agrifood emergencies. The guide introduces the PPEP, a voluntary, multistage process that incorporates self-assessment as a key component. The handbook outlines seven core elements of emergency preparedness (governance, communication, planning, resourcing, training, exercising and continuous improvement), each with specific indicators to assess a country's emergency preparedness status. The guide also introduces the tools for structured evaluation and visual reporting. These tools enable countries to identify improvement opportunities, prioritize them, and collaborate with FAO and regional partners to build resilient emergency management systems.

Digital agriculture and AI innovation roadmap. For the global agrifood systems transformation. Rome: FAO; 2025. 48 p. ISBN 978-92-5-139932-3. This document delivers two core concepts. The first is an inclusive platform whose architecture delivers value through its four elements, namely, missions and use cases, an innovation methodology, the community of people and partners, and a streamlined set of services. The second core concept guiding the roadmap's implementation are the principles which ensure that impact is not single-sided but rather a holistic practice that brightens the community and the planet. These concepts are meant to truly improve the success rate of these initiatives and maximize the value of creation and reusability of digital assets whilst adopting common technical standards. The AI roadmap is a hands-on guide for moving from centralized agrifood systems to local needs-based, safe, interoperable, scalable and inclusive innovation initiatives and approaches.

UNITED NATIONS EDUCATIONAL, SCIENTIFIC AND CULTURAL ORGANIZATION (UNESCO)

The ethics of climate engineering. Paris: UNESCO Publishing; 2025. 76 p. ISBN 978-92-3-100829-0. For the first time, a Report by the UNESCO's COMEST (World Commission on the Ethics of Scientific Knowledge and Technology) presents a comprehensive global ethical perspective on climate engineering, with a thorough assessment of the ethical, social, and cultural implications of these technologies. Based on the identified ethical challenges, COMEST also puts forth concrete recommendations for Member States and all relevant stakeholders – including scientists, policymakers, affected communities, and the public – to address the research and potential development of climate engineering technologies in a transparent and inclusive manner.

Revisiting the relations between science and society in the light of the COVID-19 pandemic. Paris: UNESCO Publishing; 2025. 76 p. ISBN 978-92-3-100828-3. The pandemic of COVID-19 showed how deeply our world is shaped by science and innovation – and how science, in turn, is shaped by society. This Report by UNESCO's World Commission on the Ethics of Scientific Knowledge and Technology of UNESCO (COMEST) explores this dynamic, presenting ethics as essential for credible and sustainable science. It emphasizes the importance of dialogue among diverse voices, addressing the hopes, fears, and needs of civil society, while ensuring that people understand the decision-making processes. This is the core of ethics as a key enabler in strengthening trust and collaboration. To address these challenges, the Report calls upon policymakers, scientists, educators, and the public to foster a resilient and ethically sound scientific ecosystem. It presents recommendations to build a more inclusive, trustworthy, and impactful relationship between science and society, benefiting present and future generations.

JOINT UNITED NATIONS PROGRAMME ON HIV/AIDS (UNAIDS)

2025 global AIDS update – AIDS, crisis and the power to transform. Geneva: United Nations Programme on HIV/AIDS; 2025. 120 p. This report shows that at the end of 2024, just before a collapse in funding triggered a crisis in the global AIDS response, the remarkable efforts of communities and governments had brought down the numbers of new HIV infections by 40% and of AIDS-related deaths by 56% since 2010. But it also shows that huge gaps in HIV prevention remained, with 1.3 million new infections in 2024 – almost unchanged from the year before. HIV programmes across the world are struggling from the sudden, drastic reductions in funding for the global HIV response announced by the United States Government in early 2025. Those services were stopped overnight when the United States Government shifted its foreign assistance strategies. Disruptions are being felt across the HIV response and pose a huge risk of increased mortality, a surge of new HIV infections, and the development of resistance to the most used treatment regimens. Urgent action and revived solidarity are needed to sustain the progress made and prevent a resurgence of HIV.

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT (OECD)

The state of cardiovascular health in the European Union. Paris: OECD Publishing; 2025. 470 p. ISBN 978-92-64-41479-2 (print) ISBN 978-92-64-86505-1 (PDF) ISBN 978-92-64-42646-7 (HTML). This report provides a comprehensive assessment of cardiovascular disease (CVD) across EU Member States, combining the latest evidence on health outcomes, risk factors, and system performance with policy analysis and actionable recommendations. It examines trends in mortality, morbidity, and quality of life, highlighting persistent inequalities and the growing impact of demographic change and multimorbidity. Beyond describing the burden of CVD, the report explores the current landscape of prevention strategies, care integration, and digital health innovations. By aligning national efforts with EU priorities and promoting people-centered, data-driven approaches, this publication aims to support policymakers in reducing disparities, strengthening resilience, and ensure that cardiovascular health is embedded as a core priority within sustainable health systems.

INTERNATIONAL LABOUR ORGANIZATION (ILO)

Berg J, Johnston H. **AI in human resource management: the limits of empiricism, ILO Working Paper 154**. Geneva: International Labour Organization; 2025. 40 p. ISBN 978-92-2-042861-0 (print) ISBN 978-92-2-042862-7 (web PDF). This paper presents a framework for understanding and evaluating the potential benefits and possible risks or harms presented by Artificial Intelligence (AI) systems in workforce management. Following a section of the paper documenting the historical context of the Human Resources (HR) field that has given rise, first to people analytics and then to AI, the paper presents a framework based on three inter-related parameters that can help assess the quality, legality, and suitability of AI systems used in the field. These are: the system objective, the data it is built on and relies on, and how the AI system is programmed. Drawing on existing literature about how AI is being used for workforce management, the paper applies the three-parameter framework to map the contours of AI use relative to four key HR management functions where adoption of AI technologies has been prominent: recruitment, compensation, scheduling, and performance management.

WORLD HEALTH ORGANIZATION (WHO)

World malaria report 2025. Addressing the threat of antimalarial drug resistance. Geneva: World Health Organization; 2025. 213 p. ISBN 978-92-4-011782-2 (electronic version) ISBN 978-92-4-011783-9 (print version). Each year, the World malaria report serves as a vital tool to assess global progress and identify gaps in the fight against malaria. This year's report provides a critical and up-to-date snapshot of efforts to control and eliminate malaria across 80 countries, including the epidemiological situation, progress toward the global technical strategy, the funding landscape, the status of malaria interventions and of emerging biological threats. The report also presents the threat posed by antimalarial resistance and its impact in a dedicated chapter, emphasizing the need for a more coordinated and effective response that is locally tailored and supported by regulation, strong quality-assurance systems, active provider engagement, and the timely generation and sharing of high-quality drug-resistance data.

Developing national meningitis plans: an operational manual. Geneva: World Health Organization; 2025. 61 p. ISBN 978-92-4-009428-4 (electronic version) ISBN 978-92-4-009429-1 (print version). This operational manual aims to support countries in developing their meningitis plans, along the five pillars of the Defeating meningitis by 2030 global road map. It suggests an approach for the development of national meningitis plans through involvement of various stakeholders to ensure political buy-in. It also suggests activities to be included in the plan, with considerations to help fine-tuning the activities to make them context appropriate.

WHO updated recommendations on HIV clinical management: recommendations for a public

health approach. Geneva: World Health Organization; 2025. 108 p. ISBN 978-92-4-011946-8 (electronic version) ISBN 978-92-4-011947-5 (print version). This document provides an overview of the updated World Health Organization recommendations for HIV clinical management, which focus on optimizing antiretroviral therapy, preventing vertical transmission, and enhancing tuberculosis prevention among individuals with HIV. These updates are intended to support global initiatives aimed at ending AIDS as a public health threat by 2030. Key changes include the introduction of new antiretroviral drugs and regimens, revised postnatal prophylaxis and breastfeeding guidelines for managing infants at risk of vertical transmission, and the endorsement of shorter tuberculosis preventive treatments to improve efficacy and adherence. The recommendations are designed to simplify and advance HIV care, promote equity, and contribute to improved health outcomes and quality of life for individuals living with HIV. The updated guidance will be incorporated into the forthcoming revision of the antiretroviral therapy chapter within the Consolidated Guidelines on HIV Prevention, Testing, Treatment, Service Delivery, and Monitoring: Recommendations for a Public Health Approach.

Instructions to Authors

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The name of the bioresource (and identifier, if available) which provided samples/data useful for the conduct of the study should be reported in extense, either in the Material and methods section or in the Acknowledgements.

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Articles in journal

Bozzuto G, Ruggieri P, Molinari A. Molecular aspects of tumor cell migration and invasion. Ann Ist Super Sanità. 2010;46(1):66-80. doi: 10.4415/ANN_10_01_09

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Legislation

Italia. Decreto legislativo 29 ottobre, n. 419. Riordinamento del sistema degli enti pubblici nazionali, a norma degli articoli 11 e 14 della legge 15 marzo 1997, n. 59. Gazzetta Ufficiale – Serie Generale n. 268, 15 ottobre 1999.
US Social Security Administration. Evidentiary require-

ments for making findings about medical equivalence. Final rules. Fed Reg. 2006 Mar 1;71(40):10419-33.
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