ISSN 0021-2571 (print) · 2384-8553 (online) | Coden: AISSAW 61 (No. 2) | 95-159 (2025)

ANNALI
DELL'ISTITUTO SUPERIORE DI SANITÀ

Volume **61** No. **2**
2025

A SCIENCE JOURNAL *for* PUBLIC HEALTH

ISTITVTO SVPERIORE DI SANITÀ
www.iss.it

Ann Ist Super Sanità 2025 | Vol. 61, No. 2

Annali
dell'Istituto Superiore di Sanità
A SCIENCE JOURNAL FOR PUBLIC HEALTH

Publication
Annali dell'Istituto Superiore di Sanità is published quarterly and in special issues.
Freely available online at www.iss.it/annali - https://annali.iss.it

Annali dell'Istituto Superiore di Sanità is indexed in
- CAB
- CHEMABS
- EMBASE/Excerpta Medica
- FSTA
- MEDLINE
- ProQuest
- SCOPUS
- WEB OF SCIENCE

The Journal Impact Factor is 1.1

Annali Editorial Office
Scientific Communication Service
Istituto Superiore di Sanità
Viale Regina Elena 299, 00161 Rome, Italy
Tel.: +39 06 49902945 Fax: +39 06 49902253
E-mail: annali@iss.it
www.iss.it/annali - https://annali.iss.it

Papers to be presented for publication should be submitted online to https://annali.iss.it

Instructions to Authors are available online at https://annali.iss.it

Publishing support
Il Pensiero Scientifico Editore, Rome
Via San Giovanni Valdarno 8, 00138 Rome, Italy
www.pensiero.it

Subscription information & terms
Il Pensiero Scientifico Editore
Tel.: +39 06 86282324 Fax: 06 86282250
E-mail: abbonamenti@pensiero.it

Year 2025
Italy individual subscription € 57,00 | Italy institutional subscription € 67,00.
Other countries € 67,00
Each quarterly issue € 21,00

Responsibility for the contents and opinions expressed on this journal rests solely with the Author(s).

ISSN 0021-2571 (print), 2384-8553 (online)
Coden: AISSAW 61 (No. 2)

Reg. Stampa - Tribunale di Roma, n. 482 del 29 ottobre 1985 (cartaceo); n. 121 del 16 maggio 2014 (online)

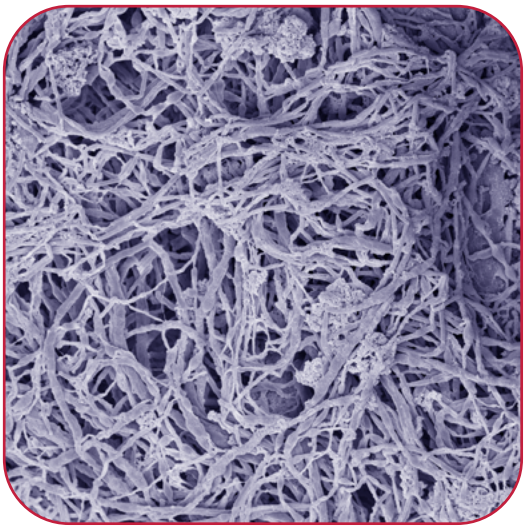
MISTO
Carta da fonti gestite in maniera responsabile
FSC® C017635

Printed in June 2025 by Ti Printing s.r.l.
Via Case Rosse 23, 00131 Rome, Italy

Ann Ist Super Sanità 2025 | Vol. 61, No. 2

Annali
dell'Istituto Superiore di Sanità
A SCIENCE JOURNAL FOR PUBLIC HEALTH

The cover image shows a filamentous biofilm of Clostridium sporogenes *enhanced by other environmental microorganisms from an oil spill site (high resolution scanning electron microscopy image, provided by the Microscopy Area, Core Facilities, Istituto Superiore di Sanità, Rome, Italy).*



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Ann Ist Super Sanità 2025 | Vol. 61, No. 2: 95-101
DOI: 10.4415/ANN_25_02_01

EpiWEAT: a new digital assessment tool for epigenetic studies

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Abstract

Introduction. Intimate partner violence (IPV) profoundly impacts women's health, increasing the risk of chronic and non-communicable diseases. Recent findings underscore the promise of epigenetic indicators to uncover the enduring effects of trauma on the human genome, especially concerning stress-related conditions such as post-traumatic stress disorder (PTSD).

Objectives. To evaluate the lasting health impacts of violence against women, developing a digital tool specifically structured to consolidate crucial details about the contexts of violence, the relationship between victim and perpetrator, and health outcomes.

Methods. A Microsoft Forms-based questionnaire was developed, organized into nine sections addressing socio-personal information, external conditions of violence, health statuses, and psychological evaluations, incorporating PTSD assessment via the International Trauma Questionnaire and depression measurement through the Center for epidemiologic studies depression scale revised (CESD-R) module. Data is securely archived, and participation includes optional consent for epigenetic analysis through blood samples.

Conclusions. The assessment tool presents a thorough tool for gathering information on IPV, evaluating health outcomes, and identifying PTSD and depression in survivors. It also aids in the collection of biological specimens for epigenetic exploration. This instrument could enhance intervention strategies and contribute to precision medicine methodologies, facilitating early detection of chronic health risks in women who have experienced violence.

Key words

- intimate partner violence
- epigenomics
- patient health questionnaire

INTRODUCTION

Violence against women is an important public health problem that represents violations of human rights and can have serious and directed consequences on the onset of noncommunicable diseases. There is now an increased understanding at the international and regional levels that health is not just an issue of development, but primarily a matter of human rights.

Intimate partner violence (IPV), i.e., violence by a partner or in the family or relationships, is the most common form of abuse. It is estimated that about 27% of women aged 15-49 years worldwide have experienced physical and/or sexual IPV in their lifetime, with large differences ranging from 16% in Southern Europe to as high as 51% in Melanesia. Overall, 13% on average have been subjected to physical and/or sexual IPV at some point within the past 12 months, with proportions ranging from 4% in Australia and New Zealand to 32% in Central sub-Saharan Africa [1].

According to research by Italian National Institute

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of Statistics (Istituto Nazionale di Statistica, ISTAT), 6 million 788 thousand women between 16 and 70 years of age in Italy have experienced some form of physical or sexual violence in their lifetime. In particular, 1,157 million involved the most serious forms of sexual violence such as rape (652 thousand) and attempted rape (746 thousand). An estimated 2,151,000 women between the ages of 16 and 70 have experienced persecutory behaviors by an ex-partner in their lifetime [2].

According to the European Injury Database (EU-IDB) [3], collecting data on the external circumstances of accidents compliant with the World Health Organization (WHO) guidelines on injury surveillance [4], the three most common forms of violence against women are: IPV (39% of cases of violence against women), violence committed by an acquaintance or friend (17%) and violence committed by a stranger (17%). In the 2021-2022 time span EU-IDB data shows that when the victim of violence is a woman, in 77% of cases the perpetrator is a man, aged 25-64 years. Fifty-five % of women victims of violence perpetrated by a man are aged 34 years or younger (*Figure 1*).

The consequences of violence on women's health can be physical and psychological. In particular, the health effects on women assume different levels of severity starting from fatal outcomes (e.g., femicide or termination of pregnancy), up to conditions of physical morbidity (mainly consequences of trauma, burns, poisoning or intoxication) and psychological problems with health conditions which include post-traumatic stress disorder (PTSD) [5], depression, substance abuse and self-harm or suicidal behavior, eating disorders, sexual disorders, etc. [6-10].

Recent scientific literature has shown how environmental factors, including violence, can alter the structure and functionality of our genome [11, 12]. Violence, and in particular intimate partner violence, is to be considered as an environmental factor "extremely negative", which significantly affects the expression of our genome and leaving traces in the DNA. Studying the epigenetic markers represents an innovative approach to understanding the effects of violence on the psycho-physical health of women. Epigenetic marker identification could indicate a connection with novel pathways that are yet to be associated with the risk of other non-communicable diseases.

Moreover, epigenetic information in parallel with psychological evaluation could create an innovative therapeutic protocol tool that takes gender differences into account and is based on precision medicine. To demonstrate the biological correlation between violence and the onset of chronic, disabling and non-communicable diseases, it is mandatory to invest in longitudinal studies where prospective data are associated with the epigenetic signature of women's DNAs. Due to the long-term effects being felt later in life, there is a threat of being seen as independent and unconnected to the violence endured.

Previous research has shown that three genes related to PTSD exhibit changes in DNA and are found to be hyper-methylated in women who have experienced violence. These changes relating to the three genes consisted of a higher degree of DNA methylation in women who had suffered violence compared to women who had not suffered violence. These epigenetic modifications in association with psychological assessment, could offer a new tool to identify innovative therapeutic protocols based on precision medicine, which, considering molecular damage association, can help prevent long-term health effects [13].

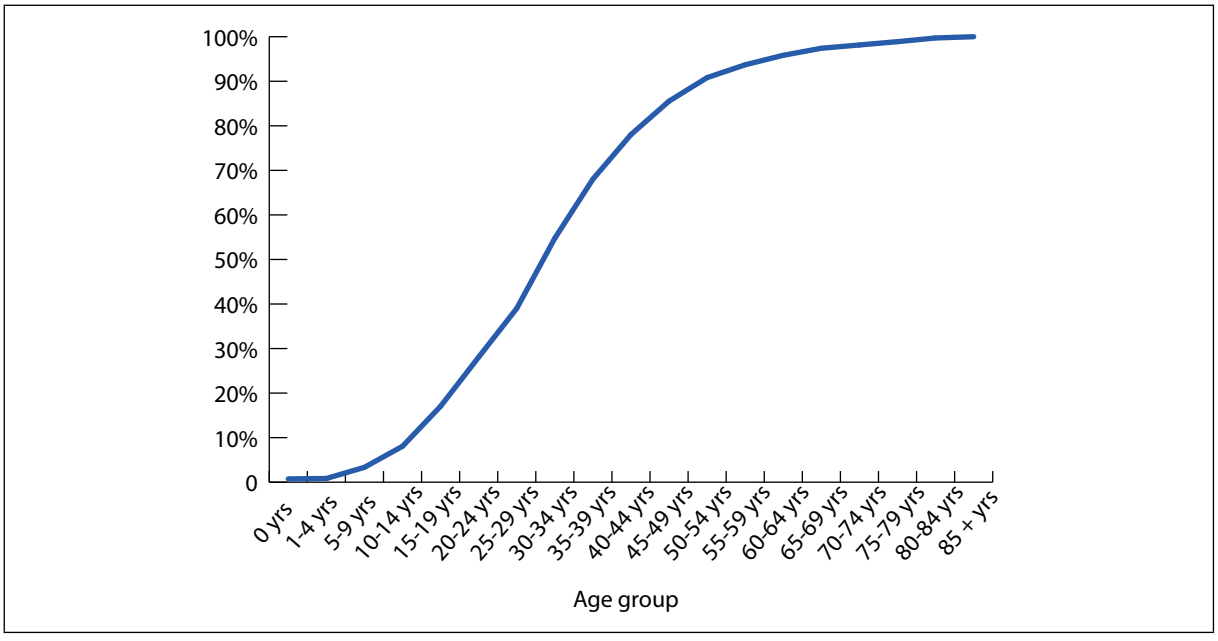

Figure 1
Admissions to Emergency Departments of women who have experienced violence. Cumulative percentage distribution by age. Source: European Injury Database.

Suffering violence represents one of the components of that causal process that leads to the onset of chronic and non-communicable pathologies. In fact, the prolonged stress caused by gender violence leads to an activation of allostatic systems with serious consequences on health. Understanding and identifying the epigenetic modifications that arise following the violence suffered is important precisely because of their reversibility. Epigenetics represents the molecular mechanism capable of connecting society to biology, the human and social sciences to the life sciences.

In order to learn more about the long-term health effects of violence against women, the Italian Ministry of Health has funded a multicenter, transdisciplinary project, "Violence against women: long-term health effects for precision prevention". This research, which adopts a transdisciplinary approach, aims to propose a series of innovative and interconnected strategies designed to ensure long-term support for women who have experienced violence. These strategies focus on the early detection of chronic, non-communicable diseases that may stem from the trauma they have endured.

One of the research lines of this project is to integrate the national guidelines [14] with a tool that allows the detection of multiple aspects of interest for the purposes of characterizing the violent event: from the context of the aggression to the victim-aggressor relationship, from the risk of recidivism to the evaluation of post-traumatic stress disorder (PTSD). This assessment tool aimed at improving innovative approaches and limiting the effect long-term impact of violence on women's health is a questionnaire developed in Microsoft Forms which is made up of 9 sections, each of them dedicated to exploring specific issues.

The purpose of this Brief Note is to illustrate and describe the digital questionnaire realized as a deliverable of the project (*see Supplementary Material available online*).

DEVELOPMENT OF EpiWE ASSESSMENT TOOL (EpiWEAT)

The use of an electronic questionnaire to collect data on violence against women plays a pivotal role in developing an effective local support model. By standardizing and centralizing the data, it ensures thorough assessments and enhances coordination among healthcare providers, law enforcement, and social services. Moreover, the data collected is essential for interpreting epigenetic results, as it provides context for biological processes influenced by environmental, lifestyle, and health factors. Without accounting for these variables, epigenetic findings may be misinterpreted.

The proposed model seeks to establish a comprehensive territorial approach to long-term care for women, based on the principles of precision medicine. The EpiWE assessment tool (EpiWEAT), aims to centralize all relevant information about the violence experienced by the woman into a single evaluation tool. This tool will support health and social services in offering the most appropriate assistance while also identifying early indicators of potential chronic conditions. The questionnaire will gather data about the context of the attack, the relationship between the victim and the perpetrator, the aggressor's age group, the risk of recidivism, and the presence of depression and PTSD in women victims of violence.

The questionnaire – currently developed in Italian but adaptations into other languages by native speakers are in progress – is organized into 9 sections (*Figure 2*).

Each section is independent of the others and the woman can stop answering the questions at the end of each section in case she doesn't feel comfortable in proceeding. More in detail, the questionnaire is structured as follows:

- *Section 1* includes the data collection phase, facility identification code, patient identification code;
- *Section 2* contains the "Socio-personal data" (patient's level of education, relationship with current partner, patient's occupation, citizenship or foreign country of birth of the patient, number of cohabiting minor children, number of cohabiting children with disabilities, number of minor children from another partner);
- *Section 3* consists of the "External circumstances of the violence" (victim-aggressor relationship, sex of the aggressor, age class of the aggressor, type of event whether single or repeated, type of violence suffered and when it occurred, type of violence suffered and for how long suffers it, assessment of the severity of the violence);
- *Section 4* evaluates the re-victimization risk through the "Severe recidivism risk assessment (danger assessment, DA-5)" [15];
- *Section 5* includes "Chronic diseases and conditions, signs and symptoms" information (eating disorders, uro-gynecological disorder, sexual dysfunction, gastrointestinal pathology, sequelae or complications of trauma, burn or poisoning, motor, sensory or functional disability, other pathology, sign or symptom);
- *Section 6* comprises "Taking of drugs" (indicate which drug(s) you take);
- *Section 7* consists of information about "Psychiatric/psychological therapy or consultancy" (psychiatric/psychological therapy or consultancy, taking antidepressants or anxiolytics, if following a path with a psychotherapist, if following a path with a psychiatrist);
- *Section 8* includes the ITQ-International Trauma Questionnaire (assessment of PTSD using the ITQ Italian version) [16] to evaluate the PTSD presence;
- *Section 9* contains the "Depression Assessment" (assessment of depression using the Center for epidemiologic studies depression scale revised (CESD-R) abbreviated depression scale; https://cesd-r.com/about-cesdr/).

At the end of the questionnaire, the woman is given the opportunity to express her willingness to join or not to join the epigenetic study, which will include the donation of a blood sample.

A closed-ended response structure was chosen primarily because the predefined options reduce the cognitive load on the women interviewed, thus increasing the response rate. In addition, data analysis is also simplified, as responses can be easily coded and processed in an automated manner, allowing for faster and more accurate statistical analysis. Another advantage is the consistency and comparability of the data, as all

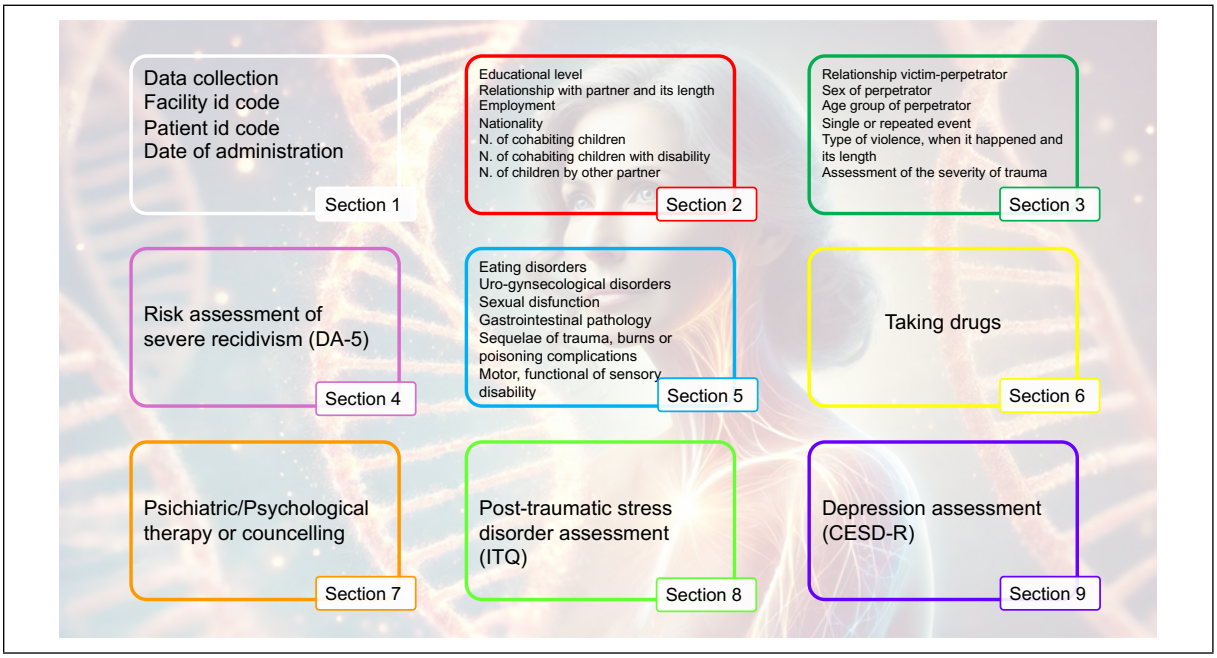

Figure 2
Epigenetics for Women Assessment Tool (EpiWEAT): diagram illustrating the structure of the questionnaire and its sections. The image in the background was created using a prompt generated with ChatGPT.
DA-5: danger assessment-5; ITQ: International Trauma Questionnaire; CESD-R: Center for epidemiologic studies depression scale revised.

women respond in a standardized manner, eliminating ambiguities and facilitating the comparison of results. Lastly, closed-ended answers also reduce the interpretation bias, offering more objective results and reducing subjective influence during data analysis. This format allows for targeted information, preventing vague or irrelevant answers, especially on sensitive topics.

Data are collected with Microsoft Forms and stored in the Microsoft cloud tenant dedicated to ISS, in full compliance with the General Data Protection Regulation (GDPR) rules. Results from the questionnaire will be accessible exclusively to the epidemiological staff of the Environment and Health Department of Italian National Institute of Health (Istituto Superiore di Sanità – ISS) involved in the project.

The assessment tool will allow greater collection of information on the circumstances of the violent event which, together with psychological assessments, will be the real assessment and prevalence tool of complex PTSD and PTSD. Furthermore, the collection of biological samples, for future studies of the epigenome, will allow the creation of a network of information and research.

The questionnaire will be accessed from any device connected to the Internet, including computers, tablets and smartphones, through a link generated directly by the application. The link will be sent to the heads of the project's operational units, who will oversee identifying the professionals (doctors/psychologists) who will manage the questionnaire's administration, after signing the informed consent.

The topic of violence is very sensitive and can evoke strong emotions or traumatic memories in respondents. When filling out a self-administered questionnaire, there is no immediate support available for the person who may be overwhelmed by intense emotions or discomfort. For this reason, the administration of the questionnaire will be conducted by a professional, who may be able to provide immediate psychological support if signs of stress or trauma emerge. The questionnaire can initially be administered when the woman is admitted to the Emergency Room, provided she is able and willing to complete it at that time. If she is unable to complete it during admission due to medical or emotional reasons, it may be administered later, for instance, during the first follow-up visit scheduled a few days after admission. This approach ensures flexibility in the timing, allowing for a sensitive and supportive process that respects the patient's physical and emotional state.

From tests with a convenience sample, the questionnaire proved to be an easy assessment tool to administer, with comprehensible questions and a duration compatible with stressful situations. The complete compilation takes an average of 20 minutes, but the possibility of stopping the administration at the end of each section and resuming it later minimizes the emotional impact on the woman victim of violence.

This questionnaire will be administered by a professional, such as a psychologist, for several important reasons related to the sensitivity of the topic and the need to ensure the well-being of the person involved. First, violence is an extremely delicate issue that can trigger strong emotions, including fear, anxiety, shame, or guilt. A professional can provide immediate emotional support and promptly intervening if the woman shows signs

of distress or suffering during the questionnaire. This helps prevent the person from feeling overwhelmed or abandoned while dealing with traumatic memories.

Furthermore, a professional can create a safe and protected environment, fostering a relationship of trust where the woman feels free to speak without fear of judgment or negative consequences. This atmosphere of trust is essential for ensuring truthful and in-depth responses. Another important aspect is the psychologist's ability to recognize signs of trauma. Victims of violence often exhibit intense emotional reactions or trauma-related disorders, and only a professional can identify these reactions and adjust their approach to avoid re-traumatizing the interviewee.

Some questions, in this questionnaire, can be highly intimate and difficult, and handling such questions requires particular care and sensitivity. A professional can guide the woman through these questions, explaining their purpose and reassuring her that she is not obliged to answer if she feels uncomfortable. This helps to reduce distress and ensures better comprehension of the questionnaire.

Another reason why the involvement of a professional in questionnaire administration is crucial concerns the quality and accuracy of the data collected. Violence is a complex issue, and a professional has the skills to formulate and collect information accurately, avoiding distortions or misinterpretations. Additionally, if information arises that indicates immediate danger or a need for urgent help, the professional can intervene by guiding useful resources, such as domestic violence centers or legal and psychological support.

Lastly, a professional knows how to manage incomplete or ambiguous responses, adjusting the interview without forcing answers, and understanding the power and control dynamics that often accompany violent situations.

DISCUSSION

Despite increasing awareness of gender-based violence, its impact on women's long-term health remains understudied and understood. The physical, emotional and psychological scars left by violence often linger for years, yet medical and public health systems often lack the means to assess the full extent of these impacts. Chronic conditions, such as PTSD, can develop as a result of prolonged trauma, yet the complex relationship between violence and long-term health remains underrecognized and unaddressed in most medical settings.

The EpiWEAT, designed to evaluate and gather data on violence against women, holds significant potential for both immediate and long-term applications in healthcare, social services, and law enforcement. By centralizing critical information about the context of violence, the victim-perpetrator relationship, and the presence of physical and psychological symptoms, this tool provides a comprehensive resource for professionals to offer tailored support to women experiencing violence. In the short term, the tool's use will facilitate more accurate risk assessments, ensure better coordination between healthcare providers, and guide the implementation of preventive measures aimed at mitigating long-term health consequences, particularly chronic and non-communicable diseases linked to violence.

The EpiWEAT could be expanded to other settings beyond Italy, allowing for broader geographic applicability. While currently designed to operate in the Italian context, with the potential for adaptations into other languages, its framework offers a flexible model that could be tailored to different cultural, social, and healthcare systems. As such, the tool could be implemented internationally, providing data that can inform global health policies aimed at improving the outcomes of women suffering from violence. Additionally, this tool could serve as a valuable component in large-scale longitudinal studies, contributing to a deeper understanding of the relationship between violence, epigenetics, and long-term health effects.

In terms of practical use, the integration of the tool into the routine practice of healthcare professionals, social workers, and law enforcement officers would be critical to ensure its success. Training professionals in its use and interpreting the data gathered will be an essential step toward establishing the tool as a routine part of the support infrastructure for women who have suffered violence. This also provides an opportunity for continuous improvement of the tool, through feedback from end users and the incorporation of emerging scientific evidence into future iterations.

The broader implications of this tool lie in its potential to influence healthcare practices and policies related to violence against women. By linking the psychological and physical effects of violence to biological markers, it opens a pathway for the integration of precision medicine into the management and care of women who have experienced violence. This approach acknowledges the need for gender-sensitive care and offers a model for addressing the long-term health needs of survivors in a more holistic manner. Additionally, by incorporating epigenetic analysis, this tool could help to highlight the biological impact of violence and contribute to an evolving understanding of the mechanisms underlying trauma, potentially influencing how healthcare systems approach trauma-informed care.

In the future, multisectoral public health interventions are required, where strong scientific bases are integrated with rigorous statistical analysis, as well as clinical and regional welfare analysis. In a few years it will be possible to make a specific prevention, starting from the epigenomic profile of the woman who has suffered violence by preventing the long-term outcomes caused by the onset of non-communicable, chronic, and disabling pathologies such as cancer or cardiovascular or autoimmune diseases. Precision prevention medicine can guarantee specific care to help women who have experienced violence become more resilient.

CONCLUSIONS

In conclusion, there is strong evidence to suggest that this questionnaire will provide valuable insights and raise awareness about violence, helping to shift priorities in managing the everyday challenges associated with various forms of illness for women who have experienced abuse and violence. The interdisciplinary approach to

analyzing violence against women brings together a range of theoretical perspectives, such as public health, epigenetics, psychology, and sociology, each offering a unique lens through which to understand this complex issue. The goal is to stimulate new, innovative research that bridges these disciplines, ultimately leading to the development of public health policies aimed at improving the well-being of women in the medium term.

By integrating personal data with epigenomic analysis, we can significantly enhance our understanding of individual health profiles, thereby refining precision prevention strategies. Combining genetic data with factors like exposure to violence, lifestyle choices, environmental influences, and behavioral patterns will provide a clearer picture of a person's health risks, allowing for more tailored preventive measures. For example, epigenetic markers can reveal how a person's environment or decisions have influenced their gene expression, potentially contributing to diseases like cancer or heart disease. When these insights are paired with personal health information, such as medical history, family background, and real-time health monitoring, they open the door to more personalized, proactive interventions. The future of precision prevention relies on this integration, as it enables targeted risk assessments and more effective preventive measures. It will not only help predict the conditions someone is at risk for but also when they might develop them and how they will respond to various treatments or interventions.

Ethics Committee

The EpiWEAT was approved by the National Ethics Committee of the Italian National Institute of Health (Istituto Superiore di Sanità, ISS), Rome, Italy (Protocol number: PRE BIO CE n. 4113 del 30 January 2025).

Acknowledgments

The Authors thank the Italian Ministry of Health for the technical and financial support: CCM – Azione Centrale Project "Violenza sulle donne: effetti a lungo termine sulla salute per una prevenzione di precisione". Furthermore, the Authors thank all participants of the Operational Units (Unità Operativa, UOs) that are involved in this project: UO1 - Antonella Crenca (Dipartimento Ambiente e Salute, Istituto Superiore di Sanità); UO2 - Loredana Falzano (Centro per la Salute Globale, Istituto Superiore di Sanità), Sara Mellano (Servizio Relazioni Esterne Centro Rapporti Internazionali, Istituto Superiore di Sanità), Antonella Pilozzi (Centro Nazionale Prevenzione delle Malattie e Promozione della Salute, Istituto Superiore di Sanità), Mariella Nocenzi, (Dipartimento di Comunicazione e Ricerca Sociale, La Sapienza Università di Roma); UO3 - Paolo Bailo (Sezione di Medicina Legale, Università degli Studi di Camerino); UO4 - Pierluigi Daniela, Fabio Pastorelli, Enzio Berbessi, Elena Cazzulo (Ente ospedaliero "Ospedali Galliera", Dipartimento funzionale Emergenza Accettazione - DEA); UO5 - Sabrina Pizzulo, Cinzia Tellarini, Maria D'Alessandro, Giovanna Savarese (Unità Operativa Complessa, Tutela della Salute della Donna e dell'Età Evolutiva della ASL Roma 2), UO6 - Paola Iovino (Dipartimento di Studi Politici e Sociali, Università degli Studi di Salerno), UO7 - Giulia Sannolla (Gruppo di lavoro per la governance della rete socio-sanitaria per il contrasto della violenza all'infanzia della Regione Puglia).

Authors' contributions

MG, AC and SG conceived and designed the study and wrote the manuscript. DLB, EC, PM, GC, MGFB, AP, PC revised and edited the manuscript. All Authors revised the manuscript for important intellectual content and agreed with this article's contents.

Conflict of interest statement

The Authors declare no competing interests.

Received on 7 November 2024.
Accepted on 11 February 2025.

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Ann Ist Super Sanità 2025 | Vol. 61, No. 2: 102-108
DOI: 10.4415/ANN_25_02_02

Comparison of corticosterone responses to acute stress in mice following different serial blood collection methods

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Abstract

Background. Accurate evaluation of glucocorticoid concentrations during serial blood collection in rodents is often hampered by the stress response elicited by the procedure itself. The optimal method to minimize stress and impact on animal welfare remains debated.

Methods. Hence, we compared corticosterone concentrations in adult mice serially bled by using the retro-orbital sinus puncture or the tail vein incision methods, either with or without exposure to an acute restraint stress.

Results. Corticosterone concentrations were significantly affected by the sampling method, with higher peaks and sustained hypercortisolemia in mice bled with the retro-orbital sinus puncture, pointing to the tail vein incision method as preferable for serial blood collections. Mice bled using the tail vein incision reached similar corticosterone peaks regardless of exposure to acute stress.

Conclusions. Our findings suggest that tail vein incision can be used to evaluate neuroendocrine reactivity without exposing mice to restraint procedures. This would improve animal welfare practices in experimental protocols.

Key words

- restraint stress
- glucocorticoid
- retro-orbital sinus
- tail vein
- animal welfare

INTRODUCTION

When studying the hypothalamic-pituitary-adrenal (HPA) axis, it is crucial to minimize variables that could influence experimental outcomes, in particular glucocorticoid levels. Rodents are highly sensitive to environmental changes, physical handling, restraint and pain [1, 2]. These factors, which are inherent to blood collection procedures, may impact HPA axis activity, affecting results of blood assays and compromising animal welfare. Consequently, plasma concentrations of corticosterone, the main glucocorticoid in rodents, are frequently artificially elevated, creating inaccurate experimental outcomes [3].

Methods for measuring corticosterone concentrations that cause minimal disturbance to the animals during collection pose limitations that prevent their use as an alternative to blood sampling. For instance, there is a significant, not easily quantifiable, delay between the appearance of corticosterone in feces and changes in blood corticosterone. Similarly, corticosterone levels in hair samples can only inform about chronic conditions. Furthermore, neither of these two non-invasive methods is sensitive enough to detect acute (e.g., over minutes/hours) or minor corticosterone fluctuations [3].

Since there is no suitable alternative to blood sampling for evaluating acute changes in corticosterone levels in rodents, it is crucial to choose a collection method that lessens stress-related artifacts and minimizes pain and distress, as ethically and legally required by the principle of refinement [4]. Multiple sites are routinely used for blood collection, including the retro-orbital (or retro-bulbar) sinus/plexus, lateral tail vein, jugular vein, saphenous vein, heart, sublingual vein and facial (or submandibular) vein. As for the lateral tail vein, blood droplets can be obtained through different methods, including tail vein puncture (i.e., inserting a needle into the vein), tail vein incision (i.e., using a blade, also known as tail nick) and tail snip (i.e., amputation of the tip of the tail, also known as tail clip). These tail sampling procedures involve different levels of handling/restraint and potentially different levels of pain, which can confound the results of corticosterone analyses and impact animal welfare [5, 6].

The retro-orbital sinus/plexus method is widely performed as it allows a skilled experimenter to rapidly obtain blood samples of good quality (e.g., not subjected to hemolysis); however, multiple side effects have also been reported following the application of this tech-

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nique. For example, Mahl and co-authors [7] reported, in rats, higher degree of tissue damage and stress associated with blood sampling from the retro-orbital plexus than from the sublingual vein (both performed after isoflurane anesthesia). However, Teilmann and co-authors [8], who compared, in mice, retro-orbital sinus and facial vein punctures (both without anesthesia), found that the impact of the latter was more severe, as indicated by increased plasma corticosterone levels, while the level of tissue trauma was comparable. Further, Tsai and co-authors [9] suggested that, in mice, retro-orbital bleeding (without anesthesia) causes the least stress (also compared to tail vein puncture), whereas jugular vein bleeding and facial vein bleeding cause the most stress and saphenous vein bleeding causes the most lasting damage. Hence, the advantages and disadvantages of this method remain controversial.

Although several studies have compared the different blood collection methods and the associated effects on corticosterone levels, most consider only one or two time points. Only few comparative studies on serial blood collection have been conducted. Frohlich and co-authors [10] compared serial blood collection (once weekly for 6 consecutive weeks) by retro-orbital (after isoflurane anesthesia) and facial vein (without anesthesia) methods in mice and found that the latter caused substantial morbidity and mortality compared with the former. Opposite results were found by Jo and co-authors [11] using the same collection methods, under the same anesthesia regimen, but with different timing (on alternate days for 2 weeks).

In the present study, we compared plasma corticosterone levels during serial blood collection by the retro-orbital sinus puncture and tail incision methods. The latter is a commonly used alternative, which poses different advantages (e.g., light handling) and drawbacks (e.g., poor-quality samples). It also allows the blood flow to be started and stopped easily, facilitating the collection of small volumes of blood (typically 30-40 µl), a crucial aspect when it is necessary to take repeated samples from the same subject at multiple time points over few hours [6]. Conversely, when applying the retro-orbital puncture technique, it is fundamental to carefully monitor the amount of blood drawn in order not to exceed the recommended 10% circulating blood volume (NC3Rs Guidelines 2021, https://nc3rs.org.uk; NIH Guidelines 2022, https://oacu.oir.nih.gov).

The tail incision method is applied in unrestrained, freely moving mice, which appear almost undisturbed by the collection process. Many investigators favor this technique in behavioral studies as it is often considered a stress-free procedure [12, 6]. Hence, the first objective of the present study was to assess whether corticosterone levels would remain low with repeated collections over 2 h.

To study the reactivity of the HPA axis, after baseline blood sampling, animals are generally exposed to an acute stress (usually restraint stress) [13] and then subjected to additional blood collections over few hours to assess corticosterone peak concentrations and time course. However, to our knowledge, there are no comparative studies that assess corticosterone response following acute restraint stress using different serial blood collection methods. Hence, the second objective of the present study was to ascertain whether stress hormone profiles (in terms of peak responses and/or return to baseline) following restraint stress were affected by the sampling procedure, by comparing the retro-orbital sinus puncture and the tail incision methods.

MATERIALS AND METHODS

Ethics statement

All experimental procedures were approved by Institutional Animal Survey Board on behalf of the Italian Ministry of Health (license n. 409/2018-PR) and performed in full accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes and Italian Law (D.lgs. 26/2014).

Animals and rearing conditions

Experimental subjects were 20 CD1 male mice of approximately twelve weeks of age provided by Charles River (Calco, Lecco, Italy). Mice were housed in pairs in 33×13×14 cm polycarbonate cages with metal tops and with sawdust bedding and left undisturbed for four weeks prior to the experiment.

All animals had *ad libitum* access to tap water and food (Mucedola, Settimo Milanese, Milan, Italy) and to environmental enrichment in the form of shelter material (Nestlets®, Ancare, Bellmore, New York, USA). Animals were housed in an air-conditioned room (temperature 22±1 °C, relative humidity 45±5%), on a 12-h reversed light-dark cycle (lights off at 7:00 am).

Experimental design

In order to investigate the impact of different blood collection methods on the physiological stress response (i.e., plasma corticosterone concentration), mice were subjected to the collection of small blood samples at different time points from either the tail (TAI group) or the retro-orbital sinus (ROS group). In addition, for each method, half of the animals, after baseline sampling, were exposed to an acute restraint stress procedure (ARS group) by placing them, for 25 min, in transparent conical polypropylene tubes (3.0 cm outer diameter, 11.5 cm length; 50 ml Falcon®, Corning, New York, USA), perforated in various points (including the cap) to allow breathing/transpiration and to position the tail; at the end of the restraint stress, mice were returned to their cages. The remaining half were not subjected to the stress procedure and were therefore returned to their home-cages immediately after baseline sampling (CTRL group).

Thus, mice were randomly divided into four groups: i) mice bled from the tail and returned to the home-cage after baseline sampling (TAI-CTRL group); ii) mice bled from the tail and exposed to the 25-min restraint stress (TAI-ARS group); iii) mice bled from the retro-orbital sinus and returned to the home-cage after baseline sampling (ROS-CTRL group); iv) mice bled from the retro-orbital sinus and exposed to the 25-min restraint stress (ROS-ARS group).

Blood collection

Two mice were simultaneously taken to an adjacent room by two skilled experimenters and bled (t0) by either method (see below for details). The time elapsed between the experimenter entering the facility room and the completion of baseline blood sampling was less than 3 min [5]. After 25 minutes mice were bled again (t25) and then relocated to their home-cage. Additional samples were taken 35 min (t60) and 95 min (t120) later. To prevent potential confounders (e.g., corticosterone diurnal fluctuations, variations in technical expertise), testing was performed within a 2-hour time window (between 10:00 am and 12:00 am), simultaneously in all mice and by experimenters with extensive experience, ensuring a high level of technical proficiency and reproducibility.

For each sampling, approximately 30-45 µl blood was collected; hence, the total volume collected over 2 h did not exceed the recommended 10% of the circulating blood volume that can be safely removed at one time (ranging from 110-140 µl for a 20 g mouse to 170-210 µl for a 30 g mouse; NC3Rs Guidelines 2021, https://nc3rs.org.uk; NIH Guidelines 2022, https://oacu.oir.nih.gov).

Blood sampling from the tail

During each sampling, the animal was placed on the standard metal cage's lid and let free to move [6, 14, 15]. For baseline sampling (t0), the experimenter made a small nick (approximately 2 mm wide×0.5 mm deep) in the tail with a callus cutter blade (Credo, Haan, Germany), perpendicular to the tail vein, approximately 2 cm from the tip of the tail. Blood droplets were directly collected into capillary tubes (Microvette® 100 EDTA K3E, 100 µl, ref. 20.1278, Sarstedt AG & Co. KG, Nümbrecht, Germany). The subsequent sample (t25) was usually collected from the same tail incision, after gently removing the clot. Additional samples (t60 and t120) were generally collected from incisions at different locations, working towards the base of the tail in 0.5 cm increments. The blood flow was encouraged by gently stroking the tail and, usually, blood flow stopped spontaneously when stroking was stopped.

Blood sampling from the retro-orbital sinus

During each sampling, following application of a drop of ophthalmic anesthetic, the animal was held with the nondominant hand using the thumb and forefinger, and the skin around the eye was pulled taut [16]. A capillary tube (disposable glass Pasteur pipettes, VOLAC®, ref. D810) was inserted into the medial canthus of the eye (30-degree angle to the nose) through a slight thumb pressure and twisting motion that was enough to puncture the tissue and enter the orbital sinus. Once the required volume of blood was collected, the capillary tube was gently removed. Bleeding was stopped by applying gentle finger pressure over the puncture site. This procedure was repeated for all four samples (t0, t25, t60, t120). From the pipette, blood was immediately transferred into EDTA-coated tubes (Microvette® 100 EDTA K3E, 100 µl, ref. 20.1278, Sarstedt AG & Co. KG, Nümbrecht, Germany).

Plasma corticosterone measurement

Samples were cool centrifuged (2,500 rpm, 20 min at +4 °C) and the plasma stored at –80 °C until assayed. Corticosterone concentration was measured using a commercially available Corticosterone Enzyme-Linked Immunosorbent Assay kit (ADI-900-097, Enzo Life Sciences, Farmingdale, New York, USA) as previously described [17].

Briefly, samples (6 µl) were analyzed in duplicate. Corticosterone concentrations were determined based on the corticosterone standard curve (range 32-20,000 pg/ml) incubated under similar conditions in each assay. Only data derived from duplicates with <20% coefficient of variation were included in the analysis. The sensitivity of the assay was 27.0 pg/ml. Light absorbance was read with a light absorption microplate reader (AMR-100, Hangzhou Allsheng Instruments, Hangzhou, China) at 405 nm. Data were elaborated by sigmoidal 4-parameter logistic curve fit using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Statistical analysis

Corticosterone concentrations at different time points were analyzed using repeated measures ANOVA, with "bleeding technique" (two levels: tail *vs* retro-orbital sinus) and "stress exposure" (two levels: control *vs* acute restraint stress) as between-subjects factors and repeated measures ("time", four levels time point: t0, t25, t60, t120) as within-subjects factor.

Total corticosterone secretion was calculated using the area under the curve with respect to ground (AUC_G) formula [18, 19] and was expressed in arbitrary units. The AUC_G was analyzed using ANOVA with "bleeding technique" (two levels: tail *vs* retro-orbital sinus) and "stress exposure" (two levels: control *vs* acute restraint stress) as between-subjects factors.

Multiple post hoc comparisons were performed using Tukey's post hoc test where appropriate [20]. All statistical analyses were conducted using the software StatView 5.0.1 (SAS Institute Inc., Cary, North Carolina, USA). Data are expressed as mean±standard error of the mean (SEM). Statistical significance threshold was set at $p \leq 0.05$.

RESULTS

As expected, all groups exhibited similar basal plasma corticosterone concentrations (t0). In the absence of a main effect of "stress exposure" ($F(1,16)=1.221$, $p=0.2856$), the repeated measures ANOVA yielded both a significant main effect of the "bleeding technique" ($F(1,16)=63.536$, $p<0.0001$) and a tendency towards a significant "stress exposure" by "bleeding technique" interaction ($F(1,16)=3.604$, $p=0.0758$).

Noticeably, the subsequent prompt physiological response ("time": $F(3,48)=79.747$, $p<0.0001$) was primarily induced by the stress associated with the bleeding procedure rather than by the exposure to the restraint stress ("bleeding technique"×"time": $F(3,48)=17.661$, $p<0.0001$; "stress exposure"×"time": $F(3,48)=1.179$, $p=0.3274$).

Multiple comparisons on the significant "stress exposure" by "bleeding technique" by "time" interaction

(F(3,48)=3.262, p=0.0293) evidenced that mice bled from the tail and mice bled from the retro-orbital sinus showed a different pattern of response over time, regardless of the stress exposure (*Figure 1*).

Specifically, TAI subjects showed an increase in corticosterone concentrations at either point t25 (CTRL) or t60 (ARS); such rise steadily declined at point t120 in both TAI groups. Moreover, visual data inspection suggested that while control subjects bled from the tail (TAI-CTRL) showed the highest corticosterone level at point t60, TAI-ARS mice appeared to show the corticosterone peak already at point t25, i.e., immediately after the end of the restraint procedure (*Figure 1*). By contrast, ROS subjects showed a significantly more pronounced corticosterone response compared to TAI mice at all time points (t25, t60, t120) in CTRL mice and at points t60 and t120 in mice subjected to the restraint procedure (ARS). No significant differences were found between ROS-CTRL and ROS-ARS groups. In addition, in both ROS groups, the steady increase in corticosterone concentration continued well beyond point t60, possibly reaching the peak of response at point t120 (*Figure 1*).

Finally, analysis of total corticosterone secretion (AUC_G) revealed a similar pattern. Indeed, differences in the AUC_G were primarily induced by the stress associated with the bleeding procedure rather than by the exposure to the restraint stress ("bleeding technique": F(1,16)=53.091, p<0.0001; "stress exposure": F(1,16)=1.449, p=0.2462; "stress exposure"×"bleeding technique": F(1,16)=2.348, p=0.1450). In particular, both ROS-CTRL and ROS-ARS mice showed significantly increased AUC_G response compared to TAI-CTRL and TAI-ARS mice respectively (*Figure 2*).

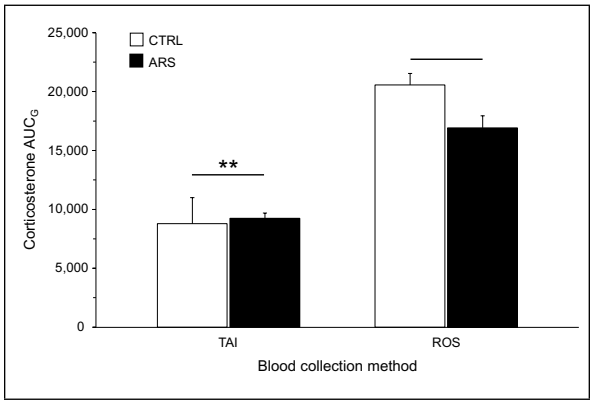

Figure 2
Plasma corticosterone area under the curve with respect to ground (AUC_G; expressed in arbitrary units) in mice bled from either the tail (TAI group) or the retro-orbital sinus (ROS group) and either returned, after baseline sampling, to the home-cage (CTRL group) or exposed to an acute restraint stress (ARS group); **p≤0.01, main effect of the bleeding technique (TAI *vs* ROS; n=10 per group).

DISCUSSION

Here we compared plasma corticosterone concentrations in mice serially bled by using the retro-orbital sinus puncture or the tail incision, either exposed, after baseline sampling, to an acute restraint stress or left undisturbed in their home-cages. We found that the differential increase in corticosterone concentrations was primarily induced by the stress associated with the two bleeding procedures rather than by the exposure to the 25-min restraint stress.

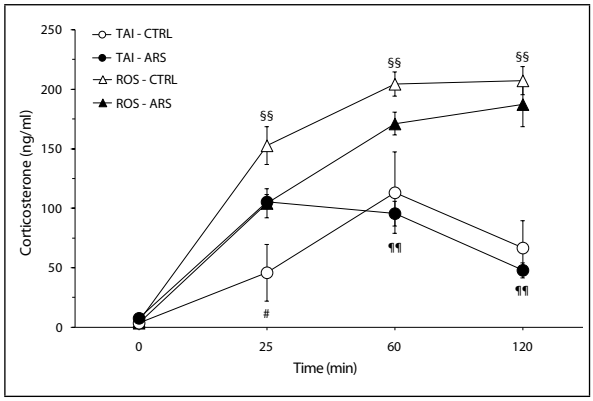

Figure 1
Plasma corticosterone concentrations (ng/ml) at different time points (at baseline and after 25, 60 and 120 min) in mice bled from either the tail (TAI group) or the retro-orbital sinus (ROS group) and either returned, after baseline sampling, to the home-cage (CTRL group) or exposed to an acute restraint stress (ARS group); §§p≤0.01, TAI-CTRL *vs* ROS-CTRL; ¶¶p≤0.01, TAI-ARS *vs* ROS-ARS; #p≤0.05, TAI-CTRL *vs* TAI-ARS (n=5 per group).

In terms of corticosterone peak concentrations, the impact of the two sampling methods was profoundly different, with concentrations in mice bled by retro-orbital sinus puncture almost doubling those of mice bled by tail incision. The marked response observed in mice bled by the retro-orbital sinus could be ascribed to the necessity of firmly restraining the subject for the duration of the blood sampling at each time point and/or to the greater invasiveness of the technique. This interpretation is supported by the absence of a decline in corticosterone concentrations following the ROS procedure even after 120 minutes from the first sampling. However, this procedure is traditionally considered to only cause transient distress and, therefore, the application of a topic ophthalmic anesthetic is usually considered sufficient. By contrast, in rats, because of the presence of a venous plexus rather than a sinus, the use of general anesthesia is widely recommended.

Although general anesthesia is effective to avoid exposure to pain and distress potentially associated with blood sampling procedures, it does not prevent the elevation of plasma corticosterone concentrations. For example, Vachon and Moreau [21] found that (i) corticosterone increased significantly, with a peak at 30 min, in both anesthetized non-cannulated and non-anesthetized jugular-cannulated rats after repeated blood sampling over 2 h, (ii) corticosterone was significantly lower at the 60 and 120 min time points in awake cannulated rats compared with rats undergoing repeated isoflurane anesthesia. Furthermore, mice subjected to both retro-orbital sinus puncture during isoflurane anesthesia and to isoflurane anesthesia alone (no puncture of the si-

nus) showed higher plasma corticosterone concentrations compared to tail incision and tail snip, both performed by means of a restraint device [22]. Finally, Kim and co-authors [3] found that tail snip and retro-orbital sinus puncture in anesthetized mice were associated with higher basal plasma corticosterone levels compared to tail snip in non-anesthetized mice (up to a 20-fold increase). Hence, this elevation was related to the intraperitoneal injection of the anesthetic (ketamine/xylazine) rather than to the bleeding procedure itself. In the present study, we did not find any difference between sampling methods at baseline, likely because the retro-orbital sinus puncture was not performed under general anesthesia.

Mice bled from the tail and mice bled from the retro-orbital sinus showed a different pattern of response over time. While the latter exhibited a steady increase in corticosterone concentrations, which possibly reached the peak 120 min after the baseline, the former showed peak concentrations either at 25 min (mice exposed to restraint stress) or at 60 min (control mice not exposed to restraint stress) time points, followed by a steady decline 120 min after baseline in both groups. In this respect, it should be noted that during bleeding by tail incision, the animal was free to move on the cage's lid. However, transportation of the cage to the adjacent room [4], removal from the home-cage at each sampling point and/or the transient pain associated with the tail incision appeared to have been sufficient to alter secretory patterns of circulating stress hormones, thus inducing a corticosterone response also in mice not exposed to the restraint procedure. Indeed, the two groups of mice bled by tail incision reached similar corticosterone peak concentrations, although they differed regarding time-to-peak, as detailed above.

Based on our results, repeated blood sampling by tail incision does not appear to be a stress-free procedure as previously suggested [6, 12]. These authors found that corticosterone concentration following serial blood collection was either only slightly increased [12] or not increased at all [6] above the baseline of the first sample. However, this conclusion was based on samples collected 80, 100, 120, 150, 180 and 240 min [12] and 24 and 48 h [6] after baseline sampling, when a potential procedure-induced corticosterone elevation might have already returned to baseline. On the contrary, in the present study, we monitored the stress response at an earlier stage, collecting samples 25, 60 and 120 min after baseline sampling. This may have allowed us to evidence an early corticosterone elevation (within 60 min) in control mice (i.e., not exposed to restraint stress) bled by the tail incision method.

Similar results to those achieved in the present study, in terms of both peak concentrations and time course, were obtained by Kim and co-authors [3] using the tail snip method. Specifically, they found that repeated collections (30, 60, 90, 120 min) in freely moving mice induced a significant 10-fold increase in plasma corticosterone after 30 min, which remained at similar levels for the duration of the study, with only a slight decrease at 90 and 120 min. They also found a similar 10-fold increase in corticosterone levels in mice briefly restrained (for less than 2 min) during the bleeding procedure when a second sample was collected 120 min after baseline [3]. Interestingly, Harikrishnan and co-authors [23] found reduced nest building activity in the home-cage, reduced activity in the open field test and increased anxiety in the elevated plus maze test both in anesthetized mice following retro-orbital sinus puncture and in mice bled by tail vein incision while contained in a restraint device.

Notwithstanding the reasons for the more pronounced and prolonged peak in mice bled from the retro-orbital sinus, the exposure to the 25-min restraint stress (immediately after baseline sampling) had no additive effect on the corticosterone response. We believe that the extremely stressful nature of the ROS procedure itself leads to a ceiling effect, thus preventing the detection of a further elevation in corticosterone following ARS exposure. Nevertheless, we cannot exclude that increasing the sample size would allow to detect additional differences between groups. By contrast, in mice bled from the tail, acute stress exposure produced an anticipation of the corticosterone peak from 60 min to 25 min after baseline, that is immediately after the end of the restraint procedure. Based on these data, we conclude that (i) the restraint stress procedure should not be combined with the retro-orbital sinus method as it does not produce any additive effect on the stress response, (ii) in line with the principle of refinement of animal use in laboratory research, combined use of tail incision and restraint stress procedures should be avoided unless it is necessary, for reasons related to the experiment scopes, to anticipate the peak of response (from 60 to 25 min after baseline sampling).

Apparently minor methodological differences in bleeding methods (e.g., using a needle to puncture the tail vein *vs* using a blade to make a tail incision; containing the animal in a restraint device *vs* letting the animal free to move) can have a substantial impact on corticosterone concentrations. The type of material employed for blood collection tubes can also have an impact since hormones tend to adhere to plastic surfaces, potentially distorting subsequent quantitative evaluation (see [24] for a review with historical elements comparing glass and plastic tubes). Hence, results obtained in the present and in the above-mentioned studies strongly support the necessity for accurate reporting of bleeding methods in publications to allow for experimental reproducibility, interpretation of comparative reports and intra-/inter-laboratory variability [25].

Considering strain- and sex-related differences in stress responses, future studies should explore whether our findings extend to females as well as to other strains. Additionally, to capture the full complexity of the stress response, different stressors and multiple physiological outcomes should be incorporated.

CONCLUSIONS

In conclusion, our results support the adoption of the tail incision method, particularly for use in behavioral studies where use of general anesthesia and/or the implantation of indwelling cannulae is not desir-

able. Moreover, we believe that bleeding mice from the retro-orbital sinus should be avoided or performed only under general anesthesia, not only in rats but also in mice.

However, our results also evidenced that the tail incision method is not a stress-free procedure as previously suggested [12, 6]. In particular, this study demonstrates that repeated blood sampling by tail incision in control mice has a considerable impact on the animals' stress response, which should be carefully considered in studies examining the effects of stress and/or assessing behavioral phenotypes potentially affected by stress [23]. Further, as corticosterone levels do not remain low after repeated collections via tail incision in unrestrained, freely moving mice, we conclude that this sampling method can also be used for the study of the acute stress response without the necessity of exposing the animals to a restraint stress procedure. Our findings highlight not only the potential confounding effects of widely used experimental techniques on stress-related endpoints but also their relevance in the context of refinement. By acknowledging and mitigating these effects, animal welfare will be improved while enhancing the reliability of stress-related measurements.

Acknowledgements

The Authors would like to thank G. Russo and A. Leonardo for practical assistance and A. Di Virgilio for technical assistance with blood sampling.

Authors' contributions

Conceptualization: CM, FF, FZ; formal analysis: MB, FZ; funding acquisition: FZ; investigation: CC, FF; supervision: FZ; visualization: MB; writing – original draft: FZ; writing – review and editing: CM, FF.

Conflict of interest statement

The Authors have no relevant financial or non-financial interests to disclose.

Funding

No funding was received for conducting this study. For the preparation of this manuscript, partial financial support was received from the Italian Ministry of Health (Ricerca Finalizzata, Young Researchers Grant, GR-2019-12370173).

Received on 9 December 2024.
Accepted on 11 February 2025.

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Ann Ist Super Sanità 2025 | Vol. 61, No. 2: 109-115
DOI: 10.4415/ANN_25_02_03

The authorization process of observational studies in Italy: exploring two decades of Ethics Committee approval data

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Abstract

Introduction. The recent guideline from the Italian Medicines Agency (Agenzia Italiana del Farmaco, AIFA) on observational studies prompts a broader reflection on the impact of regulations on clinical research and real-world evidence. While regulations are necessary to ensure ethical and scientific standards, their effectiveness in improving research quality is unclear. It is also uncertain whether these regulations strengthen clinical research or create bureaucratic obstacles.

This quantitative, "before and after" study investigates the impact of the 2008 AIFA guideline and the 2018 General Data Protection Regulation (GDPR) on the complexity of ethical evaluation processes. As a secondary outcome, we also aimed to investigate whether the duration and probability of suspensions were influenced by intrinsic study characteristics (study design, rare disease, genetic data, post-authorization safety study).

Materials. The study analyzed the ethical evaluation process of 112 observational multicenter studies with 2,875 submissions from 2002 to 2022, included in the database of Medineos srl. The number of suspensions observed in each evaluation process was a surrogate endpoint of complexity of evaluation process.

Methods. Descriptive analyses and survival analysis were used to evaluate the total evaluation time, and a logistic model was applied to assess the probability of receiving a suspension.

Results. The median (and interquartile range) evaluation time for "pre-AIFA" submissions was 70 (41-133) days, whereas it was 75 (45-122) days for "post-AIFA" submissions. The median evaluation time was 68 (41-113) days without suspension and 127 (84-180) days with suspension. Post-AIFA submissions had a higher likelihood of suspension. The median evaluation time for "pre-GDPR" submissions was 70 (42-123) days, whereas it was 90 (63-140) days for "post-GDPR" submissions. AIFA guidelines slightly increased evaluation time and the likelihood of suspension, suggesting improved quality control. GDPR increased evaluation time due to privacy evaluations but did not affect suspension probability. Intrinsic study factors did not impact evaluation duration or suspension probability.

Conclusions. Although more extensive analyses are necessary, this study suggests that past changes in Italian regulations have affected the evaluation by the Ethics Committee (EC) and have also impacted the conduct of the observational studies. The data generated can be useful for monitoring the future impact of the recently published new AIFA guideline.

Key words

- observational study
- real world data
- ethical committee
- GDPR in clinical research
- AIFA guideline

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INTRODUCTION

"Medical research is subject to ethical standards that promote and ensure respect for all human subjects and protect their health and rights" [1].

The need of evaluation to always guarantee ethically justified research, in accordance to the Declaration of Helsinki [1], applies also to observational studies, in which subject may in any case be at risk of physical or psychological harm; even research limited to an examination of existing records, in fact, may entail a risk for the group under investigation (such as stigmatization) or it may harm people by making use of information that they regard as private. Therefore, study proposal involving human subjects must be submitted to at least one Ethics Committee (EC), and the investigators need their approval or clearance before starting the research [2].

Moreover, a valid and robust study protocol is the basis for reliable research, in particular it has to respect epidemiological principles of study design and it allows to guarantee transparency regarding methodologies used: bad science is at least poor if not bad ethics [3].

Considering all this, the value of ethical evaluation before entering into any administrative agreement is undeniable, and, on top of that, the importance of careful planning, open discussions with all concerned parties and vigorous efforts to protect confidential data, as part of good study design. But how to merge an ethical guarantee and protection of subjects' privacy with an effective and efficient authorisation process? Excessive bureaucracy, in fact, can potentially turn unethical as well as detrimental to the competitiveness of research [4-6].

In this context, the recent publication of Italian Medicines Agency (Agenzia Italiana del Farmaco, AIFA) guidelines on observational studies [7] stimulates a broader reflection on its real impact, also considering that observational studies – according to the increasing interest for real world data and realworld evidence – represent a significant and continuously increasing fraction of clinical research in Italy. A survey conducted in 2019 by the National Coordination Center for Ethics Committees (Centro di Coordinamento Nazionale dei Comitati Etici, CCNCE) showed that out of approximately 14,800 studies examined in one year by 74 ECs, approximately 50% were observational studies (7,400 studies). Among observational studies, retrospective studies represented on average more than 50% of the studies submitted to the opinion of the ECs. Out of any doubt, observational research strongly impact on ECs' activity and EC's activities affect a large proportion of clinical research in Italy. During the last 20 years the Italian legislation regarding observational studies has significantly changed: from a dramatic gap at the end of the nineties to an increasing attention to regulate both ethical evaluations and data privacy management [8-16]. However, no evidence is available regarding real world impact of new regulations on the ethical evaluation processes nor on factors which can impact EC's evaluation time.

The aim of the present work is to investigate, by means of a "*before and after*" study design, the impact of two distinct important regulations affecting observational studies in Italy entered in force in 2008 and 2018, respectively the AIFA guidelines for the classification and conduct of pharmaceutical observational studies [9] (AIFA guideline) and the European Union General Data Protection Regulation (GDPR) [15] adopted in Italy as for the Legislative Decree 101 of August 10th 2018 [16] (GDPR regulation). Main investigated outcomes were: 1) the duration of the ethical evaluation processes and 2) their complexity, measured using the surrogate end point of the number of suspensions observed in each single evaluation process. As a secondary outcome we aimed to investigate whether the duration and the probability of suspensions were somehow influenced by certain intrinsic characteristics of the evaluated studies.

To our knowledge, this is the first study investigating the impact of a new legislation on a large sample of ethical evaluations. This study was conducted with the aim to deriving valuable information about the evolution of ethical evaluation of observational studies in Italy and drawing insights for how to better evolve the current legislation.

MATERIALS

In this study we analysed two proprietary databases of Medineos srl, a company subject to the direction and coordination of IQVIA Solutions, an Italian contract research organization specialized in the design and executing of clinical observational studies. The first database contained qualitative data related to all studies conducted since 2002; the second database contained quantitative data on the ethical evaluation processes carried out by ECs on each single study. Therefore, we were able to evaluate 2,875 ethical submissions related to 112 different observational studies submitted over a period of 20 years (2002-2022) considering all the following informative contents:

- "project": each study protocol submitted for evaluation to the Italian ECs;
- "EC": the body that performed the evaluation of the project in that specific period of time. Some ECs may no longer exist when this paper is written, as a consequence of new regulations in Italy;
- "evaluation": the process executed by each single EC to evaluate a project. During the examined period (2002-2022), very often the same study protocol was evaluated by the EC of each single clinical site participating to the project;
- "date of submission": date in which the study protocol of a project was submitted for evaluation to one or more ECs. This milestone was used as the start date of the evaluation process;
- "suspension": it represents the dichotomous variable defining whether or not the evaluation of an EC undergone a suspension due to a request for further information. The variable "suspension" was used as a surrogate endpoint to define the "complexity" of the individual ethical evaluation process. Indeed, we have assumed that the lack of suspension is equivalent to a more linear evaluation process, while the presence of at least one suspension represents a sign of greater complexity in the evaluation of the single study protocol. For the purposes of our study, complexity does not have a negative meaning, as the absence of a

suspension could theoretically also mean greater approximation or superficiality in evaluating the study;
- "date of response": the date of the final evaluation by the single EC, being this "positive" or "negative". This variable was used as the end date of the evaluation process in order to measure its duration.

Moreover, we examined some additional qualitative information about specific characteristics of the projects to investigate their role as potential "risk factors" for more complexity in the evaluation process. The intrinsic, qualitative factors examined were:
- "study design": according to the methodological classification of observational studies, (i) retrospective/primary data collection; (ii) prospective/secondary use of data or hybrid;
- "rare disease": representing the dichotomous variable defining whether the project was on a rare disease or not;
- "genetic data": representing the dichotomous variable defining if the project collects genetic data or not;
- "PASS study": representing the dichotomous variable defining whether the project consists of a so-called post authorization safety study (PASS) or not.

Finally, to answer the research questions of this study and to stratify the total sample in the two arms "before and after", we calculated the following derived variables:
- "total evaluation time": calculated as the number of days between the "date of submission" and the "date of response";
- "pre/post-AIFA": which represents the dichotomous variable defining whether the project was submitted before or after the publication of the AIFA "guidelines for observational studies on drugs" on March 30th 2008;
- "pre/post-GDPR": representing the dichotomous variable defining whether the project was submitted before or after the "GDPR" on May 25th 2018.

Only the submissions with all the listed above available and reliable information were considered for the analyses.

METHODS

We performed a descriptive analysis using absolute and relative frequencies for categorical variables, and mean, standard deviation, median, 25th and 75th percentiles, minimum, and maximum for continuous variables. We then analyzed the "total evaluation time" as a time-to-event variable within the context of survival analysis modeling. The database comprised various "projects", mainly multicentric studies, each with multiple submissions of the same study protocol to different ECs.

Subsequently, we analyzed the probability of receiving a suspension from an EC using a univariate linear logit mixed effects model. The variables hypothesized to impact this probability included "Ethics Committee", "study design", "rare disease", and "genetic data" as project characteristics, and "post-AIFA" and "post-GDPR" as indicators of legislative changes. We hypothesized that both the project characteristics and the legislative changes could affect the probability of receiving a suspension (yes/no). By including all submissions of the same project as a mixed effect variable in the model, we accounted for potential intercorrelation within the same project.

RESULTS

In total, we analysed 112 different observational studies ("projects"), with 2,875 different individual submissions to Italian ECs, over a period from October 2002 to April 2022.

Projects characteristics

Of these projects, 84 (75%) had a prospective or hybrid study design and 28 (25%) retrospective; 7 (6%) were studies on a rare disease, 2 (2%) collected genetic data and 19 (17%) were PASS (*Table 1*). The mean number of sites per single project was 12 (Standard Deviation, SD 8.3) with a median value of 11. Overall, 87 (77.7%) studies had at least one submission "post-AIFA" and 25 (22.3%) with at least one submission "post-GDPR".

Submissions characteristics

Considering the submission processes, 2,075 (72.2%) were performed "post-AIFA" and 416 (14.4%) submissions were performed "post-GDPR".

Total evaluation time

The median (and interquartile range) of "total evaluation time" for submissions that did not experience any suspension was 68 (41-113) days whereas it was 127 (84-180) days for the submissions that underwent a suspension (*Table 2*).

The median (and interquartile range) for submissions "pre-AIFA" was 70 (41-133) days whereas it was 75 (45-122) days for "post-AIFA" (*Table 2*). The median (and interquartile range) for submissions "pre-GDPR" was 70 (42-123) days whereas it was 90 (63-140) for "post-GDPR" (*Table 2*).

When time to evaluation was assessed by means of a Kaplan-Meier analysis, this difference was confirmed both when taking into account pre/post-AIFA (*Figure 1*) and pre/post-GDPR (*Figure 2*). Namely, the time to evaluation for 50% of responses was shorter after AIFA guidelines and longer after GDPR.

Probability for suspension of the evaluation process

Only the factor "post-AIFA" was recognized as influential in increasing the likelihood of receiving a suspension (OR=21; 95% CI 7; 60): the odds to receive a suspension for a "post-AIFA" study was on average between 7 and 60 times higher than the odds for a "pre-AIFA" study.

Any of the other pre-identified intrinsic, qualitative factors ("study design", "PASS study", etc.) does not seem to be associated to the occurrence of suspension (*Table 3*).

Table 1
Projects characteristics (112)

	Projects analysed (N=112)
Study design	
Prospective/hybrid	84 (75%)
Retrospective	28 (25%)
Characteristics	
Project on rare disease	7 (6%)
Project with genetic data collection	2 (2%)
Post-authorisation safety studies	19 (17%)

Table 2
Total evaluation time (days) according to relevant factors

Factors		N	Median (25-75 percentile)	Min-Max
Suspensions during EC evaluation	No	2,503	68.0 (41.0-113.0)	1.0-558.0
	Yes	372	127.0 (84.0-180.0)	15.0-629.0
2008 AIFA guideline	Before	800	70.0 (41.0-133.0)	1.0-494.0
	After	2,075	75.0 (45.0-122.0)	4.0 629.0
GDPR legislation	Before	2,459	70.0 (42.0-123.0)	1.0-629.0
	After	416	90.5 (63.0-140.0)	10.0-619.0

EC: Ethical Committee; AIFA: Italian Medicines Agency (Agenzia Italiana del Farmaco); GDPR: General Data Protection Regulation.

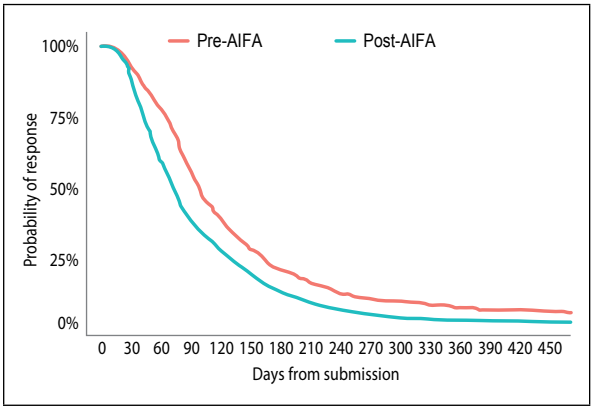

Figure 1
Kaplan-Meyer curve of time of evaluation according to the Italian Medicines Agency (Agenzia Italiana del Farmaco, AIFA) guideline emission.

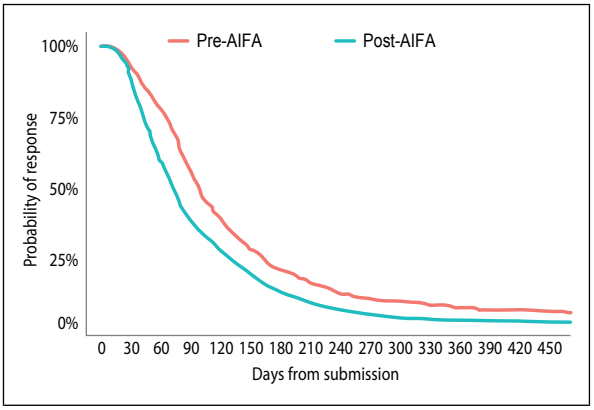

Figure 2
Kaplan-Meyer curve of time of evaluation according to General Data Protection Regulation (GDPR) legislation application.

Table 3
Results from the univariate linear logit mixed effects models for the probability of receiving a suspension of the evaluation by an Ethics Committee (CE)

Parameter		OR	95% CI	
AIFA guideline	after *vs* before	20.69	7.05	60.75
GDPR legislation	after *vs* before	0.98	0.38	2.53
Study design	Prospective/hybrid *vs* retrospective	0.80	0.32	1.98
Genetic data collection	Yes *vs* No	1.00	0.07	14.74
Rare disease study	Yes *vs* No	0.52	0.11	2.54

CI: confidence interval; AIFA: Italian Medicines Agency (Agenzia Italiana del Farmaco); GDPR: General Data Protection Regulation.

DISCUSSION

Before considering the results related to evaluation times, it is important to highlight that 67% of local ECs that received at least one submission during the observed timeframe no longer exist today, having been changed or replaced. This percentage rises to 81% when focusing on submissions prior to the 2008 AIFA guidelines. These figures provide remarkable evidence of the structural changes that have occurred in recent years, in terms of EC reorganization and procedural updates. It is also noteworthy that a transformative phase is still ongoing, with new AIFA guidelines, including instructions for different observational study designs, anticipated as per the Ministerial Decree of 30 November 2021 [13].

Impact of AIFA guideline

When considering our sample in its entirety – both suspended and non-suspended protocols – the AIFA guidelines led to a slight increase in the median number of days for evaluation (from 70 to 75 days).

On the other hand, the adoption of the AIFA guidelines was recognized as highly influential in increasing the likelihood of receiving a suspension. Our interpretation is that the guidelines provided clear rules and requirements for observational studies, leading ECs to exercise a higher level of control, thereby increasing suspensions. However, suspensions can be seen as indicative of ECs needing to request additional information when evaluating submissions that did not fully meet

AIFA specifics, and suspension itself can be viewed as a "positive" benchmark for improving the quality of observational studies.

As a consequence, the increase in suspensions could also explain why the expected 60-day evaluation time is not usually respected. This issue is not limited to the immediate period following the introduction of the AIFA guidelines but persists over a longer timeframe (15 years).

According to the AIFA guidelines, the maximum expected evaluation time for a satellite center's EC evaluation should be no more than 75 days (45 days waiting for the coordinating center's EC opinion plus 30 days for the local evaluation in the satellite center). However, even for studies that did not receive a suspension, we note that both before and after the publication of the AIFA guidelines, 45% and 43% of submissions, respectively, exceeded this maximum limit of 75 days. Data related to the AIFA guidelines can be interpreted as evidence of the resolution transposition by ECs in terms of the technical evaluation of observational studies. The AIFA guidelines introduced a set of rules on the classification, planning, and conduct of observational studies in pharmacological research, aiming to make a significant contribution to improving the quality assurance of all observational studies [13]. According to the AIFA guidelines, each observational study must be based on a defined protocol, which must include: the research hypothesis, expected results, type of observational study, choice of sample size, information to be collected, possible involvement of the facility and/or healthcare professionals, required resources, origin of funding, modalities of participation, and information addressed to the patients [4]. Despite these widely agreed-upon and accepted rules, a survey conducted by Gregori *et al.* [17] on 6 ECs right after the publication of the AIFA guidelines, for a post-hoc comparison of 364 protocols presented as observational before March 2008, revealed that a fairly high percentage (20-40%) did not comply with the new specifics introduced by the AIFA guidelines. This may be interpreted as proof of the past need for enhancement in protocol quality, which the AIFA sought to address by defining a minimum set of common requirements for all ECs.

Impact of GDPR legislation

Considering the introduction of GDPR legislation, we observed a significant increase in evaluation time (from 70 to 90 days) without a corresponding increase in the probability of suspensions. This controversial result could be interpreted as the need for privacy evaluations by other competent bodies at the local level, impacting the evaluation duration but not resulting in suspensions solely due to privacy issues. In other words, the examination of privacy concerns appears to be a bureaucratic activity leading to minimal adjustments (e.g., modifications to informed consent at the local level), but not necessitating the suspension of the study due to the intrinsic characteristics of the study protocol.

Contrary to our observations, Benfatto *et al.* [18] highlighted the valuable contribution of GDPR in reducing the number of changes necessary for final submission approval and saving time in 822 clinical trial protocols. It is worth noting that the cited review focused on experimental clinical trials, whereas our analysis specifically refers to observational study protocols, with more than 25% being retrospective studies. This essential difference suggests that the observed increase in evaluation time might be linked to the observational nature of the studies, including retrospective designs. Retrospective studies require a higher effort to evaluate privacy aspects, such as data collection from deceased or untraceable patients for whom consent is not achievable.

In this regard, the Italian National Coordination Centre for Ethics Committees (CCNCE) recently released a document specifically addressing the ethical and regulatory issues in handling personal health data in observational research [19], further supporting our hypothesis.

Impact of intrinsic study factors

Regarding the intrinsic factors of the study, we did not observe any impact on evaluation duration or the probability of suspension. This suggests that our findings on the timing and complexity of ethical assessments are generally linked to the observational nature of the protocols analyzed, rather than the inherent characteristics of individual studies.

It is surprising that the inclusion of genetic data in the protocol did not result in an increased probability of suspension, considering that the AIFA guideline states: "Observational studies are not considered to be those in which the examinations are aimed at pharmacogenetic and/or pharmacogenomic studies" [9]. This peculiarity highlights the gap that still exists between a methodologically inadequate regulatory definition of observational studies and the practical approach applied by both researchers and evaluators.

Our study has some limitations: (i) we used a surrogate outcome (suspension) to define evaluation complexity; (ii) we assumed that suspensions indicate a deeper evaluation of study protocols by ECs, implying that an increase in suspensions represents an enhanced ability of ECs to evaluate observational study protocols. Additionally, our study was conducted using a database of sponsored observational studies from a single contract research organization, representing a partial view of Italian practice. However, we believe that the large dataset provides a good approximation of the trends in observational research in Italy over the past 20 years.

In addition, the replicability of these results strongly depends on legislative conditions [20]. A study proposal involving human subjects must be submitted to at least one EC for approval before research can begin. A valid and robust study protocol, adhering to epidemiological principles, ensures transparency and reliability. We believe that having study designs and protocols reviewed by peers is a recommended practice to identify potential flaws or areas for improvement. It is beneficial to have peers review the study from various perspectives, such as statistical, operational, methodological, and quality aspects. This multi-faceted review process can help identify potential issues and improve

the overall robustness of the research. The new AIFA guidelines could potentially alter the timelines of the authorization process, which will be further investigated in the future.

CONCLUSIONS

Our findings show that changes in the regulatory environment significantly impact the conduct of clinical research. Specifically, we observed that both the AIFA guideline and GDPR legislation have controversial effects, increasing evaluation times but also apparently enhancing the thoroughness of ethical evaluations.

The results of this analysis serve as valuable indicators for assessing the effectiveness of past Italian regulations on observational studies, particularly in terms of ethical authorization timing and its consequential effect on the competitiveness of Italian research globally. These findings also provide a useful reference for measuring the impact of the new AIFA guidelines in the near future. Indeed, these new guidelines have been eagerly awaited by the local scientific community, with the hope that they will enhance the competitiveness of Italian clinical research in the field of real-world evidence generation while ensuring high standards of ethics and scientific quality.

Ethical evaluations naturally require time. To expedite the process, it might be beneficial to organize more frequent meetings of ethics committees. While this could increase costs, as committee members need to be compensated, it could ultimately speed up the evaluation process. We are confident that in the future, cost reductions can be achieved through the use of remote meetings and technological systems. Our research does not imply that the work of ethics committees delays research.

In particular, scientific societies highlighted to the legislator the main priorities: the need for updated methodological references and the establishment of standard processes with clearly defined rules and timelines. This would promote uniformity not only for observational studies on drugs but also for all types of studies (medical devices, other therapies, disease, and epidemiology) using observational methodology.

The results of this study can therefore be used to help evaluate the impact of the new guidelines in the coming months, considering the recent major transformation in the organization of Territorial Ethics Committees (TECs). This evaluation will help determine the need for further actions to harmonize clinical research based on observational methods in Italy. No more time can be wasted if Italy aims to secure a leading role in European research based on real-world data.

Conflict of interest statement

The Authors declare no conflict of interest.

Received on 14 November 2024.
Accepted on 20 February 2025.

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Ann Ist Super Sanità 2025 | Vol. 61, No. 2: 116-124
DOI: 10.4415/ANN_25_02_04

Antenatal care services and pregnancy outcomes during the COVID-19 pandemic in Milan, Lombardy, Italy

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Abstract

Background. During the COVID-19 pandemic maternity healthcare seeking and provision reduced worldwide. We explored the indirect effects of the pandemic on key pregnancy outcomes and access to antenatal care services.

Methods. Observational cross-sectional study on all pregnancies between years 2019-2020 in Milan metropolitan area (Lombardy, Italy). Multiple logistic regression analysis was used to assess the access to antenatal care (ANC) services (timing of first contact, ultrasound examinations (US) and ANC contacts) and pregnancy outcomes (preterm births, perinatal deaths and surgically treated ectopic pregnancies). Data were retrieved from both administrative (public healthcare) and self-reported sources (public and private services).

Results. The first antenatal contact was slightly delayed in pandemic year 2020. Adequate levels of antenatal care were maintained according to self-reported data, though a decrease in public healthcare was noted (administrative data). Perinatal death and preterm birth risk did not worsen, while it increased for surgically managed ectopic pregnancies.

Key words

- pregnancy
- COVID-19
- prenatal care
- maternal health services
- public health

BACKGROUND

Italy was one the European country most affected by the COVID-19 pandemic in 2020, especially in the study area located in Milan metropolitan area, Lombardy [1]. On 8th March 2020, the Lombardy region issued severe containment measures which were later extended to a nationwide lockdown until 18th May 2020. A second outbreak, with ensuing restrictions, took place between October 2020 and January 2021. Indeed, restrictions due to the pandemic took place in many nations, with different starting date and durations, on a global scale. The two pandemic waves had profound impact on the national health service, with staff reorganization and pause or postponement of all elective surgical activities and outpatient clinics. The extraordinary burden on the healthcare system concerned the delivery of all services, including women's health services. For example, it is known that decreased or delayed surgical procedures, cancer screening tests and outpatient appointments led to a significant decline in cancer diagnosis and overall poorer outcomes for oncologic patients [2]. Being pregnancy essentially time-sensitive, pregnant women were particularly vulnerable to altered or delayed access to care. With childbirth representing the first reason for hospital admission [3], maternity services are known to be a key index in healthcare system policies. During the COVID outbreak maternity care was regarded as non-deferrable both by national [4-6] and international recommendations [7, 8]. However, the modifications in social and healthcare policies inevitably influenced access to care with consequences on perinatal outcomes [9, 10]. Reduced maternity healthcare-seeking and provision were reported in the majority of studies [11-15]. Several changes in key maternal and perinatal outcomes were noted worldwide. Overall, maternal mortality, stillbirths and ruptured ectopic pregnancies increased, with substantial differences between high income (HIC) and low or middle income countries (LMIC) [16]. In HIC stillbirth figures were found to be either increased [17-19] or unchanged [20], while several reports pointed towards a decrease in preterm births (PTB) [16, 18, 20]. Alongside pregnancy care, also abortion care was deemed essential both by major societies [21, 22] and national authorities [5]. However, in Italy no details were provided on how to maintain access to voluntary termination of pregnancy (TOP), leading to unequal abortion care in the country [23]. Furthermore, the pandemic increased social disparities and highlighted

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the difference in access to care between autochthonous pregnant women and immigrants [24, 25]. Overall, the indirect effects of the restrictions brought by the pandemic waves were manyfold, sometimes even conflicting.

Therefore, the aim of the study was to explore how the COVID-19 pandemic and ensuing lockdown impacted on pregnancy outcome and access to care in Milan metropolitan area.

MATERIALS AND METHODS

An observational cross-sectional study was conducted. The population included in the study is the female population residing in the provinces of Milan and Lodi and served by the Health Protection Agency of Milan (Agenzia per la Tutela della Salute, ATS). Eligible women had an admission to hospital either for birth, spontaneous abortion or voluntary TOP between January 2019 and December 2020. Information regarding maternal access to care and pregnancy outcomes was retrieved combining data from the datawarehouse (DWH) which structures all healthcare administrative data sources and from birth certificates ("Certificato di assistenza al parto", CedAP [26]) filled out at delivery based on self-reported information. The information coming from the Civil Registry (Nuova anagrafe regionale, NAR) of women was integrated with the information from the permanent georeference system, developed and maintained by the Epidemiology Unit of the ATS of Milan. Thus, it was possible to integrate the information from the Population and Home Census of 2011 and, in particular, the deprivation index, based on census tract. The deprivation index is a composite measure on aggregate data considered a proxy of social disadvantage. It incorporates five socioeconomic indicators such as low level of education, being unemployed, living in rent, overcrowding, single-parent family. It is categorized into five quintiles, from the least (first quintile) to the most (fifth quintile) deprived [27, 28].

An information system was created for the current evaluation integrating all the described sources, with deterministic record linkage using the anonymized individual code present in the data warehouse systems of the ATS of Milan.

Data retrieved comprised access to routine antenatal care (timing of first antenatal contact, number of ANC contacts, ultrasound examinations and invasive procedures), pregnancy outcomes (birthweight, gestational age at birth, livebirth, stillbirth or neonatal death, mode of conception), spontaneous abortion, TOP and associated modality (medical *vs* surgical) and management of ectopic pregnancy (medical *vs* surgical). Stillbirth was defined as the absence of fetal cardiac activity on ultrasound examination after 24 weeks of gestation. Neonatal death (NND) was defined as death among livebirths during the first 28 completed days of life. Perinatal death comprised both stillbirths and NND.

First, data were analyzed using descriptive statistics, computing mean and standard deviation for continuous variables, frequency distribution for categorical variables. Categorical variables were compared between pre-pandemic year 2019 and pandemic year 2020 using the chi-square test, continuous variables using the Mann-Whitney U test. Then the change in access to ANC between the two years (pre-pandemic *vs* pandemic) was modelled using logistic regression, considering thresholds aligned with national guidelines [3], that identify at least four ANC contacts and two ultrasound (US) examinations in normal pregnancy. Therefore, poor antenatal care was defined as less than four appointments and less than two US examinations in pregnancies ended with delivery of a liveborn or stillborn. Inadequate timing of first ANC contact was defined as first booking after the 14th week of gestation. Considered covariates were: year (pre-pandemic *vs* pandemic), maternal country of birth (foreigner *vs* Italian), place of residence (Milan *vs* hinterland), index of deprivation (ID), pregnancy from assisted reproductive technology (ART) *vs* spontaneous pregnancy, age subgroup (less or equal to 25, 26-30, 31-35, more than 35). The census-based ID was categorized in quintiles, with the fifth quintile representing the most deprived municipalities [28]. Italian citizenship, Milan residence, an ID of 1, spontaneous pregnancy, pre-pandemic year 2019 and age subgroup of 30-34 were considered as reference. Analyses were carried out on three different datasets: the whole two-years cohort (n=55,590 pregnancies) for timing of first ANC contact; a sub-cohort of all ectopic pregnancies (n=363) for surgically managed ones; a sub-cohort of all pregnancies ended in the delivery of a liveborn/stillborn (n=43,638 pregnancies) for preterm births, perinatal deaths, appropriate levels of ANC contacts and US examinations. Missing data were assumed to be missing at random and not included in the analyses. All analyses were done using SAS Enterprise version 9.4. Ethics committee approval was not required as the data were collected as part of the service and pseudonymized data were included.

RESULTS

A total of 55,590 conceptions were analyzed in the study period, 43,638 births and 11,952 abortions respectively. Population characteristics are shown in *Table 1*. The population is slightly younger in the prepandemic period ($p<0.001$), with a slightly higher proportion of unemployed ($p<0.001$), low-education mothers ($p<0.001$), low-education father ($p<0.05$) and mother with foreign citizenship ($p<0.05$), while the figures of father work status, women living in Milan and deprivation indices were comparable (p-values 0.3, 0.3 and 0.4).

There was a decreasing trend in conceptions, ranging from 30,007 pregnancies in 2019 to 25,583 in 2020 (*Table 1*). Abortion figures decreased accordingly (*Supplementary Table 1 available online as Supplementary Materials*): from 3,504 (50.4%) TOP in 2019 to 2,428 (48.6%) in 2020 and from 3,268 (47%) spontaneous abortions in 2019 to 2,389 (47.8%) in 2020. No change in TOP modality was noted, for medical and surgical treatment did not significantly differ over the two years (p-value 0.6). Ectopic pregnancies in need of surgical treatment increased in 2020: 126 (69.2%) as opposed to 113 (62.5%) in 2019, though the change in management did not reach significance (p-value 0.2) (*Supplementary Table 1 available online as Supplementary Materials*).

Table 1
Distribution of population characteristics over the study period

Population characteristics	2019 (n=30,007)	2020 (n=25,583)	p value
Maternal age class			
25 years	3,401 (11.4%)	2,599 (10.2%)	<0.001
26-30 years	5,678 (19%)	4,800 (18.8%)	
31-35 years	9,539 (31.9%)	8,455 (33.1%)	
>35 years	11,316 (37.8%)	9,686 (37.9%)	
Mother, work status			
Employed	14,364 (47.9%)	13,172 (51.5%)	<0.001
Unemployed	6,864 (22.9%)	5,789 (22.6%)	
Unknown	8,779 (29.2%)	6,622 (25.9%)	
Mother, education level			
Low	3,881 (12.9%)	3,129 (12.2%)	<0.001
Middle	8,259 (27.6%)	7,284 (28.5%)	
High	9,078 (30.3%)	8,542 (33.4%)	
Unknown	8,789 (29.2%)	6,628 (25.9%)	
Father, work status			
Employed	19,939 (66.4%)	17,762 (69.4%)	0.3
Unemployed	1,289 (4.3%)	1,199 (4.7%)	
Unknown	8,779 (29.3%)	6,622 (25.9%)	
Father, education level			
Low	5,034 (16.8%)	4,334 (16.9%)	0.04
Middle	8,787 (29.3%)	7,846 (30.7%)	
High	7,047 (23.5%)	6,489 (25.4%)	
Unknown	9,139 (30.4%)	6,914 (27%)	
Town of residence			
Milan	13,091 (43.7%)	11,046 (43.2%)	0.3
Outside Milan	16,916 (56.3%)	14,537 (56.8%)	
Citizenship			
Italian	19,804 (66%)	17,274 (67.5%)	<0.001
Foreign	10,203 (34%)	8,309 (32.5%)	
Deprivation Index			
Very affluent	3,184 (10.6%)	2,824 (11%)	0.4
Affluent	5,271 (17.6%)	4,449 (17.4%)	
Average	7,086 (23.6%)	5,963 (23.3%)	
Deprived	7,139 (23.8%)	6,098 (23.8%)	
Severely deprived	5,716 (19%)	4,786 (18.8%)	
Unknown	1,611 (5.4%)	1,463 (5.7%)	

Data are presented as number of cases and percentages. Chi-square test for categorical variables.

Birth figures also mirrored the decrease in conceptions, from 23,115 in 2019 to 20,616 in 2020 (*Supplementary Table 1 available online as Supplementary Materials*). Of all births, preterm deliveries significantly decreased in the study period: from 1,370 (5.9%) in 2019 to 1,120 (5.5%) in 2020. Stillbirth figures also reduced in 2020 (15 deaths, 0.07%), unlike year 2019 in which there were 38 (0.16%) stillbirths. There were 4 neonatal deaths (0.02%) in 2019 and 6 (0.03%) in 2020 (*Supplementary Table 1 available online as Supplementary*

Materials). Though decreased, there was no significant change in trend for ART *vs* spontaneous conceptions (p-value 0.8).

Supplementary Table 2 available online as Supplementary Materials shows outpatient access to care, according to administrative and self-reported data. There were less US examinations in 2020 according to administrative data (mean 3.5 *vs* 3.7 in 2019), while women reported an increase from a mean of 5.8 US examinations in 2019 to 5.9 in 2020. Information regarding the week of first US examination came from administrative data only and showed a significant delay from 15.8 weeks' gestation in 2019 to 16.2 in 2020. ANC contacts mirrored the same trends: there was a decrease according to administrative data (mean 4.7 in 2019 *vs* 4.6 in 2020), while according to self-reported data ANC contacts did not decrease (mean 7.2 ANC contacts both in 2019 and 2020). Similarly, a delayed first ANC contact was noted only according to administrative data (mean 17.1 weeks' gestation in 2019 *vs* 17.6 weeks in 2020) and not by self-reported data (7.9 weeks' gestation both in 2019 and 2020). Finally, all invasives procedure decreased altogether with the decrease in conceptions. Chorionic villous sampling figures maintained from 2019 to 2020, while there was an increase (though not significant) in amniocentesis: from 1.9% in 2019 to 2.1% in 2020.

Results indicated that pandemic year 2020 resulted into higher odds of delayed first antenatal booking (aOR 1.08; 95% CI 1.04-1.13 and aOR 1.19; 95% CI 1.05-1.34 respectively) according to both administrative and self-reported data sources (*Table 2*). There was increased risk of inadequate antenatal care contacts according to administrative data only: at multiple analysis, year 2020 showed an aOR of 1.09 (95% CI 1.04-1.14) for less than four ANC contacts. Results are shown in *Table 2*. The recommended number of ANC contacts was associated with foreign citizenship (aOR 0.31; 95% CI 0.30-0.33), younger age (aOR 0.83; 95% CI 0.75-0.91) and growing deprivation in-

Table 2
Logistic regression analysis for antenatal care services (timing of 1st contact, number of ANC contacts and US examinations) in pregnancy and associated covariates, both administrative and self-reported data

	1st ANC contact >14 weeks		<4 ANC contacts		<2 US examinations	
	Administrative OR (95% CI)	Self-reported OR (95% CI)	Administrative OR (95% CI)	Self-reported OR (95% CI)	Administrative OR (95% CI)	Self-reported OR (95% CI)
Year						
2019	1#	1#	1#	1#	1#	1#
2020	1.08 (1.04-1.13)	1.19 (1.05-1.34)	1.09 (1.04-1.14)	0.82 (0.74-0.91)	1.36 (1.27-1.45)	0.71 (0.57-0.88)
Residence						
Municipality of Milan	1#	1#	1#	1#	1#	1#
Outside	1.28 (1.23-1.34)	1.16 (1.02-1.32)	1.44 (1.36-1.52)	0.80 (0.72-0.89)	0.89 (0.83-0.96)	1.26 (1.00-1.59)
Citizenship						
Italian	1#	1#	1#	1#	1#	1#
Foreign	0.72 (0.68-0.75)	5.03 (4.35-5.81)	0.31 (0.30-0.33)	2.07 (1.85-2.30)	0.54 (0.50-0.59)	1.95 (1.55-2.45)
Age class						
<25 years	0.94 (0.87-1.02)	3.02 (2.53-3.60)	0.83 (0.75-0.91)	1.64 (1.39-1.93)	0.75 (0.65-0.87)	1.97 (1.44-2.69)
26-30 years	0.91 (0.86-0.97)	1.40 (1.17-1.68)	0.84 (0.79-0.91)	1.14 (0.99-1.32)	0.79 (0.71-0.87)	1.01 (0.74-1.37)
31-35 years	1#	1#	1#	1#	1#	1#
>35 years	0.94 (0.90-0.98)	1.28 (1.08-1.53)	1.05 (0.99-1.11)	1.13 (0.99-1.29)	1.12 (1.04-1.21)	0.93 (0.70-1.24)
Deprivation index						
Very affluent	1#	1#	1#	1#	1#	1#
Affluent	0.93 (0.87-1.01)	1.39 (1.01-1.92)	0.84 (0.76-0.92)	1.19 (0.96-1.47)	0.81 (0.73-0.91)	1.22 (0.76-1.96)
Average	0.95 (0.88-1.02)	1.53 (1.12-2.08)	0.86 (0.78-0.94)	1.04 (0.84-1.28)	0.76 (0.68-0.85)	0.91 (0.57-1.46)
Deprived	0.81 (0.75-0.87)	2.24 (1.66-3.02)	0.76 (0.69-0.83)	1.09 (0.89-1.35)	0.63 (0.56-0.70)	1.02 (0.64-1.63)
Severely deprived	0.75 (0.69-0.81)	0.75 (0.69-0.81)	0.59 (0.53-0.65)	1.37 (1.18-1.69)	0.53 (0.46-0.60)	1.33 (0.84-2.11)
Pregnancy						
Spontaneous	1#	1#	1#	1#	1#	1#
ART	0.88 (0.81-0.97)	0.23 (0.11-0.45)	1.01 (0.90-1.13)	0.98 (0.74-1.29)	0.91 (0.78-1.07)	0.61 (0.29-1.30)

ANC: antenatal care; US: ultrasound; #reference category; ART: assisted reproductive technology.

dices (for class 5, aOR 0.59; 95% CI 0.53-0.65). On the contrary, self-reported data showed that pandemic year 2020 was not associated with less than the recommended number of ANC contacts (aOR 0.82; 95% CI 0.74-0.91), while it were the foreign (aOR 2.07; 95% CI 1.85-2.30), the younger (aOR 1.64; 95% CI 1.39-1.93) and the more deprived women (aOR 1.37; 95% CI 1.18-1.69) at higher risk of not meeting recommended standards (*Table 2*). Pandemic year 2020 was associated with increased figures for less than two US examinations in pregnancy (aOR 1.36; 95% CI 1.27-1.45) according to administrative data, a result not confirmed by self-reported data (aOR 0.71; 95% CI 0.57-0.88) (*Table 2*). Women living outside Milan, of younger age and foreign citizenship showed significantly reduced figures for ultrasound examinations according to self-reported data (aOR 1.26; 95% CI 1.00-1.59, aOR 1.97; 95% CI 1.44-2.69, aOR 1.95; 95% CI 1.55-2.45, respectively) (*Table 2*). *Supplementary Table 2 available online as Supplementary Materials* shows numerosity of outcome measures (first antenatal booking, ANC contacts and US examination).

Pandemic year 2020 was not significantly associated with preterm births (aOR 0.91; 95% CI 0.83-0.98), while foreign citizenship (aOR 1.38; 95% CI 1.26-151) and ART pregnancies (aOR 2.66; 95% CI 2.32-3.06) significantly increased the risks of preterm delivery (*Table 3*). Moreover, pandemic year 2020 was not significantly associated with perinatal deaths (aOR 0.52; 95% CI 0.30-0.91). Results are shown in *Table 3*. *Supplementary Table 1 available online as Supplementary Materials* shows numerosity of outcome measures (preterm births, perinatal deaths, surgical treatment of ectopic pregnancy).

The only significant factor associated with surgically managed ectopic pregnancy was indeed the pandemic year with an aOR of 2.55 (95% CI 1.16-5.63), as shown in *Table 3*. The pandemic period as covariate with associated aOR across all logistic regression analyses is shown in *Figure 1* (administrative data only).

Table 3
Multiple logistic regression analysis for key pregnancy outcomes (preterm births, perinatal deaths and need of surgical treatment of ectopic pregnancy) and associated covariates

	Preterm birth	Perinatal death	Surgical treatment of ectopic pregnancy
	OR (95% CI)	OR (95% CI)	OR (95% CI)
Year			
2019	1#	1#	1#
2020	0.91 (0.83-0.99)	0.52 (0.30-0.91)	2.55 (1.16-5.63)
Residence			
Municipality of Milan	1#	1#	1#
Outside	0.99 (0.91-1.08)	1.67 (0.94-2.96)	1.24 (0.53-2.86)
Citizenship			
Italian	1#	1#	1#
Foreign	1.38 (1.26-1.52)	1.74 (1.00-3.05)	1.10 (0.44-2.76)
Ageclass			
<25 years	1.03 (0.88-1.22)	1.12 (0.43-2.91)	0.65 (1.00-4.38)
26-30 years	0.88 (0.78-1.00)	0.64 (0.27-1.56)	0.84 (0.31-2.28)
31-35 years	1#	1#	1#
>35 years	1.20 (1.09-1.33)	1.62 (0.88-2.99)	1.22 (0.48-3.09)
Deprivation index			
Very affluent	1#	1#	1#
Affluent	1.26 (1.07-1.48)	1.79 (0.58-5.53)	3.04 (0.64-14.4)
Average	1.12 (0.95-1.31)	1.32 (0.42-4.14)	1.48 (0.31-7.13)
Deprived	1.32 (1.12-1.55)	1.21 (0.38-3.85)	1.54 (0.31-7.54)
Severely deprived	1.17 (0.98-1.38)	1.59 (0.50-5.07)	1.96 (0.42-9.10)
Pregnancy			
Spontaneous	1#	1#	1#
ART	2.67 (2.32-3.06)	0.61 (0.15-2.54)	1.00 (0.38-2.64)

#reference category; ART: assisted reproductive technology.

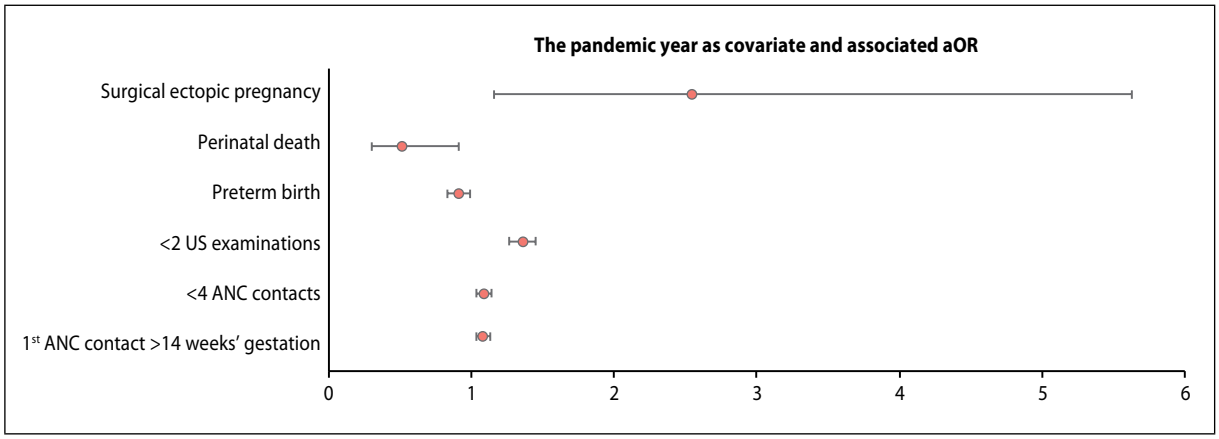

Figure 1
The pandemic period as covariate and associated adjuster odds ratios across all logistic regression analyses (only administrative data results for antenatal care services). Results are shown as adjusted odds ratios (aOR) with corresponding 95% confidence intervals. ANC: antenatal care; US: ultrasound.

DISCUSSION

In this study covering all pregnancies in Milan and Lodi metropolitan areas, we found that in pandemic year 2020 there was a small delay in the first antenatal contact according to both administrative and self-reported data. Furthermore, pandemic year 2020 was associated with less than the recommended numbers of both ANC contacts and US examinations according to administrative data, while this result did not maintain for self-reported data. Finally, year 2020 showed a rise in surgically managed ectopic pregnancies, while preterm births and perinatal deaths were not increased compared with pre-pandemic year 2019.

Administrative data results concerning reduced access to antenatal services agree with a previous report in UK where more than two-thirds of units reported a reduction in antenatal appointments [29]. There is also the systematic review and meta-analysis by Townsend *et al.* that confirmed reduced antenatal care contacts during the pandemic: quantitative data from seven studies showed that overall there was a 38.6% drop in care appointments during the pandemic period, with moderate heterogeneity (I^2=54.6%) [13]. Accordingly, there were reports of diminished maternity contacts also in Italy: from March to May 2020, only 28.4% of facilities all over the country continued to provide outpatient routine visits and examinations as usual, while 59.4% reduced the number of visits and 12.2% ceased all activities [11]. Data were collected via a national survey, with most of healthcare facilities that responded located in Lombardy or Veneto (the most affected Italian regions). However, there was a low response rate (5.4%) and most of the facilities in which visits were ceased were community-based. In contrast to these previous reports, our study provides quantitative data coming from administrative datasets and self-reported data collected for birth certificates.

Our results point towards reduced referral to ANC services and US examinations in the pandemic period according to administrative data, a finding not confirmed by self-reported data. While administrative data mainly cover national health service usage, self-reported data likely reflect usage of both national health and private services. Results show that the younger, of foreign citizenship and more deprived women were more likely to meet recommended standards according to administrative data; on the contrary, the same women were more likely to unmeet the standards according to self-reported data. A plausible explanation is that the less wealthy subgroups mostly refer to hospital based public health services, as evidenced by administrative data results, while the wealthier refer also to private health services. Self-reported data showed that overall the more disadvantages subgroups were indeed more likely to fail recommended standards of antenatal care. Anyhow, self-reported data prove that on the whole pandemic year 2020 was not significantly associated with inadequate levels of antenatal care, with most women attending more than 4 ANC contacts and 2 US examinations in pregnancy.

These findings agree with the ones of a cross-sectional survey conducted at 3 maternity care centers in Italy where, overall, there was a good compliance to prenatal care services [25]. Still, also in the study of Vilca *et al.* immigrants were less likely than Italians to comply with prenatal services [25].

In the present study, key indicators of maternal and fetal outcomes such as perinatal deaths and preterm births were significantly reduced in pandemic year 2020. The majority of perinatal deaths was driven by stillbirths. Stillbirths are known to be closely associated with poor access to adequate antenatal and obstetric care. Our data of not increased stillbirth rates are concordant with the systematic review and meta-analysis by Yang *et al.* [20], and with national data published by Rusconi *et al.* [30], Esposito *et al.* [9] and Salerno *et al.* [31]. However, reports of stillbirth rates during the COVID-19 pandemic remain contradictory: whether these variations reflect real differences due to national lockdowns, or perhaps differences in stillbirth rates and/or study designs is still unclear [18]. Reports of higher stillbirth rates come from both LMIC [32, 33] and HIC

[17, 19, 34, 35]. The Authors suggested the rise in stillbirth may have resulted from reduced access to hospital care, as mirrored by the fall in triage attendance [12, 19]. Though we did not evaluate emergency hospital attendance, we found that women reported to have met the recommended standards of routine antenatal care during pandemic year 2020 with ensuing lockdowns. This is a plausible explanation for unchanged stillbirth rates, though larger studies are needed to evaluate rare outcomes such as stillbirth.

We also found a significant reduction in preterm birth: pandemic year 2020 was associated with an aOR 0.91 (0.83-0.99, 95% CI) for PTB. The reduction of preterm births during the COVID-19 pandemic has been described before [34, 36, 37]. Preterm births are closely related to neonatal mortality, which also did not differ significantly in our cohort. Again, there is conflicting evidence which highlights important differences between LMIC and HIC. Overall, a systematic review and meta-analysis reported increased neonatal death in LMICs and decreased in HICs, consistently with the observed trends in preterm birth. This reduction in HICs appears to be driven by a reduction in spontaneous preterm birth [16]. The interrupted time series and meta-analyses by Calvert *et al.* used harmonized data from 52 million births in 26 countries and showed small reductions in preterm birth during the first three months of lockdown [18]. The study by Rusconi *et al.*, covering 84.3% of the births in Italy, demonstrated a decrease in PTB [30]. Our data fit accordingly. However, the effect of the pandemic on PTB is difficult to evaluate, given the multiple possible causal pathways. A first distinction should be drawn between spontaneous and iatrogenic preterm birth. Though the former seems reduced, maybe due to lifestyle changes [38], the latter is known to be increased in COVID infected women [39]. Nonetheless, the evidence of an overall reduction of PTB is notable given that only a small fraction of women experienced COVID infection while lockdown measures were essentially universal at the early stages of the pandemic. Finally, we found that the OR for preterm birth were significantly increased by foreign citizenship and ART conceptions. This is in accordance with previous findings of ethnic disparities [40] and pregnancy by ART [41] as established risk factors for preterm delivery.

Lockdown restrictions likely impacted most on access to emergency services. The systematic review and meta-analysis by Chmielewska *et al.* that found increased ruptured ectopic pregnancies during the pandemic included three studies [16], of which two were from Italy [19, 42]. Possible explanations could be women's hesitance to seek medical attention or the reduction of early first-trimester scans. In accordance with previous reports, our data showed both a significant increase of surgically managed ectopic pregnancies (aOR 2.55; 1.16-5.62 95% CI) and increased odds for later referrals (aOR 1.08, 1.04-1.13 95% CI and aOR 1.19, 1.05-1.34 95% CI for administrative and self-reported data respectively).

The number of abortion requests and procedures across the study period was generally reduced, together with the decrease in conceptions. These findings are consistent with previous ones [43]. Notably, the decrease in conceptions was already noted in the years before the study period, as mentioned in yearly birth reports [44]. Whether attributable to the decrease in conceptions and/or an indirect effect of the pandemic, the drop of TOP procedures was noted also in France [45, 46] and Sweden [47]. Abortion figures in other countries like Belgium [48], Israel [49] and the United States [50] were unchanged. Overall, evidence points towards an adequate response by healthcare services in HIC. Our data fit accordingly. Furthermore, we did not observe a change in abortion modality between pre-pandemic year 2019 and pandemic year 2020. This agrees with the findings of Guzzetti *et al.*, who found the procedures used (medical or surgical) were equally distributed among the considered timespans [51].

This study has several strengths and limitations. On one hand, there is the large sample size and the coverage of the entire period affected by the restrictions brought by the COVID-19 pandemic in Milan metropolitan area, Lombardy, one of the most populous and affected Italian regions. Moreover, the study covers both administrative data derived from the datawarehouse database and self-reported data filled out at delivery (birth certificates).

On the other hand, limitations are brought by the retrospective nature of the study and the lack of information regarding COVID-infected women and its direct impact on perinatal outcomes (especially preterm birth and stillbirth). Another important limitation concerns information regarding obstetric care outside the one offered by the national health system, a common reality in Italy especially for gynecologic and obstetric services [25]. Such information can only be indirectly inferred from self-reported data. For example, the fact that higher figures of ANC contacts were mostly seen in foreign and deprived women (administrative data results) led to the speculation that the wealthier population continued to use private health services. However, these must be regarded as indirect conclusions and need external validation.

CONCLUSIONS

In conclusion, both livebirths and legal terminations of pregnancy were decreased in pandemic year 2020, mirroring a decrease in conceptions. During the pandemic there was a delay in the first antenatal contact. However, adequate levels of routine antenatal care were maintained throughout 2020 according to self-reported data, though a decrease in national health system utilization was noted. There was no increase in perinatal deaths or preterm births, but there was a rise in surgically managed ectopic pregnancies. Overall, our results align with previous findings of the effects of the COVID-19 pandemic on maternal and perinatal outcomes.

Conflict of interest statement

The Authors declare that they have no conflict of interests.

Funding

The Authors did not receive support from any organization for the submitted work.

Authors' contributions

Conceptualization: AGR, MTG, MM; data curation: MTG; formal analysis: MM; writing – original draft preparation: MM; writing – review and editing: CB; supervision: AGR, MGV, CB. All Authors have read and agreed to the published version of the manuscript.

Received on 20 September 2024.
Accepted on 24 March 2025.

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Ann Ist Super Sanità 2025 | Vol. 61, No. 2: 125-131
DOI: 10.4415/ANN_25_02_05

Psychological distress and its impact on the onset of lasting neurological symptoms during the pandemic: evidence from the Italian Twin Registry

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Abstract

Introduction. Neurocognitive disorders are typical of older people. Psychological distress increased during the pandemic, particularly in young people. Although often underestimated, the impact of psychological distress on neurological disorders should be considered. As part of a longitudinal study conducted by the Italian Twin Registry (ITR) on the health effects of COVID-19 pandemic, we explored the onset of lasting neurological symptoms in relation with pre-existing psychological symptoms and/or SARS-Cov-2 infection.

Methods. Online surveys on adult subjects of the ITR: in June 2020 we investigated symptoms of depression, anxiety and post-traumatic distress and, in December 2021, the onset of six persisting neurocognitive symptoms. SARS-CoV-2 infection was examined in both surveys. Associations of psychological symptoms and of viral infection with subsequent neurological manifestations were tested through logistic regression analysis.

Results. Among 1,784 participants (mean age 46.6), 42.8% reported neurological symptoms and 15.7% SARS-Cov-2 infection. Odds of neurological manifestations increased in participants with depressive or anxiety symptoms (ORs: 1.44 to 3.72), and in those with COVID-19 (ORs: 1.73 to 2.32). Anxiety symptoms explained more cases of cognitive symptoms (26.9% to 37.9%) than COVID-19 (9.1% to 15.5%). Smell/taste changes were strongly associated with viral infection (OR: 43.2).

Conclusions. During the pandemic, widespread psychological distress contributed more than COVID-19 to the appearance of some cognitive symptoms in a relatively young population. Our findings indicates that preservation of neurological well-being cannot ignore mental health interventions.

Key words

- depressive symptoms
- anxiety
- neurological symptoms
- cognitive symptoms
- COVID-19

INTRODUCTION

The prevalence of neurological disorders increased over time, and this trend is expected to continue as the population ages [1]. At a time when the predictions of a worldwide increase in neurodegenerative diseases were shifting the focus to the search for solutions, studying the risk factors and the conception of adequate prevention programs [2, 3], the COVID-19 pandemic exploded, this research path was interrupted, as energies and attention were diverted to COVID-19 related studies.

Although COVID-19 primarily affects the respiratory system, it is a multi-organ disease, often involving the nervous system and brain [4, 5]. Cognitive dysfunction, for example, identified as brain fog, has been widely reported as COVID-19 sequela during the pandemic period: memory difficulties or impaired executive functions, such as focusing and planning, were common phenomena even in non-hospitalized individuals [4, 6]. Other neurological manifestations were reported worldwide: headache was among the five most common symptoms

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of COVID-19 infection, as well as fatigue [7-9]. The discovery that COVID-19 was associated with neurological sequelae spurred researchers to study this issue. Yet, these symptoms were not new, especially the neurocognitive ones, since they had already been described in the literature as subjective cognitive decline, that need to be monitored, as it may be normal for age, reversible, indicative of a psychological disorder, or point to an organic disease or dementia. On the other hand, the pandemic has led to a sharp increase in mental health problems among the population [10], even among those without the COVID-19 disease [11, 12], although positive mental changes have also been described [13]. It is known that neurological and psychological issues (such as depression and anxiety) could share common risk factors and pathogenesis [14, 15]. Moreover, previous research has shown that depression increases the risk of developing dementia and cognitive difficulties [16]. This existing link between mental health and cognitive difficulties – as also demonstrated by long-COVID syndromes [6] – is a topic that needs to be further explored.

As we had already observed an increase of depressive symptoms soon after the first Italian lockdown (March-May 2020) [17], we hypothesized that having psychological distress could facilitate the appearance of neurological symptoms. Therefore, we were interested in understanding whether the neurological manifestations described as lasting sequelae of COVID-19: i) arose de novo during the pandemic in a non-clinical population, ii) were associated with a previous SARS-CoV-2 infection, iii) were associated with pre-existing symptoms of depression and/or anxiety and/or post-traumatic stress. We addressed these aims in the context of a longitudinal survey [18] that covered the period between February 2020 and February 2022, performed on a large sample of individuals enrolled in the Italian Twin Registry (ITR), a population-based registry of volunteer twins [19].

METHODS

Study design and participants

This study is part of a longitudinal investigation on the effects of COVID-19 pandemic on physical and mental health that was carried out by a survey in three waves (June 2020, i.e., at the end of the Italian lockdown, December 2020, and December 2021). All adult twins (age 18-92 years) enrolled in the ITR, residing in Italy at the time of the pandemic and contactable by email, were invited to participate. For the purposes of this study, only data collected in the first and third wave surveys were used, and only participants responding to both surveys were included in the analyses.

The longitudinal survey, in which this specific study is nested, was reviewed and approved by the Ethical Committee of the Italian National Institute of Health (Istituto Superiore di Sanità, ISS) (PRE BIO CE n. 0020797, June 6, 2020). Participants provided written informed consent to participate in this study.

Measurements

Recruited twins filled out survey questionnaires using the LimeSurvey platform. Participants were given approximately a 2-month window to reply to each iteration of the survey, such that the last wave was closed in February 2022. Socio-demographic characteristics of the respondents were investigated during the first wave of the study; each wave investigated COVID-19 positivity (defined by molecular or antigenic swab), the month and year of diagnosis and whether hospitalization or admission to Intensive Care Unit had been required. In the third wave, participants reported if, since the beginning of the pandemic (February 2020), they have ever suffered for at least one month from any of the six following symptoms: headache, memory problems, difficulty concentrating, excessive tiredness, confused/heavy/empty head (also referred to as brain fog), reduction or loss of taste and smell. From now on, these six symptoms will be collectively defined as "lasting (or enduring or persisting) neurological symptoms", while we will use the term "cognitive symptoms" to refer only to memory problems, difficulty concentrating and confused/heavy/empty head. To avoid misinterpretation of the question, and in line with other articles [20], the investigated symptoms were deliberately presented in a straightforward and easy-to-understand wording. If appropriate, the month and year of onset had to be reported. One-month duration for the lasting symptoms was chosen according to NICE guidelines [21].

Validated self-reported questionnaires on mental health were administered at each wave: 1) Patient Health Questionnaire (PHQ-9) [22], for symptoms of depression in the previous two weeks; 2) State-Trait Anxiety Inventory (STAI-6) [23], that measures the presence of current symptoms of anxiety; 3) Impact of Event Scale-Revised (IES-R) [24], to assess pandemic-related subjective distress.

Higher total scores of the three above-mentioned scales corresponded to a poorer mental health status. Participants that scored above the thresholds of 10 for PHQ-9, 40 for STAI-6, or 33 for IES-R, were categorized as having symptoms of depression, anxiety, or post-traumatic stress disorder, respectively.

Statistical analysis

Descriptive statistics were used to analyse the characteristics of the sample. Continuous variables were presented as mean±standard deviation (SD) and compared by paired or unpaired Student's t-test. Categorical variables were showed as percentage and significant differences were examined using Chi-squared test. All analyses used twins as individuals.

The psychological assessment of twins – as measured in June 2020 – was described by the mean score of each scale, alongside the prevalence of symptoms of depression, anxiety, and stress, and was used for subsequent analyses.

The association between each lasting neurological symptom with COVID-19 exposure or with the measures of psychological distress was tested with logistic regression. Gender, age, area of residence, marital status, and educational level were included in the model as covariates. All subjects who reported having had enduring neurological symptoms before psychological assessment and before the diagnosis of COVID-19 were excluded from the multivariate analysis. P-values

and standard errors were adjusted for the non-independence of observations because of twin relatedness.

Furthermore, the proportion of cases of each lasting neurological symptom that can be attributed to depression, anxiety and Coronavirus infection within the entire population, was also estimated. All analyses were performed using Stata software version 16 (Stata Corporation, College Station, TX, USA), and p-value <0.05 were considered statistically significant.

RESULTS

Overall, 1,784 twins took part in both surveys (June 2020 and December 2021). Mean age was 46.6 years (range 18-92) and 64.5% were female. Socio-demographic characteristics are shown in *Table 1*.

In the whole observation period (February 2020-February 2022) 42.8% of participants reported having had one or more persistent neurological symptoms, and most of them traced the onset to the early months of the pandemic (February-April 2020) (*Figure 1*).

In the following months, instead, the appearance of these symptoms was almost evenly distributed; an exception was given by persistent change of smell and/or taste that closely followed the distribution of COVID-19 cases (*Figure 1*). Excessive tiredness was the most frequent symptom (28.0%), followed by difficulty in concentrating, headache, confused/heavy/empty head, memory problems, and alteration of smell or taste (*Table 2*).

In the whole time frame, 270 participants (15.7%) had contracted COVID-19 with a chronological distribution of cases (*Figure 1*) that closely resembles that of the national pandemic. Most of the COVID-19 cases (96%) in our sample were treated at home. A SARS-CoV-2 infection preceded the appearance of excessive tiredness, difficulty in concentrating, headache, confused/heavy/empty head, and memory problems in a minority (11.1-16.4%) of the participants; instead, COVID-19 was very frequent (72.4%) before the onset of persistent dysgeusia/dysosmia (*Table 2*). In most cases, SARS-CoV-2 infection preceded of less than one month the onset of lasting neurological symptoms (from 76% for memory problems to 97.4% for reduction/loss of taste and/or smell). Participants reporting any of the above neurological symptoms, apart from memory problems, were significantly younger than those who did

Table 1
Socio-demographic characteristics of participants (N=1,784)

Characteristics	N	(%)
Sex		
Male/female	634/1,150	35.5/64.5
Age		
<35	413	23.15
35-49	588	32.96
50-64	561	31.45
65+	222	12.44
Marital status		
Never married	727	42.2
Married	796	46.2
Widowed/divorced/separated	200	11.6
Education		
Up to lower secondary education	93	5.2
Upper secondary education	721	40.6
Bachelor or equivalent	198	11.2
Master or equivalent	541	30.5
Doctorate or equivalent	222	12.5
Area of residence in Italy		
North	985	55.4
Centre	518	29.1
South and Islands	276	15.5

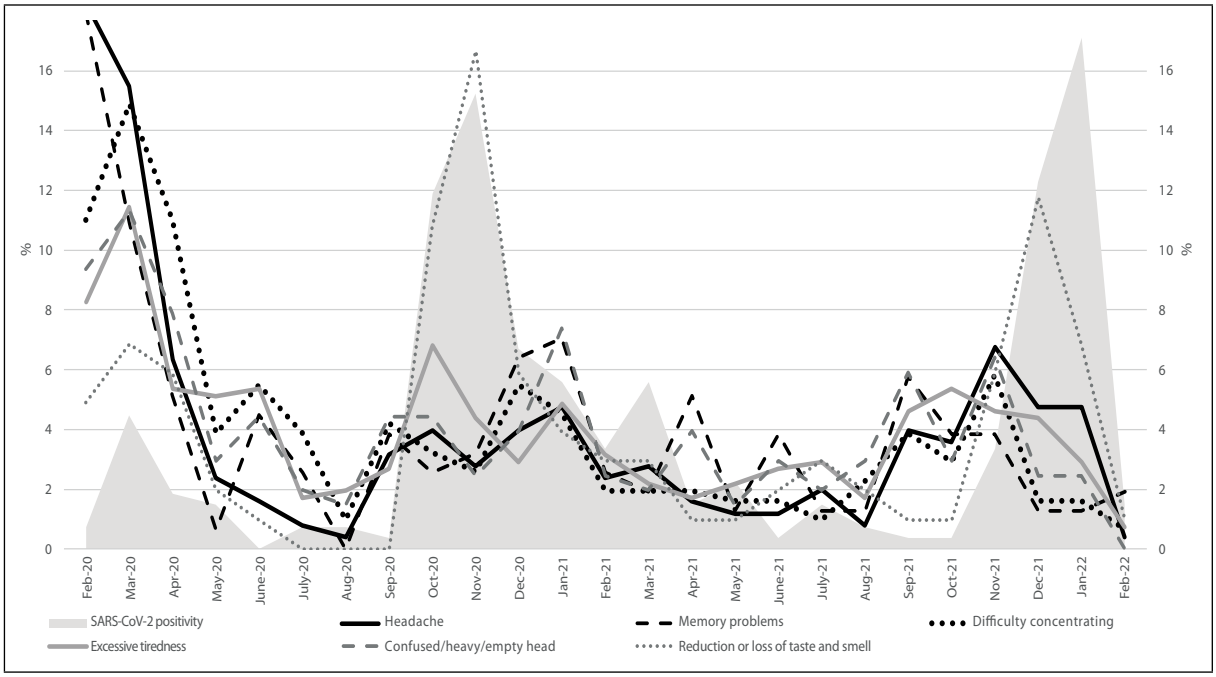

Figure 1
Monthly onset of SARS-CoV-2 positivity and of lasting neurological symptoms among participants: February 2020-February 2022.

Table 2
Individuals with or without lasting neurological symptoms (onset February 2020 - February 2022): overall number and broken down by previous SARS-CoV-2 infection

Neurological symptom	N	Yes N (%)	COVID-19 preceding onset of lasting symptom	
			Yes, N (%)	No, N (%)
Headache	1,661	282 (17.0)	39 (14.1)	238 (85.9)
Memory problems	1,662	188 (11.3)	25 (13.8)	156 (86.2)
Trouble concentrating	1,662	365 (22.0)	39 (11.1)	314 (88.9)
Excessive tiredness	1,669	468 (28.0)	66 (14.2)	398 (85.8)
Confused/heavy/empty head	1,654	242 (14.6)	39 (16.4)	199 (83.6)
Reduction/loss of taste and/or smell	1,649	112 (6.8)	76 (72.4)	29 (27.6)

not suffer from them (headache: mean age (interquartile range) 41.7 (20.4) *vs* 48.4 (23.6), p<0.001; trouble concentrating: 41.7 (21.8) *vs* 48.6 (23.7), p<0.001; excessive tiredness: 42.0 (20.8) *vs* 48.8 (23.8), p<0.001; confused/heavy/empty head: 42.7 (22.0) *vs* 47.7 (23.2), p<0.001; smell/taste problems: 42.6 (22.5) *vs* 47.3 (22.4), p<0.032). Women were more likely than men to have had each symptom (p<0.001, data not shown), with the exclusion of taste or olfaction modifications.

In the June 2020 survey, we assessed anxiety, depressive symptoms, and pandemic-related subjective distress. *Supplementary Table 1 available online as Supplementary Materials* shows the average scores and prevalence of clinically significant symptoms for each scale. Half of the participants scored above the threshold at the STAI-6 scale for anxiety symptoms; 12.5% and 9.2% of respondents obtained above cut-off scores for depression and stress, respectively.

To explore the psychological profile, beyond the Coronavirus infection, as risk factor for the appearance of the enduring neurological symptoms, we excluded from the subsequent analysis the participants who had declared the beginning of the neurological symptoms before the psychological assessment in June 2020, or that had contracted COVID-19 after the onset of the neurological symptoms. In the multivariate analysis (*Table 3*) the presence of depressive symptoms was significantly associated with headache, memory and concentration problems, confused/heavy/empty head and excessive tiredness, but did not affect the onset of lasting dysgeusia/dysosmia. Similarly, participants with anxiety symptoms were 1.44 to 2.26 times more likely to show subsequently cognitive problems, as well as tiredness. The above effects were net of the role of SARS-Cov-2 infection that, as expected, doubled the risk of suffering from all the neurological symptoms, and increased the

Table 3
Odds ratios of experiencing lasting neurological symptoms estimated by the logistic regression analysis

	Headache		Memory problems		Difficulty concentrating		Excessive tiredness		Confused/heavy/ empty head		Reduction or loss of taste and smell	
	OR	*95% CI*	OR	*95% CI*	OR	*95% CI*	OR	*95% CI*	OR	*95% CI*	OR	*95% CI*
Sex (male)												
Female	2.27***	*1.49-3.47*	2.10**	*1.28-3.44*	1.72**	*1.20-2.48*	1.74***	*1.28-2.37*	1.78**	*1.17-2.70*	1.14	*0.66-1.95*
Age (<35 years)												
35-49	1.00	*0.62-1.63*	1.20	*0.63-2.25*	0.99	*0.63-1.56*	0.87	*0.58-1.29*	1.04	*0.64-1.71*	1.10	*0.53-2.27*
50-64	0.73	*0.42-1.25*	1.61	*0.85-3.07*	1.23	*0.75-2.02*	0.85	*0.55-1.32*	0.95	*0.54-1.65*	0.93	*0.41-2.11*
65+	0.28*	*0.10-0.75*	1.15	*0.45-2.90*	0.44	*0.18-1.09*	0.65	*0.35-1.20*	0.67	*0.29-1.55*	0.24	*0.06-1.01*
Area of residence (North)												
Centre	1.54*	*1.01-2.33*	1.08	*0.68-1.72*	1.17	*0.79-1.72*	1.17	*0.85-1.60*	1.35	*0.87-2.08*	1.23	*0.69-2.20*
South and Islands	2.98***	*1.87-4.74*	1.45	*0.82-2.55*	1.70*	*1.09-2.64*	1.49*	*1.01-2.22*	2.27***	*1.42-3.61*	1.72	*0.86-3.44*
COVID-19 diagnosis	2.29***	*1.47-3.57*	2.22**	*1.36-3.61*	1.73*	*1.13-2.65*	2.02***	*1.39-2.93*	2.32***	*1.48-3.63*	43.8***	*23.4-82.1*
Anxiety symptoms	1.28	*0.87-1.88*	2.26***	*1.43-3.58*	1.95***	*1.36-2.81*	1.44*	*1.07-1.95*	1.77**	*1.17-2.65*	1.14	*0.66-1.96*
Depressive symptoms	2.33**	*1.40-3.86*	1.91*	*1.10-3.34*	2.47***	*1.60-3.83*	3.72***	*2.48-5.57*	2.47***	*1.54-3.96*	2.02	*0.97-4.20*
Post-traumatic stress disorder	1.04	*0.57-1.89*	1.19	*0.63-2.27*	1.29	*0.75-2.20*	0.88	*0.52-1.49*	1.11	*0.63-1.95*	0.75	*0.30-1.84*

Subjects who had enduring neurological symptoms before the psychological assessment and before the diagnosis of COVID-19 were excluded.
Anxiety symptoms, depressive symptoms and post-traumatic stress disorders are categorical variables (scores below or above threshold for each scale). Other covariates included in the model: marital status and educational level; OR: adjusted Odds Ratio; 95% CI: 95% confidence interval; *p<0.05, **p<0.01, ***p<0.001.

probability of having persistent alterations of taste and smell by more than forty times (*Table 3*).

The occurrence of perceived stress did not seem to increase the probability of developing lasting neurological symptoms. Female gender was confirmed to be a risk factor for the neurological symptoms investigated, with the exception of dysosmia/dysgeusia. It also emerged a significant effect of the area of residence on the majority of neurological symptoms; the age effect – observed in the univariate analysis – was reduced and remains significant only for enduring headache from which the oldest age group was protected. Marital status and educational level did not influence the onset of any neurological symptom.

We then estimated, for each persistent neurological symptom, the proportions of cases that could be attributed to the presence of anxiety or depression symptoms or to a preceding SARS-Cov-2 infection. Although the ORs estimates overlapped for the three risk factors (*Table 3*), anxiety symptoms were much more prevalent and therefore explained 1.7 to 3.4 times more cases of cognitive symptoms than those attributable to COVID-19 and depression symptoms (*Supplementary Table 2 available online as Supplementary Materials*).

DISCUSSION

Neurological sequelae of COVID-19 have been demonstrated by many studies, performed mainly in hospitalized people [25, 26]. However, neurological consequences have been described also in mild COVID-19 cases. This study began with the aim of researching and describing the onset of neurological symptoms, known as sequelae of COVID-19, in a sample of the Italian population during the COVID-19 pandemic. About 40% of our responders experienced enduring neurological symptoms: the majority reported having experienced two or more. Excessive tiredness, difficulty concentrating, and headache were the most common, followed by confused/heavy/empty head, memory issues, and reduction or loss of taste and smell. These findings are consistent with previous literature regarding COVID-19 patients, that were more likely to experience a constellation of symptoms, among which fatigue and cognitive difficulties were very common [4, 27].

The detection of lasting cognitive symptoms in a large proportion of a relatively young general population was an unexpected phenomenon, especially because our survey was conducted in a non-clinical setting. Of note, although COVID-19 infection represented a risk factor for showing neurological symptoms, possibly because of endotheliitis of brain vessels and encephalopathy [4], still a larger portion of our sample manifested cognitive difficulties without COVID-19 or any specific risk factor involved. This was especially evident during the lockdown period (March-May 2020), when the prevalence of coronavirus infections was limited to some areas of northern Italy, but neurological symptoms were reported throughout Italy. We therefore hypothesized that the drastic change in lifestyle, due to the particularly restrictive measures adopted in Italy, had a strong impact on the appearance of the neurological symptoms, as a consequence of the psychological distress. Indeed, Italy was among the first European countries to introduce a strict nationwide lockdown and kept it in place for longer than other countries, introducing regional restrictions and then gradually relaxing them [28]. This was also suggested by previous data on difficulties in falling and/or staying asleep during lockdown [18], and on the significant increase of the PHQ-2 mean score in post-lockdown (June 2020) compared to pre-lockdown period [17]. In fact, from the longitudinal analysis of the data, it clearly emerged that having symptoms of depression or anxiety in June 2020 increased – in the following months – the risk of developing the enduring neurological symptoms, excluding alterations of taste and/or smell. Although suggestive, the temporal association observed does not prove a cause-effect relationship: neurological symptoms may have a psychosomatic basis, potentially arising or worsened by psychological distress, also through unhealthy behaviors (i.e., alcohol, smoking, substance abuse, eating disorder); otherwise, psychological symptoms may have simply preceded neurological manifestations. In this line of thought is also the observation that the worsening of psychosocial functioning during pandemic was associated with pre-existing mental disorders [29].

It also emerged that the risk estimates of neurological symptoms, due to preceding anxiety or depression symptoms, are similar to those due to a previous SARS-CoV-2 infection. Risk factors with similar individual association (i.e., OR) but with different prevalence explain different proportions (i.e., attributable fractions) of the outcome: thus, in our population, anxiety symptoms (prevalence 50.1%) explained a more relevant proportion of cognitive symptoms compared to that due to COVID-19 (prevalence 14.3%).

We also show that women and those living in southern Italy were more likely to have neurological symptoms. Women tend to participate to surveys more than men, and generally report more symptoms than men [30], and some biological factors could be suggested as causes. For instance, there are sex differences in immunological responses and expression/activity of certain enzymes accountable for the development of autoimmune or inflammatory diseases and post-infectious sequelae (such as endotheliitis). This was particularly important when it came to long-COVID symptomatology [4, 31, 32]. Some symptoms such as headache and fatigue are known to be particularly frequent in women [1, 33, 34]. Also, sex hormones are known for influencing neurocognitive manifestations, especially after menopause [35, 36]. In addition, a pre-COVID-19 study reported that neurological manifestations were higher in women, both in incidence and prevalence [1]. Furthermore, it must be kept in mind that the lower participation of men may have prevented the detection of some effects related to male gender.

A higher prevalence of neurological symptoms in southern Italy could be due to environmental and cultural factors, like higher unemployment rates or poverty, health disparities, and lower quality and access to care, compared to other regions [37, 38].

This study has some limitations: the non-random sampling of the participants, affects the generalizability of our results. However, the analysis of respondents and non-respondents showed no substantial differences in age, gender, residence, or education. Moreover, we cannot rule out that some participants with lasting neurological symptoms had not been diagnosed with COVID-19, due to the limited availability of swabs in the first months of pandemic, and we could not investigate whether other causes (i.e., vascular, internal, neuromuscular, neurodegenerative diseases, etc.) were at the origin of the new-onset persistent neurological symptoms. In addition, since the study does not have a further follow-up, it is not known whether neurological symptoms have regressed, still persist, or have evolved.

CONCLUSIONS

The pandemic crisis has highlighted the widespread presence of the neurological symptoms investigated. Among the associated risk factors, previous anxious symptoms precede the highest percentage of cognitive symptoms in our population. While it is true that the impact of mental health issues due to the pandemic may have been different across countries and contexts, we believe that lasting neurological symptoms deserve to be monitored over time and appropriately classified as they may regress, but they may also reveal an underlying organic or psychiatric disorder, or progress to cognitive decline. Finally, during a pandemic emergency, health policies aimed at containing the spread of the infection should also consider mental well-being especially in younger age groups, and thus also preserve brain health.

Acknowledgements

We express our gratitude to participating twins of the Italian Twin Registry and to Cristina D'Ippolito, Maurizio Ferri, Sabrina Alviti, and Miriam Salemi for their technical assistance and management of data collection.

Statement of ethics

This study was reviewed and approved by the Ethical Committee of the Istituto Superiore di Sanità (PRE BIO CE n.0020797, June 6, 2020). Participants provided written informed consent to participate in this study.

Conflict of interest statement

The Authors have no conflicts of interest to declare.

Funding sources

This study was supported by intramural funds of the Istituto Superiore di Sanità.

Authors' contributions

LV: conceptualization, interpretation of the data, drafting and reviewing of the manuscript; LN: conceptualization, interpretation of the data, drafting and reviewing of the manuscript; GG: interpretation of the data and drafting of the manuscript; AG: interpretation of the data and critical reviewing; EM: conceptualization, acquisition, analysis and interpretation of the data, drafting and reviewing of the manuscript. All Authors approved this version of the manuscript and are accountable for the accuracy and integrity of the work.

Data availability

Data underlying this article are available from the corresponding author upon reasonable request and agreement on the terms of data use and publication of results.

Received on 26 June 2024.
Accepted on 3 March 2025.

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Ann Ist Super Sanità 2025 | Vol. 61, No. 2: 132-140
DOI: 10.4415/ANN_25_02_06

Description of an onsite school-based intervention aimed at increasing influenza vaccination uptake among children in an Italian Local Health Authority

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Abstract

Introduction. Onsite school-based intervention represents a key strategy to increase influenza vaccination uptake and improve knowledge of children, parents and school staff. This study aims to quantitatively describe an intervention in Local Health Authority Roma 1.

Methods. Vaccination was offered to children aged 2-6 years. A quantitative descriptive analysis of vaccination coverage and population variables was performed.

Results. 29 schools were included. Out of 2,424 eligible children, 405 were vaccinated (16.7%). Of these, 218 (53.8%) were male and 187 (46.2%) female, mean age 4.4 years old. 177 (43.7%) received one dose, while 228 (56.3%) were vaccinated for the first time. Of these, 150 students (65.8%) also received the second dose. 148 other people (parents, teachers and older children) decided to join the campaign, thus being vaccinated.

Conclusions. Community-based interventions in school settings increase adherence to health promotion campaigns. It is necessary to continue researching and investing in such activities.

Key words

- vaccine hesitancy
- onsite vaccination
- influenza
- children
- vaccine delivery

INTRODUCTION

Influenza vaccination has proved to be one of the main strategies to prevent seasonal influenza and to reduce its health, social and economic impact [1, 2]. Thus, it is important to promote strategies aimed at increasing coverage among target populations that present a risk for influenza-related complications, such as adults over 65 years of age, individuals with high-risk medical conditions, pregnant women and children [3, 4], even in the healthy pediatric population [5-7]. Indeed, the latter group considered generally reaches the highest rates of contagion, with an important burden in terms of illness, hospitalization and health complications [8-12]. For example, an Italian study reports that, over the 2013/14 - 2016/17 influenza seasons, children of 0-4 and 5-14 years of age had an estimated influenza-like illness (ILI) rate of 295.6 per 1000 and 160.3 per 1000, respectively, compared to 30.3 per 1000 observed in adults aged ≥65 years, with a relevant influenza attributable excess death rate [13]. Moreover, the American Centers for Disease Control and Prevention (CDC) reported during the 2018-2019 season a higher percentage of ILI in children 0-4 and 5-14 (10.2% and 21.6%) than in adults aged ≥65 years (8.7%), as well as of hospitalization (14.7% and 26.7% *vs* to 10.4%). In addition to the direct impact on health, children are the main source of spreading influenza to others, particularly within households [14, 15]. Therefore, annual childhood influenza vaccination is expected to directly protect those at highest risk of infection and produce external benefits by reducing disease transmission, illness, and complications [8, 9, 14]. While pediatric influenza has an important economic impact,

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both in terms of direct and indirect costs [16], vaccination has been shown to be cost-effective [17], thus confirming the importance of this practice in the pediatric population.

Despite the proven efficacy and effectiveness of pediatric influenza vaccination [1], coverage rates in Italy remain low [18], due to vaccine hesitancy and poor perception of the risk related to the virus in pediatric age [19]. In addition, logistical difficulties such as the need to accompany the child to the vaccination center or pediatrician represent further important barriers [20-22]. Therefore, it is important to promote a type of intervention that can, on the one hand, disseminate appropriate information about vaccination increasing the health literacy of parents, children, and people who are in contact with children (such as teachers and educators) and, on the other hand, promote vaccination adherence by overcoming logistical problems. Thus, highly integrated community programs based on a multidisciplinary approach to disease prevention and control are valuable organizational models for engaging the population and promoting healthy lifestyles [23].

In this context, the aim of this study is to describe the on-site school intervention implemented in the Local Health Authority (LHA) Roma 1, Italy, and to analyze the results of vaccination uptake reached among children.

METHODS

Study design, setting and intervention period

The Vaccination Department of the LHA Roma 1 promoted an onsite school-based intervention in health district 14, which corresponds to municipality XIV of the city of Rome, aimed at increasing adherence, knowledge and influenza vaccination uptake among children aged 2-6 years old during the flu vaccination campaign 2021/2022, from November 2021 to January 2022. A multidisciplinary team designed an intervention to involve community stakeholders like schools and parents, through an active call for school involvement, training activities through webinars, and onsite vaccination in schools.

School involvement

Kindergartens insisting on the territory of municipality XIV were identified through records available on the municipality of Rome and from the administrative flows and of the LHA Roma 1, as well as derived from the COVID-19 emergency management and through direct knowledge related to other projects developed in the LHA Roma 1. Thus, the total number of kindergartens identified and involved was 50.

Target population and type of vaccination

Vaccination was offered to children aged 2-6 years with the Fluenz Tetra® vaccine. This is a live attenuated, quadrivalent nasal spray suspension vaccine, indicated and recommended by the CDC and the World Health Organization (WHO) for influenza prophylaxis in people aged 2-49 years [24, 25]. This vaccine, available in Italy from the 2020/2021 season, is administered to children and adolescents aged 2-18 years old as a single 0.2 mL dose divided into two nostrils (0.1 mL administered to each nostril). In previously unvaccinated children a second dose is recommended at least 4 weeks after the first dose [26].

Primary and secondary endpoints

The primary endpoint of the present paper is to describe an effective and reproducible organizational model aimed at promoting vaccination among children aged 2-6 years with a multidisciplinary school-based intervention designed to increase both influenza vaccination coverage and the knowledge and engagement of children, parents and teachers.

The secondary endpoint was the vaccination of school staff, teachers, parents and other students aged >6 years old, with the injectable flu vaccine (Flucelvax®).

Participation and ethical approval

Vaccination was offered free of charge to all the participants, both children and adults. Participation in the project by both schools and parents with their children was completely voluntary, and consent could be withdrawn at any time. No further approval by the ethics committee was needed, as the flu vaccination campaign, for which the LHA is responsible each year, was conducted by the same health professionals working in the vaccination centers, following the same standards in terms of privacy. Moreover, at the end of the campaign, the participants' file was anonymized and then sent to the data analysts.

Data analysis

We performed descriptive analysis calculating the vaccination coverage (first and second dose) and the variables of our population such as median age (25° and 75° percentile, interquartile range), gender, and school distribution. All the analysis was performed using the Stata software, version 14, StataCorp limited partnership (LP), College Station, Texas (TX).

RESULTS

Description of the school-based intervention

The intervention took place from November 2021 to January 2022 and was aimed at the kindergartens in the territory of health district 14. The project included an initial phase of school involvement. Next, a vaccination promotion intervention was conducted with parents and teachers through two webinars, in which healthcare workers explained the project and gave information on pediatric influenza vaccination. Finally, an onsite school-based intervention was conducted in schools that had joined the initiative. At the end of the intervention period, a focus group among all the health staff involved was organized to discuss the barriers and facilitators. *Figure 1* shows the different phases and institutions involved in the project.

Phase 1: schools' involvement

The Vaccination Unit of the LHA Roma 1 contacted schools' representatives by phone and email, to briefly share information about the project, present the intervention methods and timing, and planned the online

webinars. In this context, 50 schools were contacted and 29 (58%) confirmed their participation in the project, potentially involving a total of 2,424 children aged between 2 and 6 years old, with the number varying per school from a minimum of 20 to a maximum of 298 children. *Figure 2* shows the distribution of the schools in the district 14 territory.

Phase 2: online webinars and informed consent

Two webinars were organized, the first addressed only to school staff and the second open to parents. During the first online meeting, the school staff was involved in the intervention design and organizational and logistic aspects were discussed. Healthcare professionals shared information about the importance of the influenza vaccination in the healthy population, and school staff proposed topics of interest to explore with families.

Thus, a second meeting was organized following the received indication, dealing with the epidemiology of influenza, risks and benefits of vaccination in children, timing and schedule, and vaccine mechanisms of actions and compositions. Moreover, the consent form was shown and explained, together with the intervention design. Finally, parents were instructed to follow the vaccination schedule, which included one or two doses depending on the children's vaccination status. For naive children, they were exhorted to complete the

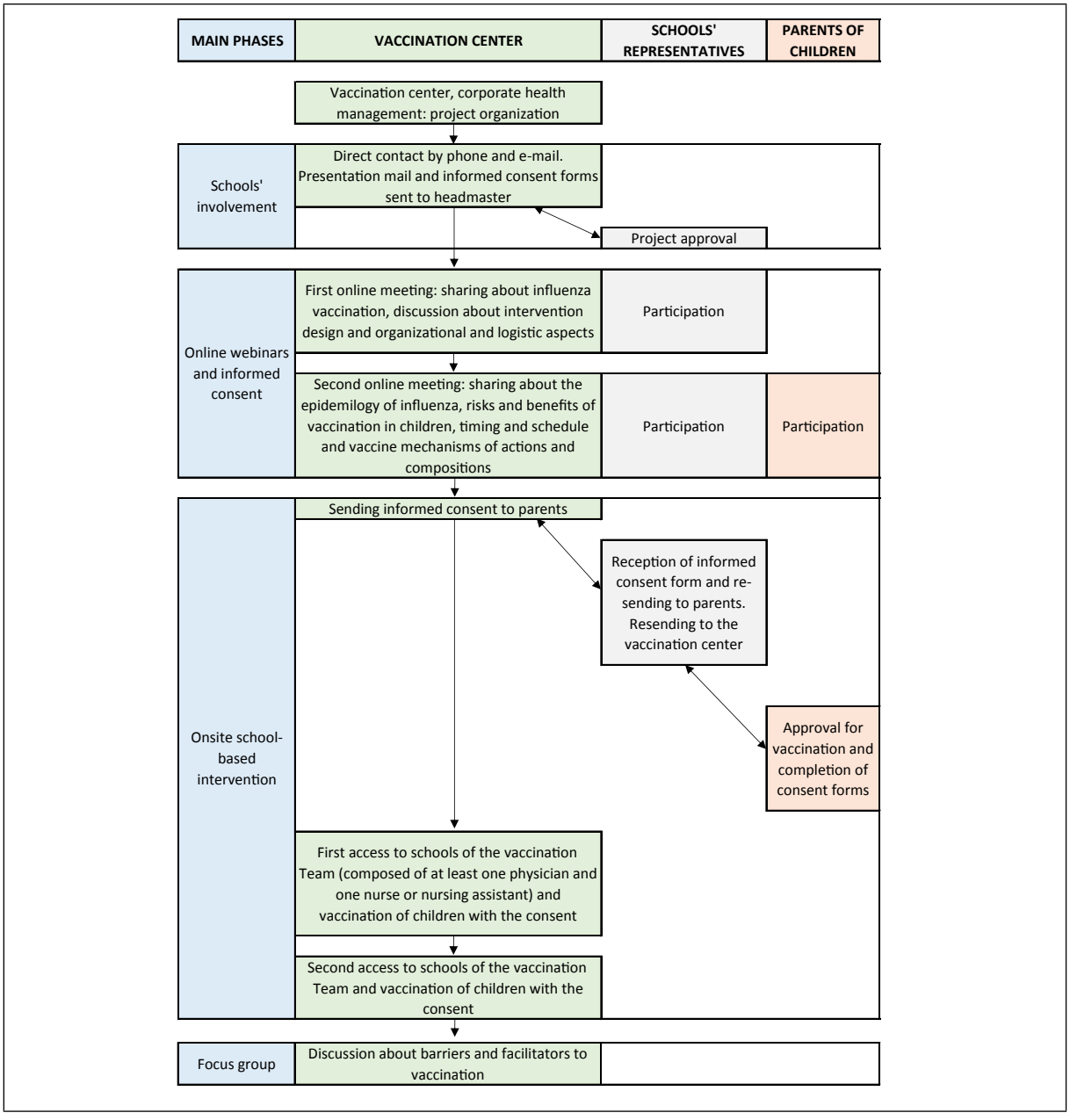

Figure 1
Project flowchart, including schools' involvement, webinars, onsite intervention and evaluation of the activity.

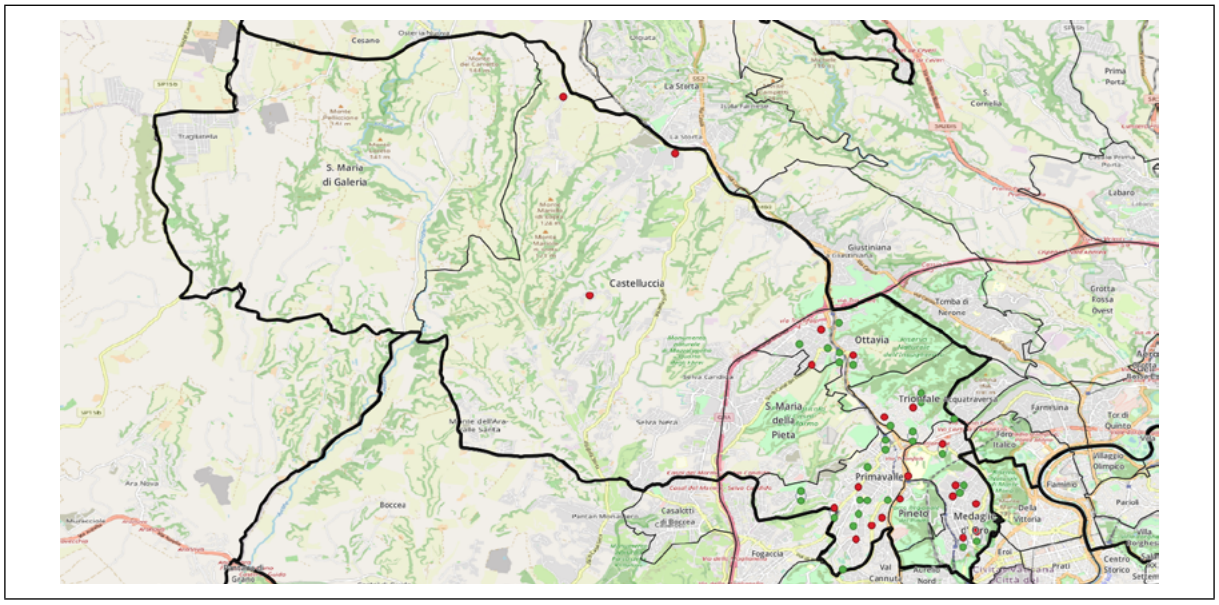

Figure 2
School distribution in the health district 14 of the Local Health Authority Roma 1.
Red dots correspond to schools that were contacted but did not participate, while green dots correspond to schools that confirmed their participation to the project.

vaccination cycle, with a second dose at school and, where it would not be possible (due to school closure or the child's absence on the day of the visit) at the family pediatrician or by going to the vaccination center. Almost 150 parents participated in the webinar. The day after the meeting, informed consent was sent to the schools, and then distributed from schools to parents to be filled out and signed. In this way, children could be vaccinated without the presence of their parents on the day the intervention was scheduled.

Phase 3: vaccination team (VT) and onsite school-based intervention

The VT consisted of at least one physician and one nurse or nursing assistant. The LHA scheduled the site visit based on the availability of the VT, in agreement with schools. At least two accesses were scheduled for each school, to vaccinate those children who were not present at school on the first access and to administer the second dose to those children who had not previously been immunized. The school was asked to prepare a separate room with chairs and a table so that health personnel could work safely without entering the classrooms. One week, before access, each school had to communicate the number of children to vaccinate. On the day of the intervention, Fluenz Tetra® vaccines were properly prepared to transport, along with some doses of injectable influenza vaccine. The VT went to schools according to the shared planning, with all the necessary equipment (i.e., drugs to manage allergic reactions), including COVID-19 personal protective equipment (PPE) that must be changed for each school. All schools provided a reserved room where health personnel could wear PPE, evaluate the consent forms, and administer vaccines. If the consents were complete and without contraindications, enrolled children were invited to the room. By engaging them through storytelling, the administration was carried out and the children were allowed to return to their classrooms. The VT waited 15 minutes after the last administration before leaving the school. The same process was followed for vaccinations of children who were in the secondary endpoint. Regarding adult vaccination, clinical history was individually taken, and informed consent was signed on-site.

In the intervention period, COVID-19 incidence among school-aged children was rising, and in Italy, quarantine measures were applied to a class with one confirmed case [27]. For this reason, additional information on quarantine dispositions in the selected schools was retrieved from the LHA database.

Vaccination uptake of children aged 2-6 years

Overall, out of 2,424 eligible children aged 2-6 years, 405 were vaccinated (16.7%). Of these, 218 (53.8%) were male and 187 (46.2%) were female, with a mean age of 4.4 years old (Interquartile range: 1.5 years). Out of 405 vaccinated children, 177 (43.7%) received only one dose, as they had already been vaccinated at least once in their lives, while 228 (56.3%) were vaccinated for the first time against influenza (*Table 1*). Of these, 150 students (65.8%) also received the second dose, as per the vaccine schedule: 26 (17.3%) were vaccinated in other settings (such as family pediatricians and vaccination centers), while 124 children (82.7%) received the second dose during the second school visit. Thus, among children who have never been vaccinated in their lives, 78 did not receive the second dose.

Indeed, due to the COVID-19 pandemic wave, at the beginning of 2021, schools were closed, and it was not possible to continue the onsite campaign in this setting.

Table 1
Number and percentage of vaccinated children in the enrolled schools with a second dose

School	N. of first doses* (%)	N. of second doses (%)	Second doses made at school^ (%)	Second doses made in another setting^^ (%)
1	4 (16)	3 (75.0)	3 (100.0)	0 (0.0)
2	9 (8.7)	1 (12.5)	0 (0.0)	1 (100.0)
3	1 (5)	0 (0)	0 (0.0)	0 (0.0)
4	9 (20.5)	8 (88.9)	5 (62.5)	3 (37.5)
5	6 (10)	5 (83.3)	4 (80.0)	1 (20.0)
6	5 (4.5)	5 (100.0)	5 (100.0)	0 (0.0)
7	4 (4.8)	4 (100.0)	4 (100.0)	0 (0.0)
8	6 (8.8)	5 (83.3)	5 (100.0)	0 (0.0)
9	2 (2.7)	1 (50.0)	1 (100.0)	0 (0.0)
10	28 (9.4)	23 (82.1)	22 (95.7)	1 (4.4)
11	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
12	7 (6.9)	6 (85.7)	6 (100.0)	0 (0.0)
13	3 (10.3)	3 (100.0)	0 (0.0)	3 (100.0)
14	18 (11.6)	0 (0.0)	0 (0.0)	0 (0.0)
15	16 (23.5)	14 (87.5)	14 (100.0)	0 (0.0)
16	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
17	17 (16.2)	14 (82.4)	11 (78.6)	3 (21.4)
18	31 (16.2)	21 (67.8)	17 (81.0)	4 (19.0)
19	2 (3.9)	1 (50.0)	1 (100.0)	0 (0.0)
20	10 (10.3)	8 (80.0)	7 (87.5)	1 (12.5)
21	5 (5.4)	5 (100.0)	5 (100.0)	0 (0.0)
22	2 (3.8)	2 (100.0)	2 (100.0)	0 (0.0)
23	10 (10.0)	5 (50.0)	3 (60.0)	2 (40.0)
24	11 (27.5)	7 (63.6)	7 (100.0)	0 (0.0)
25	3 (4.3)	3 (100.0)	1 (33.3)	2 (66.7)
26	6 (14.0)	4 (66.7)	0 (0.0)	4 (100.0)
27	9 (9.3)	1 (11.1)	1 (100.0)	0 (0.0)
28	2 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)
29	2 (2.8)	1 (50.0)	0 (0.0)	1 (100.0)
Overall	**228 (9.4)**	**150 (65.8)**	**124 (82.7)**	**26 (17.3)**

*Number of fist doses administered on the total number of children in that school; ^percentage of second doses delivered in school setting out of the total number of second doses; ^^percentage of second doses delivered in other settings out of the total number of second doses.

For that reason, a highly variable range of adherence to the second dose is observed (*Table 1*). Vaccination rates vary among schools, ranging from a low of 2.8% (2 children out of 71 children enrolled in the school) to a high of 48.0% (12 children out of 25 children enrolled in the school) (*Table 2*).

Vaccination of other categories

Vaccination was also offered, free of charge and on-demand, to teachers, staff, parents and children aged >6 years old. In this context, 130 teachers, 8 parents, and 10 children (mean age of 9.8 years) were vaccinated. Notably, the parents and children turn out to be those in a class with an immunocompromised child. Since this was a free and on-the-spot offering, it was not possible to relate the number of vaccinations to the total number of teachers and parents in the schools.

Phase 4: focus group

A concluding focus group was conducted among the healthcare workers who had been involved in the intervention. Overall, the program was deemed effective and feasible. One of the most important barriers was the pandemic, which had a twofold effect: first, the use of protective equipment made it difficult to interact with children and engage them; second, quarantines precluded the organization of visits from being fruitful. The adoption of a more flexible agenda has been proposed as a possible solution to the organizational issue. Facilitators were described as connected to the trust relationship built with schools during the previous year. Thanks to the intervention, 130 teachers decided to vaccinate, and healthcare workers shared during the focus group how the mere availability of a vaccine at work can in-

Table 2
Number and percentage of vaccinated children in the enrolled schools

School	N. of registered children	N. of vaccinated children	Vaccinated (%)
1	25	12	48.0
2	103	24	23.3
3	20	1	5.0
4	44	19	43.2
5	60	9	15.0
6	111	7	6.3
7	83	16	19.3
8	68	9	13.2
9	74	6	8.1
10	298	38	12.8
11	78	4	5.1
12	101	15	14.9
13	29	4	13.8
14	155	36	23.2
15	68	22	32.4
16	43	5	11.6
17	105	24	22.9
18	191	49	25.7
19	51	2	3.8
20	97	15	15.5
21	92	8	8.7
22	52	2	3.8
23	100	16	16.0
24	40	18	45.0
25	70	5	7.1
26	43	8	18.6
27	97	23	23.7
28	55	6	10.9
29	71	2	2.8
Overall	**2,424**	**405**	**16.7**

duce workers to protect themselves. The episode of the immunocompromised child, who encouraged parents and classmates to vaccinate, was an example of how the perception of the importance of a preventive intervention is critical to increasing adherence.

In the end, healthcare workers reported that more physicians and, in general, healthcare personnel were needed to implement this public health program effectively in the coming years.

DISCUSSION

Our study shows how an active onsite vaccination campaign in schools, where children spend most of their time and are therefore easily reachable, leads to the achievement of valuable vaccination coverage. Specifically, this study describes the implementation of an organizational model offering a preventive intervention to citizens within a community health approach. Influenza vaccination uptake enhancement programs for children, such as onsite school-based intervention, represent an important preventive action [28, 29]. For example, the experience in the United Kingdom shows good vaccine uptake levels within the targeted population, which is 7.1% higher than the non-targeted population. Moreover, among the target population, almost 55% of the pilot areas achieved vaccine uptake of more than 60% [30]. Similarly, a study from Canada confirms that immunization in school-based clinics is associated with increased vaccine uptake [31]. Onsite school vaccination has also proven to be a useful tool impacting health outcomes: indeed, a Japanese study shows that the initiation of the school-based vaccination program prevented about 37,000 to 49,000 deaths per year, equal to 1 death for every 420 children vaccinated. As the vaccination of schoolchildren was interrupted, the excess

mortality rates in Japan increased [32]. The United Kingdom experience also demonstrates that general practitioner (GP) ILI consultation rates and influenza swab positivity in primary and secondary care were lower in pilot areas compared with rates in non-pilot areas across all age groups [30]. This strengthens the choice of a school-based administration to achieve high childhood vaccination uptake [33].

Recently, Roncaglia et al. observed how the vaccination coverages of children included in the school vaccination program were higher than the coverages observed in children attending other schools (67.9% *vs* 56%) [34]. Another study showed how vaccination rates were significantly higher in schools with onsite vaccination options compared to the control group, confirming the usefulness of such interventions [35]. Similarly, school vaccination campaigns in France resulted in an increase in overall coverage (mandatory and recommended vaccines) from 10.7% to 65.7% for diphtheria-tetanus-poliomyelitis-pertussis (DTaP/IPV), hepatitis B virus (HBV), measles-mumps-rubella (MMR), meningococcal C, and human papillomavirus (HPV) vaccines [36]. Another study showed how the school-based health promotion project was effective in improving immunization uptake, especially for those recommended [37].

In our context, the intervention included an important part of training to engage teachers and families. Education and information activities of children, parents and teachers is a key factor [28]. The meetings held with teachers, parents and children, in fact, encouraged the spread of knowledge countering false myths and fake news related to vaccination, fostering a two-way relationship and building trust between the LHA and citizens. Indeed, the involvement of parents and teachers in this intervention allowed us to extend the vaccination to them as well, reaching 148 people.

Several studies have shown how educational programs can increase vaccination coverage [35, 38-40]. In Germany, for example, it was observed how the onsite vaccination offered in combination with an educational intervention showed a stronger increase in vaccination rates against MMR and DTaP/IPV vaccines [39]. Hu et al. have recently shown that multifaceted strategies (including health education course to students, educational videos to parents, involving parents in student-parent collaborative homework, and messages on different occasions to remind parents of vaccination) contribute significantly to increasing influenza vaccination coverages [40]. Finally, in Spain, it was observed that such education and knowledge-sharing activities had positive results in terms of parent and teacher attitudes [41].

A further consideration is related to the type of vaccine. Having a non-invasive inhalable vaccine available, in fact, can facilitate the organization and logistics aspects and reduce possible fears of children and parents related to injectable vaccines [42]. Our study has limitations. First, we were unable to know what the vaccination adherence was in the pediatric population 2-6 years old in previous years in the area of LHA Roma 1; however, the primary endpoint of our study was to promote a multidisciplinary school-based intervention, providing a valuable and replicable organizational model. Second, our study did not investigate whether any factors (social, cultural, and economic) may influence parents' acceptance or refusal of vaccination. In fact, the variability in coverage found in schools suggests that the area where the school is located might reflect the different income and living conditions of families and this condition provides an important cue for subsequent studies to investigate these issues. Third, in Italy, the incidence of COVID-19 infections began to rise in November 2021, peaking on January 10, 2022, so all the intervention was carried out during the period of highest viral circulation, and many classes were in quarantine during the visits. For this reason, the value of vaccination coverage is underestimated because the number of those enrolled in school was used as the denominator since it was not possible to estimate the number of those present on the days of the accesses.

CONCLUSIONS

Community-based interventions increase adherence to health promotion campaigns. It is, therefore, necessary to continue investing in activities aimed at engaging the population, facilitating access to care settings and, as in this case, integrating the healthcare setting into the school setting. Being affiant to health education actions, such interventions are useful tools to increase public confidence and fight vaccine hesitancy.

Authors' contributions

All Authors contributed to the study conception and design. Material preparation and data collection were performed by LV, MTR, PL, AB and AS. LV and PL performed the statistical analysis. The first draft of the manuscript was written by LV, MTR, MDP, and AB. AS, MG, MM and PP commented on the latest version of the manuscript. SA, PP and GQ supervised the study. All Authors read and approved the final manuscript. All Authors attest they meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship.

Conflicts of interest statement

The Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and material

Data was collected and analyzed by ASL Roma 1. Data are property of LHA Rome 1 and are available for reasonable requests.

Received on 10 August 2024.
Accepted on 24 March 2025.

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Ann Ist Super Sanità 2025 | Vol. 61, No. 2: 141-148
DOI: 10.4415/ANN_25_02_07

Evaluating screen exposure in very young children: insights from the Italian Surveillance System of children aged 0-2 years

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Abstract

Objective. To describe screen exposure and its association with socio-economic characteristics in a large representative sample of children aged 0-2 years in Italy.

Materials and methods. Data from the 2022 Italian Surveillance of children aged 0-2 years, collected on 35,550 mothers, were analysed to estimate the prevalence of screen exposure. Logistic regression was used to investigate the association between exposure and potential predictors.

Results. Overall, 39.2% of children aged 2-15 months were exposed to digital screens. The exposure prevalence increased with age, ranging from 13.9% at 2-3 months to 61.9% at 13-15 months. Screen exposure was significantly more frequent among children of mothers with non-Italian citizenship, having lower levels of education, reporting economic difficulties, non-participating in antenatal classes (ACs), and residing in the center-south.

Conclusions. Most babies, particularly from low socio-economic status (SES) families, were exposed to screens in a period when this may interfere with responsive caregiving and thus with early child development. It is imperative to inform parents and caregivers about the risks of early exposure since the first months of life.

Key words

- surveillance
- screen exposure
- young children
- parental responsiveness

INTRODUCTION

The use of screen technologies (smartphones, tablets, personal computers, television and video games) has become widespread in recent years [1]. The onset of the COVID-19 pandemic has exacerbated this trend, leading to a reduction in the age in which individuals access digital technologies [2-4].

Recently, the Convention on the Rights of the Child addressed the issue of digital technologies, emphasizing the need to ensure the potential benefits while mitigating associated harms, especially for children in vulnerable situations [5].

Research on the effects of exposure to screens continues to evolve, highlighting the complexity of the issue and underscoring the public health importance of addressing screen exposure from early childhood, as it can significantly impact the psychophysical development of children because the foundations of later development are posed during these crucial early years of life [6]. In recent years, many studies have addressed digital addiction, which has become a significant public health priority [7]. To effectively research and promote interventions in this area, it is crucial to consider the life course approach [8].

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International health organizations issued guidelines around age-appropriate screentime. The World Health Organisation (WHO) recommends that children under the age of 2 should not be exposed to any screens, while screentime in children between 2 and 5 years of age should be limited to 1 hour per day, the less is better [9]. The American Academy of Pediatrics (AAP) recommends "minimizing or eliminating media exposure, other than video chatting, for children under the age of 18 months" [10]. Screentime in older children should possibly involve interactive and educational content, supervised by an adult. Children need exploration and relational exchanges with caregivers for their cognitive and emotional development, and this crucial learning can't be mediated by screen devices [1, 11, 12]. Thus, providing alternatives to passive screen consumption is strongly encouraged.

Despite these recommendations, children are being exposed to screen technologies from a very young age [1, 13]. Screens are used to distract, calm, or entertain the child, as an educational tool or simply being present in the background during other activities [1, 14].

A recent systematic review and meta-analysis found that only a minority of young children adhere to the recommended screentime limits, with a large variability worldwide. In total, 75.3% of children under 2 years of age were found to be not adhering to the guideline of no screen time [15]. Many studies have also found that the time children spend watching screens is influenced by socio-economic determinants: higher mother's level of education and higher household income correlate with less screentime [13, 15, 16].

Since screens have become such a pervasive part of children's lives, researchers have started to investigate the potential consequences [17-21]. Excessive or inappropriate screentime has been associated with delays in learning, language and self-regulation [14, 19-21].

Some argue that most of the evidence regarding screentime and developmental problems is correlational, not allowing to prove causality, as children with developmental difficulties may be more prone to engage in excessive screen time [22].

Other studies have emphasized the importance of context, beyond sheer screen time. In a narrative review Guellai and colleagues have underlined four aspects that may modulate the effect of screens on young children's cognition: the quality and age-appropriateness of media content, the caregiver's participation, the interactivity of the program and the presence of a screen in the background [1].

It is also of note that the time spent in front of a screen is subtracted from other occupations, including outdoor physical activities, exploring the environment, relational and bonding time [11, 14]. Finally, excessive sedentary screen watching may put children at a higher risk of obesity, sleep disturbances and relational problems [9, 23-26]. In the first years of life, a child's brain is extremely plastic, new competences are acquired and the capacity for future learning develops [27, 28]. It is a crucial time of opportunity for health promotion, therefore international organisations such as WHO and UNICEF have encouraged the development of national policies aimed at interventions in infancy and young childhood for a fair start in life [29, 30]. However, effective policies need data in order to plan targeted interventions, as recommended by the WHO [30].

Since 2019 the "Surveillance System for the main determinants of health in children aged 0-2 years" (Surveillance of children aged 0-2 years), promoted by the Italian Ministry of Health and coordinated by the Italian National Institute of Health (Istituto Superiore di Sanità, ISS), has been collecting information on behaviours recognized as risk or protective factors for the children's health, including screen exposure.

The main objective of this paper is to describe the daily frequency of screen exposure in children under 2 years in Italy, and to assess associated factors using data from the 2022 Surveillance of children aged 0-2 years.

MATERIALS AND METHODS

The Surveillance of children aged 0-2 years was based on cross-sectional sample surveys repeated at regular intervals among mothers of children up to 2 years of age taken to vaccination centers (VCs) to receive immunizations in the Italian regions. Mothers were enrolled in all the VCs of the regions when one of the following vaccine doses was administered to their children: first, second, third dose of mandatory vaccine against diphteria, tetanus and pertussis (DTP) or hexavalent vaccine (against diphtheria, tetanus, pertussis, poliomyelitis, haemophilus influenzae type B, and hepatitis B), and first dose of the vaccine against measles, mumps, rubella, varicella (MMRV). Four independent samples in each region were selected in correspondence with the four doses corresponding approximately to the ages 2-3 months, 4-5 months, 11-12 months and 13-15 months, according to the Italian vaccination schedule.

Mothers self-completed an anonymous questionnaire online or on paper with the support of trained health professionals during the waiting periods before or after the vaccination session.

The Surveillance collects information on several important children health determinants including exposure of children to screens (tablet, mobile phone, TV, computer). Demographic and socio-economic characteristics of participants were also collected. For further methodological details see Appendix 1 in the paper by Pizzi *et al*. previously published on Annali dell'Istituto Superiore di Sanità [31].

The present study used data of the second round of the Surveillance, conducted between June and October 2022 in the Italian regions, except for the Region of Molise and the autonomous province of Bolzano, who did not participate in the Surveillance (the Molise Region had difficulty starting the data collection while the autonomous province of Bolzano was unable to complete it), and Region of Tuscany who shared results of its ongoing maternity care survey.

Outcome

Exposure to screens (yes/no) was included in the analysis as outcome variable. Mothers were asked if their children spend time in front of a screen (TV, com-

puter, tablet and/or mobile phone) and how long per day. Children who spend time in front of a screen were considered exposed.

Covariates

The following socio-economic characteristics were included as potential risk factors: mother's age (<30, 30-34, ≥35 years), citizenship (Italian, not Italian), educational level (low, middle school or lower; medium, high school; high, bachelor's degree or higher), perceived economic difficulties (no, some/many), parity (primiparous, multiparous), attendance of an antenatal class (AC) (yes, never), geographical area of residence (North, Centre, and South Italy), and family type (mother permanently cohabiting in couple, single parent).

Statistical analysis

Frequency distributions, prevalence rates and odds ratios (ORs) with 95% confidence intervals (CIs) were used to describe data. Percentages were calculated based on cases with available information excluding missing values. From the overall sample, specific age groups were selected for the analysis of exposure to screens, irrespective of the administered vaccine dose (*Table S1 available online as Supplementary Materials*).

Frequency distributions were used to describe both socio-economic characteristics of mothers participating in the study and daily exposure to screens of children at different ages within the 0-2 years class.

Prevalence rates of exposure stratified by socio-economic characteristics were calculated. Adjusted ORs and their 95% CIs were estimated through a multiple logistic regression model in order to explore factors associated with the exposure to screens.

Statistical analyses were performed using STATA/SE version 18.0 statistical software.

RESULTS

The present study involved 35,550 mothers with a response rate of 95.7% (ranging between 89.2% and 98.6% at the regional level). The socio-economic characteristics of women participating in the study are shown in *Table 1*. More than 4 out of 10 mothers were aged ≥35 years, 12.2% were foreigners. Four out of 10 obtained a bachelor's degree and about one third reported economic difficulties. Women who never attended an AC were 38.8%, and 2.9% were single parents.

Table 2 presents the daily frequency of exposure to TV, computers, tablets, and/or mobile phones among children of different age groups. Among children aged 2-3 months, 13.9% were exposed to screens, with the majority (10.5%) spending less than one hour per day in front of a screen. The percentage of exposure increased with the age of children, affecting over 6 out of 10 children in the 13-15 months age group, with 2 out of 10 (20.1%) exposed for more than one hour per day, and 3.0% spending more than three hours per day. Three-quarters of the children (75.9%) were exposed to screens in the presence of an adult, either parent or another caregiver, 13.7% were alone or with other children during screen time, and 10.4% were exposed indiscriminately, either with adults, alone, or with other children (data not reported in *Tables*).

Table 3 shows prevalence rates stratified by socio-economic characteristics and adjusted ORs of exposure to screens among children aged 2-15 months. Children significantly more likely exposed included those with mothers who had foreign citizenship (OR=1.27; 95% CI: 1.15-1.42), lower educational level (medium: OR=1.29; 95% CI: 1.20-1.39; low: OR=1.46; 95% CI: 1.32-1.63),

Table 1
Women's socio-demographic characteristics (n=35,550)

Variables	(%)
Age	
≤29 years	21.5
30-34 years	35.0
≥35 years	43.5
Missing	*5.5*
Citizenship	
Italian	87.8
Not Italian	12.2
Missing	*5.1*
Educational level	
Low	14.8
Medium	45.4
High	39.9
Missing	*4.9*
Economic difficulties	
None	65.5
Some/many	34.5
Missing	*4.6*
Parity	
Primiparus	54.2
Multiparous	45.8
Missing	*7.8*
Geographical area	
North	45.9
Center	16.4
South	37.7
Missing	*0.0*
Attendance of an antenatal class	
Never	38.8
Yes	61.2
Missing	4.2
Family type	
Cohabiting in couple	97.1
Singol parent	2.9
Missing	*4.7*

Table 2
Children at different ages by daily frequency of exposure to screens

Exposition hours/day	Children aged 2-3 months		Children aged 4-5 months		Children aged 11-12 months		Children aged 13-15 months		Children aged 2-15 months	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Never	7,271	86.1	6,210	69.0	3,204	45.1	2,086	38.1	18,771	60.8
Yes	1,116	13.9	2,572	31.1	3,695	54.9	3,231	61.9	10,614	39.2
Less than one hour a day	*804*	*10.5*	*2,035*	*24.7*	*2,670*	*39.3*	*2,153*	*41.8*	*7,662*	*28.2*
1-2 hours a day	*256*	*2.9*	*465*	*5.3*	*857*	*12.9*	*913*	*17.1*	*2,491*	*9.2*
3 hours or more a day	*56*	*0.6*	*72*	*1.1*	*168*	*2.7*	*165*	*3.0*	*461*	*1.8*
Total	8,387	100.0	8,782	100.0	6,899	100.0	5,317	100.0	29,385	100.0

Table 3
Prevalence rates of exposure to screens and mutually adjusted odds ratios for the reported variables. Logistic regression model. Children aged 2-15 months

Variables	(%)	OR	95% CI	
Age				
≤29 years	44.1	1		
30-34 years	38.1	0.93	0.85	1.02
≥35 years	37.6	0.99	0.91	1.08
Citizenship				
Italian	38.4	1		
Not Italian	45.3	1.27	1.15	1.42
Educational level				
Low	47.4	1.46	1.32	1.63
Medium	42.0	1.29	1.20	1.39
High	33.0	1		
Economic difficulties				
None	36.1	1		
Some/many	45.6	1.33	1.24	1.43
Parity				
Multiparous	37.4	1		
Primiparus	40.3	1.21	1.13	1.29
Geographical area				
North	34.2	1		
Center	38.9	1.20	1.10	1.30
South	46.3	1.51	1.41	1.62
Attendance of an antenatal class				
Yes	34.8	1		
Never	46.0	1.26	1.17	1.35
Family type				
Cohabiting in couple	39.0	1		
Single parent	43.2	0.97	0.79	1.18

CI: confidence interval.

reported economic difficulties (OR=1.33; 95% CI: 1.24-1.43), were primiparous women (OR=1.21; 95% CI: 1.13-1.29), mothers who never attended an AC (OR=1.26; 95% CI: 1.17-1.35), and those residing in the Centre (OR=1.20; 95% CI: 1.10-1.30) and South Italy (OR=1.51; 95% CI: 1.41-1.62). No statistically significant associations were found with either maternal age or family type.

DISCUSSION AND CONCLUSIONS

Despite the international guidelines discouraging to expose children in the age range that we surveyed to any type of screen [9-13], our findings highlighted how exposure to digital technologies occurs at a very early age and increases as children grow older, in line with a growing body of literature [1, 13, 32]. The 2020 USA common sense census survey found that children under the age of 2 were exposed daily to an average of 49 minutes of screen time, compared to 2 hours 30' in children of 2-4 years and 3 hours 5' in children of 5-8 years [13]. Furthermore, during the pandemic emergency an increase in screen time has been observed in children and adults, including young children [33]. In 2016, Balbinot and colleagues published a study on parents' attitudes to screen exposure in Italian children, where more than 1/3 of parents reported the use of screen devices to keep calm their children under 12 months of age, while parents of toddlers reported an even higher use of screens for this purpose [6].

Our data showed that adult caregivers are mostly present (75.9%) during screen time, however, it is of note that the recommendation to be present during exposure only applies to children older than those we focused on in this study.

Research has shown that such young children can learn little to nothing from a screen device because they learn through interaction and social modelling that are crucial in the first years of life [1, 14].

A noticeable gradient in the association between socio-economic characteristics and screen exposure was found, indeed, mothers with a medium or low level of education as well as those reporting economic difficulties were more likely to expose their children to screens compared to less disadvantaged mothers. A US study on young children's screen time using data from 1997 and 2014, before and after the widespread availability of mobile devices, highlighted that in both years, the lower screen time group was associated with higher income and educational level of the family [16]. Also, the Common Sense Census survey concluded that the general increase in children's screen time seems to be led by the lower income group [13].

A higher occurrence of exposure was found in children of non-Italian mothers, confirming the greater vulnerability of foreign women who often live in conditions of social deprivation and suffer from the lack of a supportive family network. Similarly, women residing in the southern regions of the country exhibited a higher tendency to expose their children to screens compared to those in northern Italy, confirming the challenge of adhering to best practices aimed at protecting health during the first years of life in this area of the country [34].

Finally, screen exposure was significantly more common among mothers who did not attend an AC compared to those who did. ACs offer support to mothers and parents, particularly during pregnancy and the postnatal period [35, 36], contributing to higher rates of exclusive breastfeeding among participating mothers [37].

The main strengths of this study were the standardized and validated data collection procedures, the representative sample of the population studied, and the training provided to all professionals involved in the Surveillance.

The study limits included the lack of detailed information on exposure content/context and the device used. However, considering that screen time is universally discouraged within the age range investigated by the surveillance, inquiring about the context seemed unnecessary for the purposes of this study.

Understanding the screen time limits recommended by health organisations and being aware of the implications of digital content and context are fundamental for both parents and for all caregivers. In this regard, several initiatives are being developed in Italy [38].

Among interventions on children's screen time, restrictions and limitations may not be effective [39], therefore parents and caregivers should strive to offer children quality time alternatives to passive screen viewing, exposing them from a young age to music, reading books, interactive play and physical exploration in nature [40]. All professionals working in health and education could have a key role in informing and supporting parents and caregivers about this topic since the family environment is the first place of learning for the child [41, 42]. A critical aspect to consider is the excessive use of screen devices by parents, that has been negatively associated with parental responsiveness [43]. This aspect entails the ability to pay attention, understand, and respond to the child's needs, which are fundamental for developmental outcomes [44, 45]. Some studies suggest that screen devices themselves are not the sole cause of a lower quality parent-child interaction but rather may reflect underlying issues in the relationship [46], Increased technology use among parents was found to be associated with less parent-child interactions, increased media use by children, and child psychosocial difficulties [45], suggesting that parents should reflect upon their own media usage habits.

Since children imitate their parents' behaviour and routines, families' habits are generally transferred to the next generation, including parental media use [45].

In this regard, the ONU [5] has recently expressed itself, stressing that states should develop guidelines directed to parents, health and education service providers to promote a healthy balance between digital and non-digital activities and adequate rest periods taking into account the child's developmental needs.

In conclusion, the surveillance data highlights the widespread use of devices among children under the age of 2 in Italy. The findings also outline associated factors that could be improved, presenting intervention opportunities whose effectiveness will be measured through the data collected in future rounds of the national surveillance.

Acknowledgments

We thank all the mothers who were willing to complete the questionnaire. We thank all the Regional and Local Health Unit coordinators and all the professionals involved in the survey for their valuable work. Our sincere gratitude to the Surveillance of children aged 0-2 years Scientific Technical Committee for their contribution to the definition of methodological aspects and critical reading of the results.

Funding

The Surveillance of children aged 0-2 years is founded by Italian National Institute of Health (Istituto Superiore di Sanità, ISS).

Ethical approval

The Surveillance of children aged 0-2 years study protocol and questionnaire were formally approved by the National Ethics Committee for clinical trials of public research bodies of the Italian National Institute of Health (Istituto Superiore di Sanità, ISS) (Prot. n. PRE-4255 – 20/10/2014; Prot. n. PRE-BIOCE 10939 – 06/04/2018; Prot. n. 0015067 PRE BIO – 19/04/2022).

Authors' contributions

EP, MAS conceptualized and designed the study; MAS, analysed the data; EP, MAS, AEK wrote the first draft; SD, GT, MT, MB, EP critically reviewed the manuscript. All Authors have revised the manuscript and approved its final version.

Conflict of interest statement

None of the Authors declare competing financial interests.

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Received on 30 July 2024.
Accepted on 5 May 2025.

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Ann Ist Super Sanità 2025 | Vol. 61, No. 2: 149-156
DOI: 10.4415/ANN_25_02_08

A preliminary study of the effects of transformations induced by gamma-ray treatment on the detection of *Acheta domesticus* and *Tenebrio molitor* allergenic proteins by enzyme-linked immunosorbent assay (ELISA) techniques

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Abstract

Introduction. With the global population projected to reach approximately 9 billion by 2050, there's a growing need to explore alternative food sources. Insects have emerged as a potential solution to meet food demand, offering a substitute for conventional livestock. However, a primary safety concern surrounding these novel foods is their allergenic potential, especially given the absence of standardized testing methodologies. To mitigate this risk, food irradiation has been explored as a method to reduce allergenicity in insects intended for human and animal consumption.

Material and methods. This study utilized an enzyme-linked immunosorbent assay to determine the allergenic proteins in specific insect types after irradiation treatment.

Results and discussion. Significant differences in detectable protein levels were observed between *Tenebrio molitor* and *Acheta domesticus* samples, but no significant differences in protein content were found between food and feed samples of both species under identical irradiation conditions. Further research is required to ensure the protocol's suitability for more complex food matrices.

Key words

- edible insects
- food contamination
- food hypersensitivity
- enzyme-linked immunosorbent assay (ELISA)
- food irradiation

INTRODUCTION

The global food system faces increasing challenges as it strives to meet the nutritional needs of an ever-growing population within the constraints of environmental sustainability. It has been estimated that global population will reach 9.8 billion in 2050, so requiring an increase of livestock production of about 40% with respect to 2019 [1]. However, traditional livestock production, the main source of protein at the time, is associated with significant environmental impacts, namely high greenhouse gas emissions, extensive land and water use, and significant contributions to deforestation and biodiversity loss [2]. Insect consumption has therefore been proposed as a viable means to address these major challenges [3]. In fact, insect farming may represent a significant opportunity to reduce the impact of agriculture on climate change, mainly because insects emit fewer greenhouse gases than traditional livestock [2, 4]. Moreover, insects can efficiently convert feed into protein while requiring minimal land and water for breeding. From a nutritional perspective, insects are a rich source of micro- and macromolecules, making them a valuable addition to the human diet. Particularly, they are rich in high-quality protein whose average con-

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tent is of about 40%, ranging from 20% up to over 70%, depending on the species and the development stage at the time of harvesting [5]. Currently, edible insects are a dietary component for about 2 billion consumers, although consumption in Europe and the Americas is low [3]. Nonetheless, the global edible insects' market is expected to reach USD 16.39 billion by 2032 [6].

In Europe, edible insects are considered novel food, namely food product that does not have a history of human consumption within Europe and that was not significantly consumed before May 1997, when the first Novel Food regulation came into place. Therefore, their introduction as a food in the market must follow the Novel Food Regulation 2015/2283 [7] and its implementing Regulations 2468/2017 and 2469/2017 [8, 9]. Nowadays, four insect species, namely Tenebrio molitor (Coleoptera: *Tenebrionidae*), *Alphitobius diaperinus* (Coleoptera: *Tenebrionidae*), *Locusta migratoria* (Orthoptera: Acrididae), and *Acheta domesticus* (Orthoptera: Gryllidae), have been authorized as novel foods [10-15]. As well as being a viable alternative to many foods, edible insect proteins have also been studied as an alternative protein source in animal feed [6, 16]. It must be pointed out that insects intended for animal or human consumption are farmed animals; therefore, they cannot be collected from the wild and the general legislation for feeding other livestock should be applied [17]. Noteworthy, the parts of insects that can be used varies depending on the animal to be fed and whether it enters the food chain [6].

Despite the recognized benefits, there are several barriers to the widespread adoption of insect-based proteins [6]. Particularly, disgust, food neophobia, lack of interest, lack of information, and no prior experience have been reported as the most frequent factors affecting this repulsion [2, 6]. Also, the potential safety concerns represent a limitation to their use. Besides contamination with pathogens and heavy metals [1, 18], edible insects may be also a source of allergens. Indeed, the consumption of novel food insects may induce primary sensitization and allergic reactions to insect protein [19]. The primary allergenic proteins present in insects are tropomyosin and chitin. Current research points to tropomyosin as the leading allergen causing cross-reactivity among crustaceans, mollusks, mites, and cockroaches. Chitin, a polysaccharide forming the exoskeleton of insects, is another molecule of concern. Studies have revealed diverse effects of chitin on the immune system [20].

Tropomyosin was identified as a major allergen with cross-reactivity for subjects already allergic to crustacean and cross-reactivity with pan-allergens of the Arthropoda genus, namely crustaceans and house dust mites, has been reported too [21]. Therefore, the labeling of insect-based food products should include a statement near the ingredients list indicating that they may cause allergic reactions in individuals with known allergies to crustaceans and dust mites.

To overcome the safety issue due to the allergenic risk, food processing methods have been investigated to modulate the risk of cross-reactivity and allergenicity of edible insects. In fact, it has been demonstrated that food processing could have the potential to reduce the IgE-binding ability of allergens, thereby attenuating IgE-mediated allergic responses, by altering both the linear and conformational structure of epitopes [21]. In this context, previous studies have demonstrated that enzymatic hydrolysis and thermal treatments [22, 23] can effectively reduce cross-reactivity and allergenicity in edible insects [21]. Moreover, gamma radiation (1-15 kGy) has also been shown to effectively reduce the IgE-binding capacity of tropomyosin in shrimp [24].

Gamma radiation treatment is already used against microbiological contamination, to preserve the hygienic quality of food during storage and transport, ensure shelf life, and reduce the risk of foodborne illness [25]. It is toxicologically and microbiologically safe and nutritionally adequate for any food irradiated up to a maximum dose of 10 kGy [26]. Irradiation's primary advantage overheat processing or chemical treatments is its capacity to decrease microbial load without compromising product quality. Over the last two decades, the treatment with ionizing radiation has been used all over the EU [27] on a large variety of foods [28] including frog legs, poultry, fish, and vegetables. In the European Union the treatment is regulated by the Directive 1999/2/EC [29] that covers general and technical aspects of irradiation, the labelling of irradiated food, and the conditions for authorizing food irradiation.

However, up to date, no studies have investigated the effect of gamma radiation on the allergenicity of the edible insects authorized by the European Commission [30]; therefore, further experimental work is needed to determine optimal treatment conditions, ensuring safety without compromising the nutritional and organoleptic value of these foods. In this context, the present work was aimed at studying the effect of gamma radiation on the allergenicity of both food and feed samples of the house cricket (*Acheta domesticus*, Linnaeus, 1758, AD) and the mealworm (*Tenebrio molitor*, Linnaeus, 1758, TM). These two insect species are known for their high protein content and wide commercial distribution across various regions of the world. Furthermore, the European Food Safety Authority (EFSA) has recently completed safety assessments for their consumption. Particularly, *Acheta domesticus* is an orthopteran insect commonly classified under the family Gryllidae (Orthoptera-Insecta-Arthropoda-Invertebrates), likely originating from southwestern Asia. Adults measure between 16 and 26 mm in length. Their bodies are brownish to yellowish and pale, with three distinct dark crossbands on the head [31]. Although this species originates from Northern Africa and the Middle East, it is now distributed in eastern North America, Europe, and India, and has been introduced into Latin America. Ten allergenic proteins have been identified in cricket, namely hexamerin 1B precursor, hypothetical accessory gland protein (partial), myosin heavy chain isoform G, myosin heavy chain (MHC), putative arginine kinase, tropomyosin, and tropomyosin isoforms [32]. Among these cricket allergens, tropomyosin has high cross-reactivity with the serum of shrimp allergic patients [32]. *Tenebrio molitor* is an insect species that belongs to the family of Tenebrionidae (darkling beetles). Adults mea-

sure approximately 14 mm in length, with shiny dark brown to black bodies. Common names for these insects often reference the coloration of their immature stages. TM is a cosmopolitan species, naturally found in the temperate regions of Europe, but it is now distributed worldwide. The larvae feed primarily on stored grain products, making them a common pest. They also consume animal-based materials such as meat scraps, dead insects, and feathers. A variety of allergenic proteins have been identified in TM, including alpha-amylase, putative trypsin-like proteinase, putative serine proteinase truncated, cockroach allergen-like protein, larval cuticle protein A1A, larval cuticle protein A2B, and larval cuticle protein A3A [33, 34]. In addition, TM and AK in yellow mealworm strongly cross-react with IgE in patients with house dust mite (HDM) and crustacean allergy, which indicate their allergenicity [34, 35].

To achieve our objective, a crustacean allergen detection test (enzyme-linked immunosorbent assay – ELISA) was exploited to establish if the clinical cross-sensitization can also translate into analytical cross-reactivity.

MATERIAL AND METHODS

Insect sampling

Insect samples for both food and feed applications were provided by the food and feed supply chain operator Italian Cricket Farm (Via Vigone 20, 10060 Scalenghe, Turin, Italy). Each species was processed through dedicated production lines for either food or feed purposes.

The process for food production line (FO) includes the following stages:

1. insects were carefully selected at an age of 50 to 60 days, ensuring that females had not yet laid eggs;
2. the selected insects underwent a fasting period of 24 to 36 hours;
3. they were then humanely stunned by exposure to hypothermia in a cold room (2-8 °C);
4. after stunning, the insects were boiled for approximately 10 minutes.

Conversely, for the feed production line (FE), no restriction about age or sex was applied for insects. However, as for FO, they underwent a fasting period of 24 to 36 hours and the stunning process via hypothermia (2-8 °C).

In both lines, the final step involved drying the insects in a desiccator to achieve a relative humidity level of 5% before removal for further processing.

Sample irradiation

Whole dried insects were irradiated using a Nordion Gammacell 40 Cs-137 irradiator (Ottawa, 447 March Rd., Ottawa, ON, Canada, K2K 1X8), located at the Italian National Institute of Health (Istituto Superiore di Sanità, ISS). The irradiator operated at a dose rate of approximately 0.7 Gray per minute, with a variation of ±15%.

Irradiation treatments were conducted at room temperature, applying doses of about 1 (identifiable lower-level treatment) and 3 kGy (used in some countries for shrimp treatment). For each dose, approximately 100 grams of each sample type (AD and TM, both food and feed) was packaged in sterile polyethylene bags (Fisherbrand™ Twirl'EM™) and placed within the irradiation chamber. Following irradiation, samples were stored in a silica dryer at room temperature. To prepare samples for analysis, they were ground using a mortar and pestle, avoiding the use of a blender to minimize potential thermal effects and preserve sample integrity.

Sample preparation

Approximately 1 g aliquots of sample grinded in mortar were prepared for each group for subsequent analysis. For research purposes, the samples were labelled with a 6-character alphanumeric code according to the scheme *(Table 1)*: Species (AD/TM), Food (FO)/Feed (Fe), exposure (Not irradiated - 0 kGy, 1 kGy, 3 kGy).

A total of 69 samples were prepared: 33 for method validation and 36 for testing. Particularly, 20 AD and 13 TM samples were exploited to verify the manufacturer's validation report, while 18 AD and 18 TM samples for testing. Samples, no longer in their original packaging condition, were stored at room temperature.

Enzyme-linked immunosorbent assay (ELISA) determination

Given the lack of specific methods and certified reference materials for detecting insect presence using the ELISA technique, as well as the reported cross-reactivity of insect proteins with crustacean ones, the Ridascreen® FAST Crustacean Enzyme Immunoassay Kit (Art. R7312) supplied by R-Biopharm Italia Srl (Via Morandi, 10, 20077 Melegnano MI, Italy) was utilized to determine insect allergenic proteins. This sandwich immunoassay kit is designed for the quantitative detection of crustaceans that may be present either as ingredients or contaminants in both raw and cooked food products. It identifies crustacean proteins, mainly

Table 1
Sample code assignment

Species	Intended use	Sample labelling		
		Not irradiated	1 kGy	3 kGy
Acheta domesticus (AD)	Food (FO)	AD FO 0k	AD FO 1k	AD FO 3k
	Feed (FE)	AD FE 0k	AD FE 1k	AD FE 3k
Tenebrio molitor (TM)	Food (FO)	TM FO 0k	TM FO 1k	TM FO 3k
	Feed (FE)	TM FE 0k	TM FE 1k	TM FE 3k

tropomyosin from the troponin-tropomyosin-complex, a protein recognized as the primary allergen in this group of organisms, characterized by heat resistance, and therefore suitable for the detection of crustaceans in food samples.

The assay operates within a range of 2-160 mg of crustaceans per kg of food (ppm). The reliability of the kit was evaluated by comparing key validation parameters – such as selectivity, limits of detection (LOD), limits of quantification (LOQ), repeatability, and recovery – provided by the manufacturer with our experimental findings. The kit instructions also report minor cross-reactivity with arthropods, due to similarities in protein composition. For locusts, the manufacturer reports a cross-reactivity value of 23,600 mg protein/kg for pure samples diluted at a ratio of 1:160.

Experiments were conducted following the manufacturer's guidelines. Particularly, 1 g of a representative sample was mixed with 20 ml of preheated Allergen Extraction Buffer (GFl 1002, Gesellschaft für Labortechnik GmbH, Burgwedel, Germany) at 60 °C. The mixture was vigorously agitated and incubated for 10 minutes at the same temperature in a water bath. After incubation, the samples were cooled in an ice bath and centrifuged for 10 minutes at 2500 rpm at 4 °C using a refrigerated centrifuge (ALC 4237R, ALC Apparecchi per Laboratori Chimici, Milan, Italy). Approximately 2 ml of the extract was then subjected to high-speed centrifugation at 12000 rpm for 10 minutes in reaction caps using a microcentrifuge. One hundred microliters of the extract were added to each well for the assay.

The testing methodology utilizes microtiter strips coated with specific antibodies for crustacean proteins. When standards and samples are added to these wells, any crustacean proteins present will bind to the antibodies. During a washing step, unbound components are removed. An antibody conjugated to peroxidase is then introduced, binding to the antibody-antigen complex, forming an antibody-antigen-antibody (sandwich) complex. Any unbound conjugate is eliminated in another washing step.

Crustacean proteins are detected by adding a Substrate/Chromogen solution, in which the peroxidase conjugate catalyzes the conversion of the chromogen into a blue product. The addition of a stop solution induces a color shift from blue to yellow, and photometric measurement is conducted at 450 nm. The absorbance measured correlates directly with the crustacean protein concentration in the sample, expressed in mg/kg. The instrumental results are then converted to grams of protein per kilogram (g/kg), accounting for the sample dilutions (1:25 for AD and 1:25-1:150 for TM).

RESULTS AND DISCUSSION

Validation

The reliability of the kit was evaluated by comparing its performance with experimental results relating to key validation parameters (accurate identification of presence, limits of detection, LOD, and limits of quantification, LOQ, repeatability, and recovery) as specified by the manufacturer (verified in different zero matrices). According to the validation report provided by the manufacturer, the LOD is indicated to be 2 ppm, while the LOQ aligns with the method's application threshold of 20 ppm. In our protocol, the optical density (OD) measured for each blank sample was found to be either near or below that of the 0 ppm calibrators, and lower than the optical density associated with the manufacturer's LOQ (20 ppm). Under our experimental conditions, the calculated LOD and LOQ for the method (verified in a rice flour matrix free from shrimp), derived as mean ± 3 standard deviations (SD) and 10 SD, respectively, were determined to be 2 ppm and 19 ppm. In every instance examined, the negative control exhibited an optical density signal lower than that of the initial point on the curve (20 ppm). Each analytical session included a positive sample consisting of shrimp meat, which the selected kit accurately identified on all occasions. The manufacturer's validation report indicates a repeatability estimate of 6.6%, derived from determinations conducted on spiked samples prepared by the manufacturer. Within this research the repeatability of the measurement was assessed by analyzing the deviations observed between two determinations of the same AD sample within the same analytical session.

The repeatability data ranged from 1.4% (for food at 0 kGy) to 9.4% (for feed at 3 kGy). The average percentage repeatability value calculated from two measurements across all samples is 3.3%. These values are fully compliant with the specifications outlined in Standard EN 15633-1 [36], which stipulates that the repeatability value at the recovery level must be less than 20%. Additionally, the validation report from the manufacturer states that the reproducibility estimate is 10.3%. All reproducibility values observed for both food and feed categories across three irradiation levels (0, 1, and 3 KGy) were found to be below this threshold. An evaluation of recovery values was not conducted in this preliminary study exclusively assessed insects as a raw material while certified reference standards for AD and TM were unavailable. Based on the tests conducted, the method was deemed suitable for detecting the presence of insects by identifying crustacean proteins.

Experimental testing

Analytical measurements were conducted at different time intervals, with the day following the completion of 3 kGy irradiation serving as the starting point (T0). AD samples were analyzed at T0 (immediately after irradiation), as well as 7- and 28-days post-irradiation. TM samples were analyzed 18, 22, and 28 days after the irradiation endpoint. For each product type (AD and TM), both food and feed samples were analyzed to determine the concentration of crustacean proteins (g/kg) in untreated (0 kGy) and irradiated samples (1 kGy and 3 kGy). *Table 2* provides obtained data for AD and TM samples, respectively, across the different irradiation treatments (0, 1, and 3 kGy).

The analysis revealed varying levels of crustacean proteins in AD samples, ranging from 1.2 g/kg in irradiated feed samples (AD FE 1K) to 3.7 g/kg in not irradiated food samples (AD FO 0K). The data demonstrated a

Table 2
Crustacean content (grams per kilogram) found in non-irradiated and irradiated (1 kGy and 3 kGy) *Acheta Domesticus* (AD) and *Tenebrio Molitor* (TM) food and feed samples

	g/kg (mean ± partial dispersion)				
Sample code	**day 0**	**day 7**	**day 18**	**day 22**	**day 28**
AD FO 0K	3.7±0.02	2.9±0.07			1.3±0.02
AD FO 1K	2.9±0.06	1.9±0.12			2.1±0.09
AD FO 3K	2.8±0.12	2.5±0.02			1.3±0.03
AD FE 0K	2.1±0.01	1.5±0.04			1.5±0.02
AD FE 1K	1.5±0.01	1.2±0.04			1.9±0.04
AD F3 3K	1.9±0.12	2.8±0.35			1.2±0.11
TM FO 0K			4.7±0.17	5.4±0.15	5.6±0.17
TM FO 1K			5.4±0.25	7.4±0.05	1.2±0.03
TM FO 3K			5.7±0.01	4.5±0.05	1.0±0.01
TM FE 0K			6.3±0.17	6.6±0.07	6.5±0.12
TM FE 1K			6.4±0.13	8.1± 0.36	7.5±0.25
TM FE 3K			5.6±0.08	4.0±0.25	5.5±0.12

significant variability, particularly with respect to reproducibility. While repeatability within the same sample and analytical run was relatively low (1.4-9.4%), reproducibility across different analytical runs of the same sample was higher, ranging from 8% to 30% for both food and feed AD samples.

The data demonstrated that there were no statistically significant differences in protein concentrations between food and feed. Furthermore, no significant differences in protein content were observed between food and feed samples of both AD and TM under identical irradiation conditions (0, 1, and 3 kGy). Conversely, a significant difference in detectable crustaceans' protein levels was observed between TM and AD samples, with TM samples showing significantly higher levels (Kruskal-Wallis test, p=5.676E-05), as illustrated in *Figure 1* [37]. To the best of our knowledge, there are not many studies that have evaluated the effect of irradiation on the allergenic effect of insect proteins. In a 2023 study, Yang *et al*. concluded that high doses of radiation induce structural changes in tropomyosin (unfolding or aggregation), which result in the reduction of the IgE-binding capacity of tropomyosin.

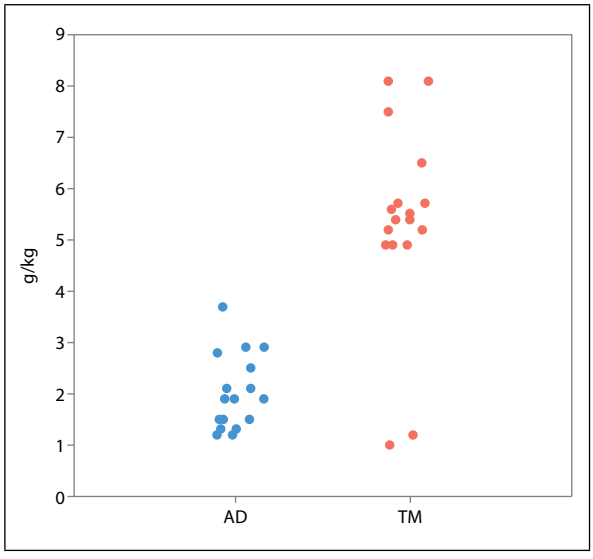

Figure 1
Density distributions (Jitter plot) of crustacean protein content (g/kg) in irradiated and non-irradiated *Acheta Domesticus* (AD) and *Tenebrio Molitor* (TM) food and feed samples.

The interpretation of this scientific evidence is complex and multifaceted. And some aspects warrant further investigation.

Firstly, the protein content and composition can vary significantly among different crustacean and insect's species. Moreover, the antibody used in the assay may exhibit varying affinities for proteins from different species. Consequently, different crustacean species may yield different results, as the calibration of the assay was performed using a limited set of representative species (Ridascreen RIDASCREEN® FAST Crustacean -2nd generation- Art. No. R7312 product information's). In addition, the inherent heterogeneity of protein content across different insect species significantly complicates the correlation between the ELISA-derived total allergenic protein levels and the precise protein composition of each specific insect matrix. Consequently, the experimental data do not elucidate whether the observed discrepancy is attributable to a genuine difference in total protein.

Numerous analogous studies have yielded inconsistent results. Rothman [38] reported protein concentrations of 42.2% ± 20.2% for adult Coleoptera, 37.3% ± 13.3% for immature Coleoptera, and 49.0% ± 23.0% for adult Orthoptera. These findings align with the study

by Rumpold [39], which reported mean protein values of 40.69% for Coleoptera and 61.32% for Orthoptera, and with van Huis [40], who reported a protein content on a dry matter basis for insects ranging from 7% to 91%. These findings were further corroborated by Churchward-Venne [41], who highlighted a protein concentration of 65.0% for AD and 58.1% for TM. Da Silva [21] reported a protein content range of 64.4%-70.7% for AD and 65.6% for TM. In two 2021 opinions [10, 11], EFSA reported a protein content for dried AD (4-5 weeks old) and TM (~11 weeks old) ranging from 59.5% to 60.8% and 55.5% to 58.8%, respectively. Yang [25] found a protein content of 46.44% for TM samples and 71.7% for AD. Subsequently, García-Vaquero [42] reported a protein content on a dry matter basis of 44.8% to 66% for TM and 62.57% to 70.7% for AD. These data are partially confirmed by Liang *et al*. [43], who highlighted a protein concentration of 40.69% for Coleoptera and 61.32% for Orthoptera.

However, a significant challenge in comparing results across these studies arises from the lack of standardized methodologies for protein quantification. Notably, a consensus on the optimal nitrogen-to-protein conversion factor for protein determination remains elusive and there is still no consensus about what the right nitrogen to protein ratio is to be used, when protein is determined by means of Dumas or Kjeldahl methods.

Furthermore, the protein and amino acid content of edible insects is quite variable, likely due to factors such as diet, developmental stage, geographic location, seasonal variations, and processing procedures [41]. Additionally, a direct comparison between literature-reported protein content values and allergenic protein data obtained from ELISA assays is complicated by the lack of comprehensive information regarding the composition of the standards and antibodies used in these assays. While ELISA kits can detect a broad spectrum of crustacean proteins, including tropomyosin and chitin, the specific quantitative contribution of each protein remains uncertain [21].

CONCLUSION

Globalization has accelerated the spread of dietary habits. In many cases, the nutritional and safety implications of consuming these novel foods have not yet been fully assessed. Insects, for example, have recently been approved as novel foods within the EU, but their consumption is already widespread globally. As a potentially more sustainable alternative to traditional protein sources, insects have garnered significant attention. However, there are still knowledge gaps in the scientific understanding of insect consumption. Existing studies often present conflicting results, highlighting the need for further research. Primarily, allergenicity is a significant food safety issue that requires further scientific investigation. The allergenic potential of edible insects is a significant concern as crustacean food allergy affects up to 4% of the population in different regions of the world. Given the high prevalence of crustacean food allergy, this poses a significant threat to individuals already allergic to crustaceans who may also react upon consuming novel insect protein-based foods. On the other hand, numerous studies have produced often contradictory results regarding the effectiveness of food technology techniques (such as irradiation) in reducing the allergenicity of a food. This pilot research delved into the food safety aspects of insect consumption and aimed to verify the possibility of using a simple analytical technique for the determination of insects in foods and to evaluate the effect of gamma irradiation on the allergenicity of *Acheta domesticus* (cricket) and *Tenebrio molitor* (mealworm) samples. At the current state of knowledge, no harmonized official analytical methods are available to determine allergenic insect proteins in food. Nor are certified reference materials and inter-laboratory proficiency schemes available. In this context, our study showed that an ELISA kit for the determination of crustaceans was able to detect certain insects (AD and TM) due to cross-reactivity and that irradiation did not significantly affect insect detection. Furthermore, no significant differences were found between samples intended for animal consumption and those intended for human consumption that underwent additional heat treatment (boiling). However, a key finding was significantly higher levels of detectable crustacean proteins in TM samples compared to AD ones, although it should be considered that the inherent complexity and heterogeneity of insect samples (both AD and TM) presented challenges in sample handling and analysis.

Research has confirmed the difficulties generally encountered in homogenizing complex matrices such as insects and dried products in general. Homogenization processes were performed manually to avoid overheating the insect aliquots, which could potentially degrade proteins. The limitation of this treatment is the possibility of obtaining finely homogenized samples. In this sense, a possible methodological evolution of this study is represented by the possibility of increasing the sample size and evaluating alternative homogenization methods. Advances in homogenization techniques for such samples can enhance the quality of analytical data, improving precision and enabling the detection of significant differences in allergenic protein levels. Further improvements in analytical performance could be achieved using more advanced analytical methods, certified reference materials, and proficiency testing. Also, future research in this area may explore the potential of using sieved insect flours. This aspect may influence the dispersion of the analytical data obtained. At last, the possibility of extending the methodology to complex foods that may contain insects as ingredients and as contaminants should be also explored. This latter assessment could represent a further step for the enhancement of scientific knowledge in this area of research and an increase in the actions carried out to protect allergic consumers.

Conflict of interest statement

The Authors declare that there are no conflicts of interest.

Received on 20 March 2025.
Accepted on 7 May 2025.

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Ann Ist Super Sanità 2025 | Vol. 61, No. 2: 157-159
DOI: 10.4415/ANN_25_02_09

Publications from International Organizations on Public Health

Edited by
Annarita Barbaro

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO)

The Third Report on The State of the World's Plant Genetic Resources for Food and Agriculture. Rome: Food and Agriculture Organization of the United Nations 2025; 374 p. ISBN 978-92-5-139675-9. The third report on the State of the World's Plant Genetic Resources for Food and Agriculture (PGRFA) presents a comprehensive assessment of the conservation and sustainable use of PGRFA, as well as the human and institutional capacities to support these efforts. Covering the period 2011–2022, it provides a comprehensive analysis of the global status and trends in PGRFA conservation and use. The report is based on information from 128 countries and four regional and 13 international research centres and the contribution from over 1 600 experts. The report provides a sound basis for recalibrating relevant policies and strategies, including the rolling Global Plan of Action for Plant Genetic Resources for Food and Agriculture.

The Second Report on the State of the World's Forest Genetic Resources. Rome: Food and Agriculture Organization of the United Nations 2025; 260 p. ISBN 978-92-5-139699-5. Drawing on data and information from 77 countries representing more than three-quarters of the world's forests, this report provides a global assessment of forest genetic resources. It highlights the crucial role of these resources in addressing food insecurity, malnutrition, poverty, climate crisis, and biodiversity loss, and examines progress in implementing the Global Plan of Action for the Conservation, Sustainable Use and Development of Forest Genetic Resources. The report reviews scientific advances and concludes with recommendations for continued action at the national, regional, and global levels to ensure that forest genetic resources are sustainably managed for the benefit of current and future generations.

Guidelines for Sustainable Aquaculture. Rome: Food and Agriculture Organization of the United Nations 2025; 68 p. ISBN 978-92-5-139497-7. The Guidelines for Sustainable Aquaculture (GSA) are designed to support Members and other stakeholders in the implementation of the 1995 Code of Conduct for Responsible Fisheries. The GSA were created in response to the rapid expansion of aquaculture, the fastest-growing food production sector in the world, driven by scientific progress, technological innovations and investment, amid a consistently increasing global demand for aquatic foods. They provide a comprehensive framework with technical advice for policymakers and stakeholders at all levels — international, regional, national and local — on how to expand and intensify aquaculture responsibly. They highlight the need to balance social, economic and ecological well-being; while increasing productivity and profitability in the sector. This rapid growth has exposed challenges to the sustainability of aquaculture and raised concerns about potential negative impacts. The GSA provide a comprehensive framework for addressing these challenges.

INTERNATIONAL SCIENCE COUNCIL (ISC)

Preparing national research ecosystems for AI: strategies and progress. Second edition. Paris: International Science Council 2025; 118 p. The report offers a comprehensive analysis of the integration of artificial intelligence in science and research across various countries and is an expansion of the previous edition published in March 2024. It addresses both the advancements made and the challenges faced in this field, making it a valuable read for science leaders, policymakers, AI professionals, and academics. This edition of the paper presents a total of 18 case studies from countries of different sizes and regions, authored by people directly engaged in these discussions in their respective countries. The countries were selected using ISC's networks and connections to identify willing contributors from diverse global regions.

UNITED NATIONS ENVIRONMENT PROGRAMME (UNEP)

The Adaptation Gap Report 2024: Come hell and high water. As fires and floods hit the poor hardest, it is time for the world to step up adaptation actions. Nairobi: United Nations Environment Programme 2024; 124 p. ISBN 978-92-807-4187-2. The UNEP's Adaptation Gap Report 2024 provides an overview of key findings related to climate change adaptation efforts. It details progress in national adaptation planning instruments worldwide, highlighting the challenges in achieving global coverage and the need for increased support, particularly in vulnerable countries. The document also examines the adaptation

finance gap, emphasizing the significant shortfall in funding required to meet developing countries' needs and the necessity of shifting towards more anticipatory and strategic investments, especially in areas harder to finance. Furthermore, it addresses the crucial role of capacity-building and technology transfer, outlining common needs identified by developing countries and the challenges in securing adequate finance and effective implementation for these essential components of adaptation.

Emissions Gap Report 2024: No more hot air … please! With a massive gap between rhetoric and reality, countries draft new climate commitments. Nairobi: United Nations Environment Programme 2024; 100 p. ISBN 978-92-807-4185-8. This report is the 15th edition in a series that brings together many of the world's top climate scientists to look at future trends in greenhouse gas emissions and provide potential solutions to the challenge of global warming. The report looks at how much nations must promise to cut off greenhouse gases, and deliver, in the next round of Nationally Determined Contributions (NDCs), due for submission in early 2025 ahead of COP30. This fifteenth Emissions Gap Report has a special focus on what is required from these NDCs to maintain the possibility of achieving the long-term temperature goal of the Paris Agreement of limiting global warming to well below 2 °C, while pursuing 1.5 °C relative to pre-industrial levels. Its core message is that ambition means nothing without action – unless global emissions in 2030 are brought below the levels implied by existing policies and current NDCs.

EUROPEAN FOOD SAFETY AUTHORITY (EFSA)

European Food Safety Authority (EFSA). **Review of the methodology used for the assessment of the short-term (acute) dietary exposure to pesticide residues in food (IESTI methodology)**. EFSA Journal 2025, 23(2), e 9233. This report from EFSA outlines a review of the methodology used for the assessment of the short-term dietary exposure to pesticide residues currently used in the EU (also known as international estimated short-term intake (IESTI) methodology) and proposes three alternative calculation methods to address some of the weak points of this current methodology.
Through comparative calculations, the report indicates that these alternatives would affect exposure estimates for different food commodities variably. Furthermore, probabilistic exposure calculations for a selection of pesticides suggest that while legal limits (MRLs) generally provide good protection, there are a few instances where the possibility of exceeding acute reference doses cannot be entirely excluded. These findings are intended to inform future discussions on refining the revised methodology's conservatism level.

European Food Safety Authority (EFSA), Salvatore S, Vericat Ferrer M. **Report for 2023 on the results from the monitoring of residues of veterinary medicinal products in live animals and animal products.** EFSA Journal 2025, 22(2), 9297E. This report summarises the monitoring data collected in 2023 on the presence of residues of veterinary medicinal products and certain substances in live animals and animal products in the EU Member States, Iceland and Norway. A total of 548,194 samples were reported to the European Commission. A total of 284,850 samples were reported in accordance with the specifications of the national risk-based control plan for production in the Member States; 13,709 were samples collected in conformity with the specifications of the national randomised surveillance plan for production in the Member States; and 5162 samples were collected in conformity with the specifications of the national risk-based control plan for third-country import. Additionally, 8741 suspect samples were reported in 2023 as follow-up of non-compliant results and 235,732 samples were collected in the framework of other programmes developed under the national legislation. Most countries fulfilled the requirements for sampling frequency laid down in Commission Implementing Regulation 2022/1646.

WORLD HEALTH ORGANIZATION (WHO)

Ethics and governance of artificial intelligence for health: Guidance on large multi-modal models. Geneva: World Health Organization 2025; 98 p. ISBN 978-92-4-008475-9 (electronic version) ISBN 978-92-4-008476-6 (print version). This guidance addresses one type of generative AI, large multi-modal models (LMMs), which can accept one or more type of data input and generate diverse outputs that are not limited to the type of data fed into the algorithm. It has been predicted that LMMs will have wide use and application in health care, scientific research, public health and drug development. LMMs are also known as "general-purpose foundation models", although it is not yet proven whether LMMs can accomplish a wide range of tasks and purposes. WHO is issuing this guidance to assist Member States in mapping the benefits and challenges associated with the use of LMMs for health and in developing policies and practices for appropriate development, provision, and use. The guidance includes recommendations for governance, within companies, by governments, and through international collaboration, aligned with the guiding principles.

WHO guideline on contact tracing. Geneva: World Health Organization 2025; 92 p. ISBN 978-92-4-010296-5 (electronic version) ISBN 978-92-4-010297-2 (print version). This guideline presents a "disease agnostic" guideline with recommendations and definitions that will be available in circumstances where disease-specific guidelines are not applicable. This

practical guideline establishes definitions for "contact", "contact person", "contact tracing" and other associated concepts. It allows for improvement of contact tracing strategies, and provides recommendations attempting to answer some, though not all, questions that arose during the Coronavirus disease 2019 (COVID-19) pandemic, and other outbreaks. The employment of this guideline begins once people have been diagnosed and the potential for transmission exists. It is not, however, intended to assist with case investigation.

WHO recommendations on maternal health: guidelines approved by the WHO Guidelines Review Committee. Second edition. Geneva: World Health Organization 2025; 198 p. ISBN 978-92-4-008059-1 (electronic version) ISBN 978-92-4-008060-7 (print version). This document provides a summary of all WHO recommendations on maternal health based on guidelines approved by the WHO Guidelines Review Committee. The summary includes promotion, prevention, and prevention of maternal complications during pregnancy, childbirth, and postnatal periods as well as management of maternal complications. Each summary includes the recommendations as well as what is not recommended. The summary is a useful resource for policymakers and program managers.

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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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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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