Study Title: Integrated environmental and clinical surveillance for the prevention of acute respiratory infections (ARIs) in closed settings and vulnerable communities (Stell-ARI)

Project name**: Stell-ARI**

CUP: **I53C22000780001**

i. Abbreviations and definitions

ACAQ = Acute Clinical Assessment Questionnaire
ARI = Acute Respiratory Infections
BCQ = Basal Clinical Questionnaire
CO_2 = Carbon Dioxide
COVID-19 = Coronavirus Disease 2019
CUP = Unique Project Code
EQ = Environmental Questionnaire
GCP = Good Clinical Practice
HAdV = Human Adenovirus
Inmates = People Who Live In prison
IVAB = Influenza A & B virus
LTCF = Long-Term Care Facility
NRRP = National Recovery and Resilience Plan
PCR = Polymerase Chain Reaction
$PM_{2.5}$ = Particulate matter (atmospheric particles - 2.5 µm)
PPE = Personal Protective Equipment
QMRA = Quantitative Microbial Risk Assessment
RFID = Radio Frequency Identification
RSV = Respiratory Syncytial Virus
RT-PCR = Real time PCR
SARS-CoV-2 = Severe Acute Respiratory Syndrome CoronaVirus 2
SVOCs = Semi-volatile organic compounds
TB = Tuberculosis
TD-GC-MS = Thermal desorption coupled with gas chromatography and mass spectrometry
THE = Tuscany Health Ecosystem
VOCs = Volatile organic compounds (volatile organic compounds)
WBE = Wastewater-Based Epidemiology
Workers = all workers in LTCF, Prison or School

1. Summary

Viral Acute Respiratory Infections (ARIs) are a broad spectrum of illnesses caused by different viruses that can infect both the upper and lower respiratory tracts. Symptoms may vary from mild to more severe clinical pictures, including fever, cough, sore throat, breathlessness, headache, and fatigue. These diseases are highly contagious and spread through various mechanisms, such as direct contacts, fomites, droplets of saliva or mucus, and aerosol emitted by infected individuals not only during sneezing and coughing, but also by speaking, singing, or simply breathing. Therefore, the monitoring of environmental contamination through the analysis of matrices (namely air and surfaces) may be useful, not only to evidence the pathogen diffusion pathways but also to assess the risk of infection in specific settings through the Quantitative Microbial Risk Assessment (QMRA).

On the other hand, the infection dynamics in general or specific populations are described by clinical surveillance that requires the implementation of specific procedures to monitor and collect data on the incidence, prevalence, severity of cases, and deaths. Several sources of information are required for this process, such as epidemiological and clinical indicators, as well as morbidity and mortality data. Therefore, surveillance systems need well-established and continuous structures and procedures, not easy to be implemented and maintained.

Wastewater Based Epidemiology (WBE) represents a complementary resource to clinical surveillance in identifying and monitoring the course of an epidemic. Infected individuals, both symptomatic and asymptomatic, can eliminate the virus through faeces, making WBE able to measure the spread of the pathogens throughout the community without possible restrictions related to the administration of clinical tests. WBE can show a pathogen circulation before the onset of cases, thus representing an early warning tool, and it can allow timely identification of new variants.

Both kinds of surveillance have advantages and disadvantages and can be affected by biases. An integrated surveillance, taking into consideration data from both clinical cases and environmental matrices, can help to better describe an epidemiological scenario. This integration assumes a particular importance in small communities of people forced to stay together for long periods of time and with personal or social vulnerability factors, as in the case of elderly people in LTCFs, inmates in prisons and students in primary schools.

Among ARIs, infections from SARS-CoV-2, influenza viruses, and Respiratory Syncytial Virus are of major interest in public health and can be tracked by both clinical and environmental surveillance. Therefore, they have been chosen for our study, together with Adenovirus infections, widely spread and often used to apply QMRA in waters and air.

Our project aims to provide insights into how a clinical surveillance can be implemented by the WBE and by the risk assessment based on environmental matrices, to help in setting up prevention and control measures in enclosed environments and vulnerable communities, such as schools, LTCFs, and prisons.

The experimental field study will last 12 months: it will be preceded by a period of method and instrument development and testing and followed by a period of data analysis and dissemination of results. The experimental study will take place in three closed communities: a Long-Term Care Facility (LTCF), a Prison and a Primary School, and it will involve residents, inmates and students. For every facility, workers will be taken into consideration for their proximity to the target populations. Those involved will be able to join the study after signing the consent form and can withdraw at any time.

The study is financed by the European Union within the framework of the National Recovery and Resilience Plan (NRRP) and, in particular, of the Tuscany Health Ecosystem (THE), aimed at strengthening the life sciences sector in the Region of Tuscany. The following departments of the University of Pisa are participating in the study: Biology; Chemistry and Industrial Chemistry; Clinical and Experimental Medicine; Surgical, Medical, Molecular and Critical Area Pathology; Translational Research and New Technologies in Medicine and Surgery.

Clinical data will be collected through pseudonymised questionnaires administered to study subjects, at the initial time of the study (baseline questionnaire) and in case of suspected ARI (acute questionnaire): they will investigate the presence of symptoms and of personal and environmental risk factors. The questionnaires, either self-compiled or by interviews, will be administered in paper form in the prison, on tablets in the LTCF and online via the Influweb platform in the primary school. Confirmatory nasopharyngeal swabs will be collected from subjects showing symptoms compatible with ARI and analysed by biomolecular tests for Human Adenovirus (HAdV), influenza virus A and B (IVAB), SARS-CoV-2, Respiratory Syncytial Virus (RSV). In addition, for students in schools, close contact data will be collected using proximity sensors (tags) that will allow contact to be tracked without tracing the identity of the participants.

Data on the environmental characteristics of the facilities will be collected by means of questionnaires administered to the managers. Wastewater from the facilities will be sampled weekly and analysed for the presence of the same viruses searched in clinical swabs, with the same methods. Air and surfaces monitoring will take place in communal areas and will include the detection of temperature, humidity, CO_2, PM2.5, VOCs, total microbial load, *Staphylococcus aureus*, and the same viruses searched in clinical and wastewater samples.

The data collected from air and surfaces will be organised in an integrated database and analysed with parametric and non-parametric statistical analyses in order to identify the most representative variables (i.e., proxy) to be monitored both at the clinical and at the environmental levels. QMRA models will be designed and applied for different viruses and settings. The WBE data will be analysed to develop and set up early warning models.

The final outcome of the project will be an integrated clinical and environmental surveillance system for close communities, including parameters and times of monitoring, early warning indexes, thresholds, and risk assessment tools.

2. Background

The global burden of acute respiratory tract infections is huge, even if often overlooked: as examples, it is estimated that influenza virus causes 39.1 million episodes of acute lower respiratory tract infection (95% uncertainty interval 30.5-48.4) and 58,200 deaths (44,000-74,200) per year and respiratory syncytial virus causes 24.8 million episodes (19.7-31.4) and 76,600 deaths (55,100-103,500) per year [1]. Moreover, SARS-CoV-2, the etiological agent of COVID-19, has caused millions of infections and deaths worldwide since its emergence in December 2019, along with social and economic disruption [2]. Also, the emergence of new human viral diseases affecting the respiratory tract continues to threaten global public health security [3].
The environmental dynamic of spreading of respiratory infections, already studied for several diseases (e.g. TB, influenza), has been deeply analysed during the COVID-19 pandemic, and can be represented by a chain of events, going from the droplet/aerosol emission, to their environmental dynamics (falling or remaining suspended), to the viability of pathogens in different matrices and conditions, to the ways and amount of exposure, to the infection/disease according to dose-response relations [4,5].

It is well known, in fact, that most respiratory viruses, e.g., SARS-CoV-2, are transported via aerosols and fomites and can, moreover, remain viable in the air and on surfaces [6, 7]. Environmental characteristics, in particular air quality and living/working conditions, are important determinants of disease, deserving of targeted surveillance activities. Transmission may be particularly high in enclosed or semi-enclosed crowded places with limited recirculation of ambient air, such as schools, LTCFs and Prisons [8]. Data on the contamination of vehicles (namely air and surfaces) and on the pathogen survival, can be used not only to evidence the diffusion pathways, but also to assess the risk of infection through the Quantitative Microbial Risk Assessment (QMRA) [9]. This is a formal process of risk assessment (based on a modified chemical risk approach) including the classical steps of hazards identification and characterization, exposure assessment and risk characterization through the formulation and application of models using fields and literature data to represent a scenario, also taking in consideration its uncertainties.

In addition to the environmental contamination, frequent contacts, especially typical of school age, are a further element in the spread of infectious diseases. Furthermore, individual risk factors and underlying chronic diseases are often associated with frail conditions, which can facilitate infections and their severity.

For ARIs, robust epidemiological and clinical surveillance systems are needed to efficiently target prevention and control measures for areas and populations at risk. However, to date, clinical surveillance has some limitations and concerns, including the availability and cost of clinical tests as well as objective difficulties in intercepting asymptomatic individuals [10]. Current public health strategies that rely solely on 'symptom onset' for the identification of infections need urgent reassessment due to an underestimation of their number owing to the presence of asymptomatic individuals, who are not promptly reported to the competent authorities. This approach allows evidence only the tip of the iceberg of viral diseases (hospitalised individuals or laboratory-diagnosed cases) to be captured. Moreover, changes in case definition and surveillance strategies can lead to data variations not connected with the real epidemiology.

WBE, firstly introduced for the polio surveillance, has shown its high potential in the occasion of COVID 19 pandemic, and it is today considered a very promising tool also for other infections as well as for the antibiotic resistance [11, 12].

In the COVID-19 pandemic scenario, WBE has been suggested as a tool to determine the extent of the viral spread in cities and acts as an early warning for SARS-CoV-2 emergence and circulation in communities [13,14]. Most people infected with SARS-CoV-2 develop mild or no symptoms of respiratory illness, but excrete viral genomes, particularly in faeces and other body fluids that reach sewages. Therefore, detecting the genome of SARS-CoV-2 in urban wastewater has been useful in capturing the full extent of the disease at a community level, although the viral genome in this matrix was not associated with infectivity [15].

WBE can show a pathogen circulation before the onset of cases, thus representing an early warning tool, and it can timely identify new variants. WBE has been extensively used, not only in cities and large areas, but also in small communities, to identify the initial introduction of a pathogen (also in absence of symptomatic infections) for the implementation of prevention and control measures [16]

Both kinds of surveillance have advantages and disadvantages and can be affected by bias. An integrated surveillance, taking in consideration data from both clinical cases and environmental matrices, can help to better describe an epidemiological scenario. This can be particularly useful in settings where people are forced to stay in enclosed environments for long periods of time, mainly if they have vulnerability factors coming from age (children or elderly), health conditions or socio-economic dis-ease.

The design and useful application of an integrated clinical and environmental surveillance requires that the target infectious diseases possess several characteristics: in particular a clear case definition with a diagnostic microbiological ascertainment and environmental pathways of circulation with the possibility of tracking them with reliable microbiological detection methods. Several ARIs have these properties: infections from SARS-CoV-2, IVAB, and RSV are of major interest in public health and have been chosen for our study. To them, HAdV have been added, because they are widely spread, usually present in sewage and easily detected, often considered as index pathogen for QMRA in waters and air [17].

The present work aims to design and validate tools for an integration of clinical surveillance and the monitoring of environmental parameters: questionnaires to evaluate symptoms and risk factors (both individual and environmental), analytical protocols and sampling strategies for environmental matrices, models for risk assessment and early warning.

Closed communities could benefit from such tools in two ways:

- Risk assessment: based on knowledge of the environmental context in which people are located and by monitoring contamination, it is possible to predict the risk of transmission of respiratory infections and the effects of preventive measures to be activated before infection is introduced in the community.
- Early warning: WBE has recently attracted great interest because of the possibility of early detection of SARS-CoV-2 circulation both in large settings (cities) and in more restricted settings such as communities (primarily, university campuses and residences for the elderly). This type of surveillance

can take place before individuals in a facility are directly involved, prompting preventive interventions (such as mask use, spacing and vaccination).

By combining risk assessment and early warning it would therefore be possible to enhance the preventive capacities of such settings, but the development and validation of such a system requires the integration of clinical-epidemiological and environmental data, which can only be achieved by conducting these surveys in parallel and in a coordinated manner.

Our study is aimed to design and apply such a system in small communities characterised by populations forced to spend long time together, even in crowded and poorly ventilated spaces. Since the onset of COVID 19, numerous studies of WBE have been carried out in LTCFs [18] and schools [19], with the aim of implementing prevention measures. although without the approach of integrated surveillance. On the other hand, data on prisons are scarce and limited to few studies [20], although these settings are at high risk for ARIs both because of the presence of particularly vulnerable groups (e.g. drug addicts and foreigners with a poor health culture), and because of environmental characteristics such as overcrowding and structural deficiencies (e.g. ventilation systems) that may favour the spread of infectious diseases.

In fact, an integrated environmental and clinical surveillance system for ARI infections is at present not in place or not formally structured in communities. This study protocol will be firstly tested in LTCFs, prisons, and primary schools, but it could then be replicated to other settings.

Our project is funded in the context of the National Recovery and Resilience Plan (NRRP) 'Italy Tomorrow', that is a vast programme of reforms, including public administration, justice, simplification of legislation, competition, taxation, accompanied by adequate investments.

The NRRP is part of the Next Generation EU (NGEU) programme [21,22]. The Plan is built around three strategic axes shared at European level: digitalisation and innovation, ecological transition, and social inclusion. It is an intervention intended to remedy the economic and social damage caused by the SARS-CoV-2 pandemic crisis, as well as to help resolve certain structural weaknesses of the Italian economy and accompany the country on a path of ecological and environmental transition.

NRRP is structured into 6 missions corresponding to the areas of intervention in Next Generation EU, with 16 components, each comprising missions, intervention lines, and investments. Ecosystems of Innovation (EI) (investment line 1.5) focuses on establishing 'territorial leaders of R&D' nationwide, networks of universities, research bodies, and qualified public/private entities promoting specialized technological activities (Duration 2022-2026). THE - Tuscany Health Ecosystem [23], led by the University of Florence, aligns with NRRP's 'Life Sciences' aim, fostering Tuscany's life sciences sector growth through collaborative efforts among public/private entities, addressing innovation needs. The THE includes 10 Spokes: our study is part of THE's Spoke 2 (Preventive and Predictive Medicine) and involves University of Pisa's Departments of Biology (lead), Chemistry, Clinical and Experimental Medicine, Pathology, Translational Research in Medicine.

3. Study objectives

General objective: to create an integrated clinical and environmental surveillance system of ARI and its determinants in community settings in order to plan prevention strategies and interventions to minimise the spread of infections.

Specific objectives:

Obj. 1. Establishment of sentinel clinical surveillance and risk factors for ARI in selected closed environments for the detection of a) individual and social behaviours, b) individual risk factors, including comorbidities and contact tracing, c) early signs and symptoms, according to diagnostic criteria for specific respiratory diseases and their evolution and d) etiological agents through confirmatory sampling and analysis.

Obj. 2. Establishment of sentinel environmental surveillance in closed environments selected for a) environmental parameters (e.g., space, crowding, microclimate conditions and ventilation), b) indoor air quality monitoring (air pollutants such as volatile organic compounds, microbiological indicators, index pathogens) and c) microbiological monitoring of wastewater.

Obj. 3. *Integration of clinical, behavioural, and environmental data to develop early warning and risk prediction models* that can be replicate and transferred to other similar settings at the regional/national/international levels.

4. Study design

This is an open, multi-setting, epidemiological cohort and environmental monitoring study that plans to include subjects among LTCF residents, inmates in the prisons, students and workers.

Duration and phases:

The project has a duration of three years: it started on 1 December 2022, will end on 30 November 2025 and will be divided into successive phases, with very strict timelines for the purpose of funding disbursement:

Phase 1: Duration 1 year (1 December 2022 - 30 November 2023). Enrolment of facilities and development of survey instruments (questionnaires and analytical methods for clinical and environmental analyses). Authorisation by the ethics committee. Information on the project at facilities to workers and health education programmes;

Phase 2: Duration 1 year (1 December 2023 - 30 November 2024). Field study for data collection;

Phase 3: Duration 1 year (1 December 2024 - 30 November 2025). Data processing and model building. Production of information material and dissemination.

The study workflow is summarised in Figure 1.

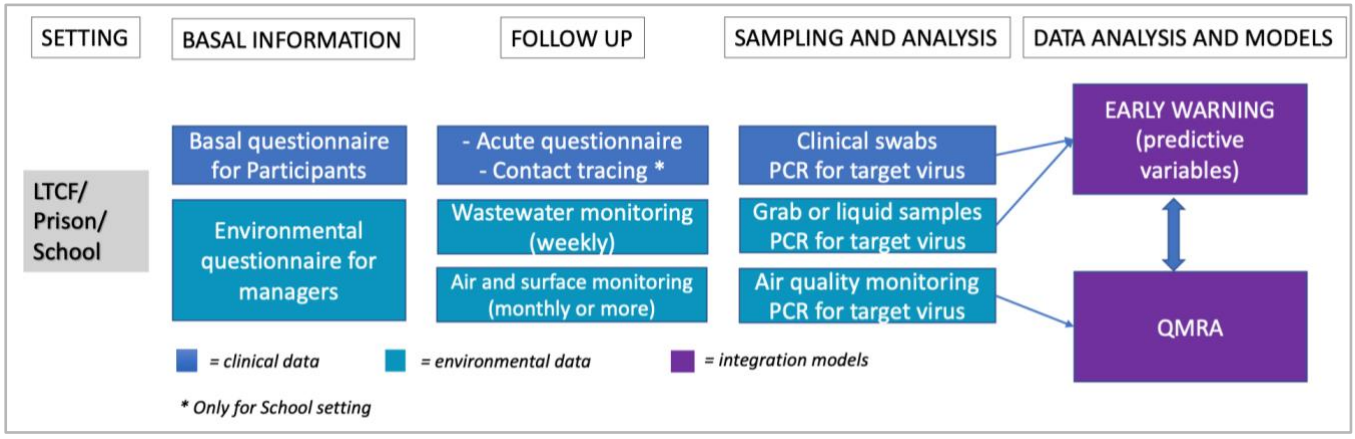

Figure 1. Scheme of the study workflow: clinical data in blue squares, environmental data in green squares, integration models in purple.

The following measurements will be carried out for each recruited population and site:

Clinical surveillance: semi-structured pseudonymised questionnaires administered in total respect for privacy to samples of participating subjects and facility staff, for the detection of individual symptoms and risk factors. In the case of suspected ARI, nasopharyngeal swabs will be taken for the identification of potentially responsible viruses (among the chosen viral targets) by means of molecular PCR tests. In addition, only for students, data will be collected during school hours regarding close contact using proximity sensors (tags) based on RFID (Radio Frequency Identification) technology (devices not mass-produced by the company Bitmanufaktur, Cambridge UK). RFID tags will enable the accurate, automated and pseudonymised detection of close encounters between individuals within the school environment. This technology offers a promising approach for monitoring and managing social interactions, especially in settings such as schools, where controlling the spread of disease is a priority.

Environmental surveillance: semi-structured questionnaires will be administered to facility managers to detect the characteristics of the spaces and their attendance. In addition, periodic monitoring of microclimate parameters and the chemical-microbiological quality of the air will be carried out. Existing or ad hoc developed electrochemical sensors will be used for this purpose. The target viruses will be detected both in air and on surfaces of common areas, with biomolecular tests. At the same time, virological monitoring of the facilities' wastewater will be performed by sample concentration and analysis with biomolecular methods.

Finally, data processing and model development will take place. The data collected from a clinical perspective will be integrated with behavioural and environmental data in order to develop and validate models for ARI alerts and risk assessment in closed communities.

5. Study setting

5.1 LTCF

The LTCF 'Le Sorgenti' is a socio-medical institution located in San Giuliano Terme, Via Di Giacomo 1, which provides care for elderly people over 65 years of age who are unable to take care of themselves

physically and psychologically as well as being unable to live independently at home. However, in special cases, it can also accommodate people under the age of 65 who present a condition of senile decline and cannot find another care facility. The LTCF offers services of medium complexity and care intensity, both temporary and permanent, and has 71 beds divided into four units: three with 20 beds each and one with 11 beds. The facility is part of the 'Centro Polifunzionale di Cura e Assistenza' (Multifunctional Care and Assistance Centre), a complex that includes various social-health units, both residential and outpatient, and is authorised by the Municipality of San Giuliano Terme to receive residents with hospitality fees paid in full by the guest. The LTCF is in a modern, 50,000 square metre building with a total surface area of approximately 7,400 square metres, on three levels (basement, ground floor and first floor), consisting of two rectangular bodies and a connecting element. The ground floor houses the administrative offices, reception, a gymnasium, medical and nursing surgeries, a room for the podiatrist and hairdresser/beauty therapist, a multipurpose room and a bar and refreshment room. The first floor comprises three nuclei: two with 20 beds and one with 11 beds, all with exclusive services. The basement is intended for support services for the residence, such as storage, technical rooms and changing rooms. The rooms, 31 in total, are equipped with private bathrooms and are divided into 32 double rooms and 7 single rooms. All rooms have centralised oxygen systems, bathrooms equipped for the disabled, ergonomic beds and provision for TV installation. The Residence has an outdoor car park and a garden set up for the use of residents and their families. In addition, there are common areas inside such as the gym and dining rooms.

5.2 Prison

The prison of Lucca, located on the eastern outskirts of the Tuscan city, was opened in the early 19th century. The prison, although very old, is equipped with new spaces in good condition. At the moment, only two of the four ordinary prison sections are in operation and are divided into two levels. In addition, there is a section for disabled inmates with special sanitary equipment. The sleeping rooms are mostly double and without an internal shower, except for one section which has triple rooms with a shower. The cells are equipped with working heating and a separate toilet, and all windows are fitted with screens.

There are no common areas in the detention sections. However, a new section, which is entirely dedicated to the treatment of inmates, was recently inaugurated. It is spread over two floors and is equipped with numerous rooms, including interview rooms, two gyms, two libraries, a large multi-purpose space, a room dedicated to digitalisation, spaces dedicated to schooling, table football and other empty rooms used according to the needs of the moment. In addition, there are two libraries within the treatment section, accessible to inmates as a reading room without time restrictions. There are spaces specifically dedicated to education and training, but no areas for work or worship for non-Catholic inmates.

5.3 School

The Comprehensive Institute ‘V. Galilei’ comprises five pre-schools, four primary schools and one secondary school, together with kindergartens and public schools. Of the four primary schools of interest, the one with the greatest number of students was chosen for the study.

The primary school 'Livia Gereschi' is a state school located at Via Viale 16, Pisa and accommodates 154 pupils divided into eight classes. The school building has two floors, and on the first floor there is a space

dedicated to physical activity and the development of motor skills. In addition, there is a multimedia room for interactive activities. At the back of the school is a multi-purpose field that provides the pupils with an outdoor space for motor activities and leisure time.

6. Study populations

6.1. LTCF

A total of 71 residents over the age of 65 and all carers will be included in the LTCF in Pisa, according to the admission criteria established for the LTCF and with different health conditions, including those with chronic diseases, degenerative diseases, physical disabilities, and other known or diagnosed medical conditions. Subjects will have different levels of functional autonomy, including those who require assistance with daily activities such as mobility, personal hygiene, mealtimes, and other routine activities. Individuals included may have different socio-economic statuses, including those with different financial circumstances or levels of education.

6.2 Prison

The prison of Lucca currently houses 76 inmates, with the presence of 68 prison police officers, 9 administrative staff and 1 educator.

The subjects under study are all inmates and workers of the prison, aged over 18, according to the national law in force for the age of majority. Subjects can have different lengths of detention, various clinical conditions (e.g. chronic diseases, infectious diseases) and with known or reported risk behaviour (e.g. drug use, violent behaviour, violations of prison regulations). Moreover, they can have different educational and socio-economic levels and different nationality and language.

6.3 School

Subjects included are students at primary school, aged between 5 and 11 and the school workers. All subjects will be included regardless of health status, language spoken or ethnic origin.

7. Inclusion criteria

7.1 LTCF

- Gender of subjects: All individuals, of all gender identities, will be allowed to participate in this study without any restrictions.

- Age of subjects: All residents and workers of the LTCF over the age of 18.

- Origin: All residents and workers of all origins will be authorised to participate in this study without any restrictions.

- Acknowledgement: those who have signed the informed consent.

7.2 Prison

- Gender of subjects: All individuals, of all gender identities, will be allowed to participate in this study without any restriction.

- Age of subjects: All inmates of the prison and all workers over the age of 18.

- Origin: All Inmates in the prison and all workers of any origin will be allowed to participate in this study without any restriction.

- Acknowledgement: those who have signed the informed consent.

7.3 School

- Gender of subjects: All students and all primary school staff of all gender identities will be allowed to participate in this study without any restrictions.

- Age of subjects: All primary school students between the ages of 5 and 11 years, and all workers over the age of 18 years.

- Origin: All students and workers of any origin will be allowed to participate in this study without any restrictions.

- Acknowledgement: For all students whose parents/guardians have signed the informed consent and for all workers who have signed the informed consent.

8. Exclusion criteria

8.1 LTCF

- Subjects under 18 years of age;

- Subjects who do not provide consent;

- Subjects incapable of understanding instructions or answering questions.

8.2 Prisons

- Subjects under the age of 18;

- Subjects who do not provide consent;

- Subjects incapable of understanding instructions or answering questions.

8.3 School

- Non-primary school students between 5 and 11 years of age, and all staff under the age of 18;

- Subjects/families who do not provide consent;

- Subjects unable to understand instructions or answer questions.

9. Study exit criteria

- Withdrawal of consent

- Death or transfer to another facility

10. Outcomes of the Study

The socio-economic impact of ARI is greatest in fragile and disadvantaged populations [24]. Our project aims to bring improvement in terms of public health: reducing the incidence of ARIs, their economic consequences on the NHS, reducing inequalities such as those due to age or marginalisation.

The expected outcomes corresponding to each specific objective are detailed below. They will be related to the Tuscany Region, but they can be generalized at national/international levels in similar settings:

1. Sentinel clinical surveillance system for acute respiratory diseases, with data collection tools (questionnaires) for clinical surveillance and risk factors tested and validated in different indoor settings: LTCFs, prisons and schools
2. Indoor air quality assessment instruments and tools validated in the above-mentioned settings; Air and surfaces microbial contamination methods and strategy, validated in the same settings; WBE methods and strategy validated for the same selected communities;
3. Integrated environmental and clinical surveillance system established and tested for closed settings, through:
    a. Assessment of the association of respiratory disease incidence with environmental and individual risk factors (A predictive model using a nomogram will be developed, validated and visualised);
    b. A QMRA model validate for different viruses and settings;
    c. Recommendations for expansion of the acute respiratory disease sentinel integrated surveillance system for early warning and pandemic preparedness.

11. Variables

In brief, data will be collected through both questionnaires and analytical procedures.

Through questionnaires, the following information will be obtained:

- Environmental characteristics of the settings (including surfaces, volumes, ventilation, and common areas), daily activities performed, and hygiene practices followed within the facilities
- Individual participant characteristics: general attributes, existing medical conditions such as respiratory infections and allergies, vaccination status, exposure to risk factors, socio-economic factors, occurrence of acute illnesses during the study period, contact with other individuals and consent for potential nasopharyngeal swab testing.

Analytical procedures will involve:

- For symptomatic individuals: detection of etiological agents via confirmatory swabs
- Air analysis: assessment of microclimatic conditions, chemical indicators, microbiological indicators, and key pathogens
- Surface Analysis: assessment of microbiological indicators and key pathogens
- Wastewater analysis: Quantification of genomic copies of target viruses.

12. Data collection: methods and tools

12.1 Clinical sampling

Biological material (surface cells, mucus and secretions from the nasopharynx and tonsils) from the respiratory tract of the study subjects will be collected by nasopharyngeal swab. that are minimally invasive, easy to perform, and painless diagnostic test used to detect pathogenic microorganisms responsible for infections of the upper respiratory tract. Sampling is carried out using a thin, sterile, single-use wadded stick. This procedure, subject to the consent of the subject in the study or his or her guardian, will be carried out by the facility workers (nursing staff in the LTCF and prison settings) and by parents and the workers themselves in the school setting. For the purposes of correct execution and non-contamination of the sample, after washing hands and wearing PPE, the individual taking the sample will explain the procedure to the subject, inviting him/her to tilt his/her head back and keep his/her torso erect. The swab will then be gently inserted through one nostril and continued along the floor of the nasal coana until it reaches the back of the nasopharynx. Once in place, the swab will be gently rotated and then held in situ for a few seconds to collect abundant nasal secretion. Taking care not to contaminate it, with the same swab, the manoeuvre will be repeated for the contralateral nostril. Once extracted, the applicator will be placed in a sterile tube containing the transport medium. After washing his hands, the sample collector will place the pseudonymised code on the tube and send it to the laboratory by a dedicated transport service.

12.2 Environmental microbiological sampling

For air and surfaces, the parameters monitored will be indicators of general/human microbial contamination (total bacterial load at 22°C and 37°C, *Staphylococcus aureus*) and the selected respiratory viruses (adenovirus, influenza virus, SARS-CoV-2, respiratory syncytial virus (RSV).

To choose the most contaminated sampling points, an extensive distribution of "exposed plates" containing a non-selective medium will be made: after 6 hour exposition they will be incubated and colony forming units will be counted.

Air samples will be collected by automatic samplers positioned in the common areas. Indicatively, 180 L of air will be sampled for bacteria, 1,000 L for viruses. The sampling method will be chosen with preliminary tests. Sampling of the most exposed surfaces will be carried out in the same areas, by means of specific swabs. Air and surface sampling will be carried out monthly. In case of disease occurrence, weekly sampling may be arranged. Once collected, samples will be transported and refrigerated to the laboratory of Environmental Hygiene and Virology of the Department of Biology of the University of Pisa for analysis.

For wastewater, the target viruses will be considered. Sampling will be performed on a weekly basis at the exit of each facility by appropriately trained personnel via a sewer drain outside the building. All necessary safety measures (e.g. use of Personal Protective Equipment - PPE) must be applied. Two types of sampling can be performed: definite volume of wastewater, allowing to determine the viral concentration at a definite time (instant samples) and passive samples, allowing to detect the presence of viruses and their amount considering longer periods. In the first case raw sewage samples will be collected, in bottles with safety caps, appropriately labelled indicating the date and place of collection. They will then be transported to the laboratory, maintaining a temperature of 4°C, and pre-treated in a thermal bath for 30 minutes at 56°C for viral particle inactivation. This will be followed by a concentration step by precipitation using PEG/NaCl, and a nucleic acid extraction step using a commercial kit (BioMérieux Nuclisens System) [25]. Cotton buds or gauze passive samples will be immersed into the flow of wastewater and left there for hours [26]. These swabs will be eluted, and the eluate concentrated as described for liquid samples.

12.3 Microbiological analyses

The air and surface samples will be analysed for bacteria using cultural methods and for the target virus with PCR. The same PCR protocols will be used for both clinical and environmental samples: they are summarised in table 1.

Table 1. PCR protocols used for the detection of viral targets.

Virus	Source	Reference
SARS-CoV-2	SARI (Surveillance of SARS-CoV-2 in wastewaters in Italy)	Verani et al., 2022 [27]
HAdV	Virobathe (Methods for the concentration and detection of adenovirus and norovirus in european bathing waters with reference to the revision of the	Hernroth et al., 2002 [28]

	bathing water directive 76/160/EEC)	
IVAB	TaqMan® Microbial Assays, Thermo Fisher Scientific (assay ID Vi99990011_po; assay ID Vi99990012_po)	Ahmed et al., 2023 [29]
RSV	TaqMan® Microbial Assays, Thermo Fisher Scientific (assay ID Vi99990014_po; assay ID Vi99990015_po)	

For wastewater samples the obtained value of genomic copies will be adjusted to estimate the daily viral emission from the community.

12.4 Environmental chemical sampling

To choose the most contaminated sampling points of the three structures under examination (LTCF, prison, and primary school), a preliminary chemical air characterization will be performed using an analytical protocol based on diffusive sampler coupled with gas chromatography and mass spectrometry (TD-GC-MS). The analytical protocol is able to monitor a wide range (C3-C30) of volatile organic compounds (VOCs) and semi-volatile organic compounds (SVOCs) [30]. Briefly, passive sampler will be placed at the different sampling points for 6 hours allowing VOCs and SVOCs to be retained by the sorbent material of the sampler. After sampling, the diffusive body of the sampler will be stored in empty sorbent tubes and then hermetically sealed with Swagelock caps avoiding any external contamination and stored at 4 °C at the Department of Chemistry and Industrial Chemistry of the University of Pisa for the necessary time. Once selected the most representative environmental area, routine chemical analysis will be carried out monthly by using an analytical protocol based on the quantitative transfer of an aliquot (0.5 L) of air samples with a suction pump into stainless steel absorbent systems packed with a suitable stationary phase. Prior to sampling, each absorbent tube will be systematically spiked with a known amount (30 ng) of labelled internal standards (ISs) ensuring accurate quantification of the analytes of interest as well as quality control of the analytical data. In order to reduce any drift in instrumental response over time, environmental samples will be analysed in batches of 20-30 tubes. Between the sampling and the instrumental analysis, the absorbent tubes containing the samples from the three structures will be hermetically sealed with Swagelock caps, so as to avoid any external contamination, and stored at 4 °C at the Department of Chemistry and Industrial Chemistry of the University of Pisa for the necessary time. The result of the chemical analysis will contain information on

the qualitative chemical composition of the air samples as well as the concentration levels of each contaminant found.

During the phase 2 of the study, an Arduino based platform will be also installed at the different sites to continue monitoring temperature, humidity, CO_2, PM2.5, and total VOCs.

12.5 Questionnaires

12.5.1 Environmental questionnaire

The purpose of the questionnaire is to understand the hygiene policies and practices currently in use in the facilities and to identify any aspects that may influence the risk of respiratory virus infections.

The environmental questionnaire (EQ) is a survey targeted exclusively to the managers of the various facilities under review: LTCF, prison and school, and it is based on the assessment of environmental structure and conditions, individual and group practices and behaviour within the facility in order to prevent the spread of respiratory viruses.

The questionnaire will cover several aspects, including personal and environmental hygiene practices, cleaning and disinfection of common areas, ventilation, air quality and daily activities. It will consist of a series of multiple-choice and open-ended questions to cover different environmental factors in order to obtain specific information on residents' behaviour, their movements and contacts within the facility. In addition, the questionnaire will assess the effectiveness of existing policies on preventing the spread of respiratory viruses, as well as the facility's ability to adopt any policy changes in a timely and effective manner. The questionnaires will be administered once at the beginning of the survey to the facility managers involved and will be administered again later if there are substantial changes in the facility. The questionnaire will take approximately 8 minutes to complete.

12.5.2 Baseline Clinical Questionnaire

The objective of the baseline clinical assessment questionnaire (BCQ) is to provide a complete and accurate picture of the participant's clinical situation, identifying risk factors and individuals at risk of developing complications from respiratory infections.

The QCB is intended for all participants and consists of a series of multiple-choice and open-ended questions covering different aspects of the participant's medical history, such as the presence of previous illnesses, risk factors for respiratory infections, previous vaccinations, respiratory and allergic symptoms, socio-economic factors, and exposure to risk factors such as smoking and alcohol consumption. The information collected by the QCB will allow a baseline assessment of the subject's condition and evaluate a possible correlation with respiratory viruses infections. The questionnaires will only be administered once at the beginning of the survey, or when a new participant is added (e.g. transfer to the facility). The questionnaire will take approximately 12 minutes to complete.

12.5.3 Acute Clinical Assessment Questionnaire

The objective of the Acute Clinical Assessment Questionnaire (ACAQ) is to collect information that will allow us to monitor the evolution of the subject's condition over time, to identify possible clusters of respiratory infections within the community of the enclosed environment, in order to take the necessary prevention and control measures to prevent the spread of infection.

The ACAQ is an essential tool for the early identification of any symptoms associated with ARIs and for monitoring the evolution of the subject's condition over time. The ACAQ is designed to identify all participants presenting with respiratory symptoms, such as cough, sore throat, difficulty breathing or fever, and is intended to provide a detailed and timely clinical assessment of participants. The questionnaire consists of a series of specific questions regarding the subject's current symptoms, the duration of symptoms, any current therapies and contact with other people who have experienced similar symptoms. The completion of each questionnaire will take approximately 6 minutes.

12.5.4 Methods and time of questionnaire administration

The Stell-ARI project, in order to identify individual symptoms and risk factors, will use two main methods to collect information and data from participants: Self-administered questionnaires and interviews. Questionnaires will be on paper or on tablet. In the case of the primary school, they will be on a web portal.

- Self-administered questionnaires, where participants will read the questions and answer them themselves. The questionnaires will be in paper, on tablet or via a web portal, simply formulated, easy to understand and fill out, and accompanied by short, clear instructions. They will be distributed with a brief explanation of the objectives of the survey, motivating the participants and asking for their cooperation. The completed questionnaires will be collected in a special urn to guarantee the anonymity of the participant and transported to the Biology Department of the University of Pisa, Via San Zeno 35/39, Pisa. In the case of administration via tablet or the "Influweb" portal (https://influweb.org/welcome), a letter of introduction and detailed instructions for completion on the platform will be provided.
- Questionnaires will be administered by an interviewer who will ask the questions and record the answers during an interview with the participants. Training will be provided for the interviewers to ensure the quality of the answers. The questionnaires may be administered either during a face-to-face interview or by means of a telephone interview with the facility manager(s).

The types of administration (self or by interviews), the support (paper, tablet or web portal) and the time the different settings are summarised in tables 2, 3, 4.

Table 2. Questionnaire administration in LTCF

Type of questionnaires	Referred to	Method of administration	Frequency
Environmental Assessment Questionnaires	Head of facility manager	Self-administered paper	1 time

Baseline clinical evaluation questionnaires	Residents of the facility	Interviewed by workers via tablet	1 time
	Facility workers	Self-administered by tablet	1 time
Acute Clinical Assessment Questionnaires	Residents of the facility	Interviewed by workers via tablet	In case of Symptoms
	Facility workers	Self-administered by tablet	In case of Symptoms

Table 3. Questionnaire administration in prison

Type of questionnaires	Referred to	Method of administration	Frequency
Environmental Assessment Questionnaires	Head of facility manager	Self-administered in paper form	1 time
Baseline clinical evaluation questionnaires	Inmates in the facility	Interviewed by workers in paper form	1 time
	Facility workers	Self-administered in paper form	1 time
Acute Clinical Assessment Questionnaires	Inmates in the facility	Interviewed by workers in paper form	In case of symptoms
	Facility workers	Self-administered in paper form	In case of symptoms

Table 4. Questionnaire administration in school

Type of questionnaires	Referred to	Method of administration	Frequency
Environmental Assessment Questionnaires	Head of facility manager	Self-administered in paper form	1 time
Baseline clinical evaluation questionnaires	Parents of students	Self-administered via the 'Influweb' platform	1 time
	Facility workers	Self-administered via the 'Influweb' platform	1 time
Acute Clinical Assessment Questionnaires	Parents of students	Self-administered via the 'Influweb' platform	In case of Symptoms
	Facility workers	Self-administered via the 'Influweb' platform	In case of Symptoms

12.6 Proximity sensors (only for students)

Proximity sensors collect information that will be stored internally in the devices (tags) and extracted at the end of data collection, via a physical connection between the sensors and the operators' computers. The data collected by each sensor represent the time-resolved proximity relationships between that sensor and the other sensors in the system, along with information on the sensor's state of motion obtained through an on-board accelerometer in the device.

In addition to representing a minimally invasive method of data collection, the tags require only minimal participation from those involved, who will be asked to wear a pin on their apron once they enter the classroom. Proximity sensors will enable accurate, automated and pseudonymised detection of close encounters between students within the school environment. This technology offers a promising approach for monitoring and managing social interactions, especially in contexts such as schools, where controlling the spread of disease and maintaining safety are priorities, allowing for both frequency and duration of contact.

All students who have given their consent will be required to wear the proximity devices every day in class for a period of 10 to 15 days.

13. Sample size

The sample size is a parameter that determines the number of individuals to be included in the study in order to capture differences based on the prevalence of the diseases under study, taking into account a certain margin of error. As this project aims to study a large set of respiratory diseases, it is not feasible to calculate the sample size based on a specific prevalence. The maximum sample size is achieved at a disease prevalence of 50%; therefore, following a cautious approach, we calculated the sample size at this maximum point. Assuming a disease prevalence of 50% and a 5% margin of error, the required sample size to achieve the objective is 400 subjects. To gather data from this number of subjects, all eligible residents, inmates, students and facility workers who meet the defined criteria will be invited to participate in the study.

14. Follow-up procedure

After the baseline survey, follow-up will be performed exclusively for the clinical assessments of the study subjects by means of ACAQ. As the administration timing cannot be predetermined, new cases will be monitored and investigated weekly in the LTCF and prison, and as they occur in the primary school. The ACAQ will accompany a clinical swab for new cases during the administration.

Since the study population might change due to new entrants or exits during follow-up, data collection will take into consideration these variations by calculating person/months of observation.

15. Data management and storage

All collected data will be pseudonymised, and no sensitive information will be requested from recruited participants.

Pseudonymization will be performed by the LTCF, and prison workers based on investigator-provided code lists. For the primary school, this process will be automatically performed by Influweb platform, which will provide codes to parents.

The collected data will be stored in a secure computerised repository for analysis, with paper forms digitally converted. Data collected via the 'Influweb' platform and 'tag' devices will be managed directly by platform workers, who will extract the data by means of a pseudonymised codes.

Electronic databases will be established and maintained to ensure data security, integrity and reliability, protecting against loss, alteration or corruption. Paper questionnaires will be archived for a minimum of 10 years at the Department of Biology of the University of Pisa in via San Zeno 35/39, Pisa, under the responsibility of the principal investigator.

The data will be processed in accordance with the provisions of the European Regulation for the processing of personal data no. 2016/679 and Legislative Decree of 30 June 2003 no. 196 ss.ii. as amended by Legislative Decree of 10 August 2018, no. 101 "Provisions for the adaptation of national legislation to the provisions of the GDPR" as well as the Deliberation of the Privacy Guarantor no. 52 of 24 July 2008 and subsequent updates.

The data, processed by electronic means, will be disseminated only in aggregate and anonymized form, such as through scientific publications, reports and academic conferences.

16. Statistical analysis

Subject characteristics will be described by means of medians and interquartile ranges for numerical variables and absolute frequencies and percentages for categorical variables. The time course of the environmental variables will be represented by means of graphical displays.

Associations between infections detected via ACAQ and confirmed by clinical swabs (where necessary) and exposure factors detected by the QCB and the environmental air and surface monitoring will be assessed employing logistic regression models taking potential confounders into account.

Statistical methods and analysis algorithms will be used to examine the collected data and identify contact patterns, interaction frequencies and other relevant metrics. These will include patterns of close person-to person encounters, interaction dynamics between different groups, and pathways of environmental exposure, using diverse indicators. The high spatial and temporal resolution infrastructure will enable monitoring the number of individual contacts, encounter durations, cumulative interaction times, and encounter frequencies between any two individuals. For each pair, we will collect the following data: occurrence and frequency of each contact, time spent in each encounter, and

cumulative contact duration per subject. In relation to the collected data, the following metrics will be obtained: number of distinct contacts, total number of contacts and cumulative contact time.

WBE data will undergo normalisation and adjustment to reduce variations arising from excretion and sewage flow. Environmental data will be treated as continuous variables, with regression models featuring splines used to estimate the time course of the measured parameters. The relationship between ARI incidence and environmental data will be explored via time series regression models. In the event of missing data, imputation models will be employed, and sensitivity analysis will evaluate the impact of outliers on results. All analyses will encompass the entire dataset or stratified by setting. All statistical tests will be two-tailed with a significance level set at $\alpha = 0.05$. Data analysis will be carried out using the R statistical software[31].

17. QMRA

Quantitative Microbial Risk Assessment (QMRA) models will be designed and applied to the four selected target viruses and for the different settings with the aim of assessing the risk of ARI based on environmental contamination measured by the microbiological air and surface monitoring. The contamination values, with the estimated frequency and duration of the exposure by inhalation and contact obtained by questionnaires, will be used to calculate the dose, i.e., the amount of pathogen assumed in a unit of time. Then, probabilities of infection and disease will be estimated by applying virus-specific dose-response relationships, as reported in existing literature. These probabilities will be expressed as functions taking into account variability and uncertainties, using the Monte Carlo Analysis, applied through Vensim software, also performing a sensitivity analysis to identify the most influential variables [32].

18. Funding of the study

In accordance with the PNRR, the period of eligibility of the expenditure of the Research and Innovation Programme “THE - Tuscany Health Ecosystem”, lasting 36 months, starts on 01/12/2022 and can be extended no later than 28/02/2026. Investment Line 1.5 (1.300 M€) provides for the creation and/or strengthening of 12 Innovation Ecosystems (THE) “territorial R&D leaders'' on the national territory with the proposing entity University of Florence - UNIFI, HUB: “Tuscany Health Ecosystem Società Consortile a Responsabilità Limitata”, the SPOKE with UNIPI involvement (budget: 17,662,130.40 €), affiliated Spoke 2 (Preventive and Predictive Medicine), scientific heads Prof. Bellina and Prof. Carducci, 1,308,657.86 €.

19. Ethical considerations and privacy

The project’s planned interventions and the administration of questionnaires at various points during the course of the study will impact the participants’ health status.

The project is committed to maintain respect for individuals and human dignity, ensuring fair distribution of the benefits and burdens, while protecting the rights, values, and interests of study participants. The project will also consider the social, cultural and historical experiences of the involved participants. The

research process will not perpetrate or endorse, directly or indirectly, any form of discrimination, stigma, or exclusion, in line with “Avoiding Harm Principle”. Risks will be assessed with respect to their physical, social, and psychological implications for participants.

In addition, the investigators ensure that the trial will be conducted in full compliance with the European legislation on clinical trials [Regulation (EU) No 536/2014] and its national transposition [DM 15 July 1997; D.Lvo 211/2003; D.L.vo 200/2007 and D.L.vo. 52/2019] and the principles of the Declaration of Helsinki in order to ensure maximum protection of the subjects involved. The principal investigator agrees that the study will be conducted in accordance with this protocol and Good Clinical Practice (GCP). The principal investigator will provide the relevant Ethics Committee and Competent Authorities with the study protocol and any related documents provided to the participant (Information Notice and Informed Consent Form). Approval of the Ethics Committee and the Competent Authority should be obtained prior to the start of any trial-related procedures and should be documented by official communication to the investigator. Should protocol modifications become necessary during the course of the study, the sponsor will submit an appropriate protocol amendment request to the Ethics Committee, and the approval process will align with the Committee's established procedures.

Participants will be invited to take part in the project following the procedure outlined in the ‘Method of administration of questionnaires’ section. Actual participation will start after individuals have signed the informed consent form, indicating their willingness to join.

It will be the responsibility of the investigators, or their representatives, to obtain informed consent from surveyed subjects (or their parents, for students) after they have been adequately informed about the aims, methods, expected benefits and foreseeable risks of the study. The investigators or designees will also inform the participants that non-participation or discontinuation will not result in any harm or negative consequences.

Participation entirely voluntary, and the signed informed consent forms will be kept by the respective facilities (LTCF, Prison and Primary school) for the duration of the study.

20. Communication and dissemination

Various dissemination strategies will be employed, depending on the specific settings and participant groups (workers, LTCF residents, inmates in the prison and primary school students), will be implemented to invite them to participate. Engagement methods will include presentations and informational events held within the respective facilities, providing preliminary knowledge about respiratory infections and their environmental diffusion.

To illustrate the project in the enrolled facilities, informative material will be produced to explain the project within the participating institution, using slides, poster, and leaflets, in at least two different languages.

Intermediate and final results will be shared through scientific publications and academic conferences. Information material concerning the use of the results will be produced for relevant stakeholders,

including the project's sponsor (Ministry of University and Research with reference to mission 4 - Education and Research - of the National Recovery and Resilience Plan, NRRP).

21. Conflict of interest

The investigators declare that they have no financial or non-financial conflicts of interest in relation to this study.

22. Responsibility and publication policies

The results of this research will be published in peer reviewed journals, scientific reports, presentations at conferences, seminars and symposia and will be used to inform public agencies and authorities about evidence-based results of the study, leading to an integrated clinical-environmental surveillance system for close communities. The data will be presented in an aggregated and anonymous manner and no information that could identify participants will be reported in any way.

However, the views and opinions expressed are solely those of the authors and do not necessarily reflect those of the European Union or the European Commission. Neither the European Union nor the European Commission can be held responsible for them.

23. Role of the Promoter and the Investigators

The role of the promoter and the investigators in the study is established in the technical annex of the Stell-ARI project, on the basis of which the financial resources corresponding to the planned commitment have been allocated. In detail, the promoter is responsible for developing the study design, the data collection tools and the analysis plan. The investigators are involved in the definition of the study design and are responsible for enrolment. Data collection will be performed by the investigators, commissioned facilities and, limited to schools, by the ISI Foundation via the Influweb platform. The promoter, with input from all investigators, is responsible for writing the scientific and dissemination reports. All investigators will be included in the authorship of the products resulting from this study.

24. Ownership of data processing

The University of Pisa with headquarters in Pisa, Lungarno Pacinotti 43, in the person of the Rector pro tempore and, limited to the school setting, shared with the ISI Foundation with headquarters in Turin, via Chisola 5, in the person of the President.

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