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Hospital-acquired bacterial infections in SARS-CoV-2 infected patients. Wasn't COVID-19 complicated enough? Prospective study on nosocomial bloodstream infections and description of NDM-1-producing *Klebsiella pneumoniae* outbreak in COVID-19 units

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Oh, mama, can this really be the end

Bob Dylan

Summary

ABSTRACT

Introduction Bacterial superinfections may complicate the clinical course of hospitalized SARS-CoV2 infected patients. However, the exact burden of bacterial complications and their impact on mortality in COVID-19 is not fully understood yet.

Aim of the first study included in this dissertation is to evaluate the burden and epidemiology of hospital-acquired bloodstream infections (HA-BSIs) in patients hospitalized with COVID-19 pneumonia, exploring in particular risk factors associated with HA-BSIs and impact of HA-BSI on mortality.

In the second study it will be described an outbreak due to NDM-1-producing *Klebsiella pneumoniae* (NDM-Kp) involving SARS-CoV-2 infected patients in ICU and non-ICU Units. Epidemiological and clinical characteristics of the outbreak, including molecular characterization of the micro-organism, will be discussed as well as the infection control measures implemented to control the outbreak.

Methods Prospective observational cohort study conducted at San Paolo Hospital in Milan, Italy. All patients admitted to hospital for symptomatic SARS-CoV-2 infection from 24 February 2020 to 31 March 2021 were included in the study. HA-BSI defined as infections occurring ≥48 hours after hospital admission. Incidence of HA-BSI was estimated as the numbers of HA-BSI episodes over 1000 patients-day of hospitalization with 95% confidence interval calculated by Poisson distribution. Factors associated with the development of HA-BSI were analysed using an unadjusted and adjusted logistic regression model. The impact of HA-BSI on in-hospital mortality and hospital discharge has been evaluated with a 1:1 matching nested study using competing-risk analysis.

The main objectives of the study were: (i) description of microbiological and clinical characteristics of HA-BSIs; (ii) assessment of the incidence and prevalence of HA-BSIs; (iii) evaluation of risk factors for the development of HA-BSIs; (iv) evaluation of the impact of HA-BSIs on length of stay and in-hospital mortality.

Results Among 1,950 consecutive patients hospitalised with COVID-19, 121 episodes of HA-BSI were observed in 101 (5.2%) patients. The incidence rate of HA-BSI was 3.5/1000patient-days (95%CI 2.3–4.3). 1,077/1,950 (55%) patients received corticosteroid therapy and 93/1,950 (5%) immunomodulators. 29% and 7% received continuous positive airway pressure (C-PAP) or non-invasive mechanical ventilation (NIMV) while 5.5% of patients received invasive mechanical ventilation (IMV). No difference in the distribution of therapies and oxygen support was noted between the HA-BSI and non-BSI groups except for a higher consumption of steroids

(73% vs 54%, p=0.001) and higher use of NIMV/IMV (44% vs 12%, p<0.001) in the HA-BSI group. At multivariate analysis, NIMV/CPAP (aOR 1.82, 95%CI 1.15–2.90, p=0.010), IMV (aOR 4.75, 95%CI 2.32–9.72, p<0.001) and corticosteroid treatment (aOR 2.15, 95%CI 1.27–3.65, p=0.005) were confirmed as independent factors associated with HA-BSI. Concerning clinical outcomes, patients with HA-BSI compared to no-BSI groups, had a longer hospital stay (28 vs 10 days, p<0.001) but a similar in-hospital mortality (HA-BSI 33% vs non-BSI 25%, p=0.091). At competing-risk analysis, an increased risk of death in patients with HA-BSI was observed (p=0.030). However, after fitting a multivariable competing-risk regression model, a trend toward an increased risk of death in patients with HA-BSI was observed even tough statistical significance was not reached (aSHR 1.80, 95%CI 0.98-3.30, p=0.057). Regarding length of stay, the 30 days cumulative incidence of hospital discharge was 54% and 75% in patients with and without HA-BSI, respectively (p=0.019); this finding was confirmed after multivariable competing-risk regression model adjusted (aSHR 0.65, 95%CI 0.43-0.85, p=0.003).

The outbreak of NDM-Kp described in the second study was observed from February 2021 to March 2021 at the San Carlo Hospital. It involved 4 Units, including COVID-ICU, for a total of 12 patients. Five of these patients developed an infection caused by NDM-Kp while the rest of them had an asymptomatic colonization. 7 out of 12 patients died. Genomic sequencing has confirmed that all the cases were due to the same strain, ST-945. The outbreak was controlled thanks to the implementation of additional infection control measures.

Conclusions In our cohort the incidence of HA-BSI was relatively low. Development of HA-BSI did not significantly affect mortality but was associated with a longer hospital stay. Patients treated with corticosteroid therapy had double the risk of developing BSI.

Concerning the outbreak, the additional infection control measures implemented, in association with those already in force, made possible the containment of the NDM-Kp outbreak. No more cases have been reported; recently few more cases of NDM-Kp have been identified in non-COVID-19 patients; epidemiological and molecular analysis are currently ongoing.

Introduzione. È ormai noto come il decorso clinico dei pazienti ospedalizzati per infezione da SARS-CoV-2 possa essere complicato da sovra-infezioni batteriche. Tuttavia, la reale incidenza, le caratteristiche cliniche e le conseguenze di tali complicazioni batteriche sull'esito del ricovero non sono ancora del tutto chiariti. In questa dissertazione verranno esposti due studi clinici condotti durante gli anni di studio del corso di dottorato in Ricerca Clinica.

Il primo studio si pone come obiettivo quello di valutare il burden e le caratteristiche cliniche e microbiologiche delle infezioni del circolo ematico acquisite in ospedale (acronimo in inglese HA-BSI) nei pazienti ricoverati per polmonite COVI-19. In particolare, sono stati analizzati i fattori di rischio per queste infezioni e l'impatto sull'outcome clinico in termini di mortalità e lunghezza di degenza.

Nel secondo studio viene descritto un outbreak intraospedaliero di infezioni sostenute da *Klebsiella pneumoniae* produttrice di metallo-betalattamasi di tipo New Delhi (NDM-Kp) che ha coinvolto alcuni reparti COVID compresa la terapia intensiva. Verranno descritte le caratteristiche cliniche ed epidemiologiche dei casi e la caratterizzazione fenotipica e molecolare dei ceppi. Infine, verranno discusse le misure di Infection Control messe in atto per limitare l'outbreak.

Materiali e metodi. Il primo è uno studio osservazionale di coorte prospettico condotto presso l'Ospedale San Paolo di Milano. Tutti i pazienti ricoverati per infezione da SARS-CoV-2 sintomatica dal 24 febbraio 2020 al 31 marzo 2021 sono stati inclusi nello studio. Le HA-BSI sono state definite come infezioni insorte almeno 48 ore dopo l'ingresso in ospedale. L'incidenza è stata stimata come il numero di episodi di HA-BSI su 1000 giornate di ospedalizzazione con il 95% di intervallo di confidenza calcolato tramite Poisson. I fattori associati con lo sviluppo di HA-BSI sono stati analizzati tramite modelli di regressione uni e multivariati. L'impatto delle HA-BSI sull'outcome clinico è stato analizzato tramite analisi competitiva del rischio dopo aver selezionato per età, sesso e ricovero in ICU 100 pazienti che non hanno sviluppato batteriemia (non-BSI) (controlli) comparati con i 100 pazienti HA-BSI (casi) con un match 1:1.

Obiettivi principali dello studio: (i) descrizione delle caratteristiche cliniche e microbiologiche delle HA-BSI; (ii) valutare l'incidenza delle HA-BSI; (iii) valutare dei fattori di rischio associati; (iv) valutare l'impatto sulla mortalità ospedaliera e la lunghezza di degenza.

Risultati. Tra i 1950 pazienti ricoverati per COVID-19, sono stati osservati 121 episodi di HA-BSI in 101 (5.2%) pazienti. L'incidenza è stata di 3.5/1000patient-days (95%CI 2.3–4.3). Clinicamente, il 55% dei pazienti è stato trattato con terapia steroidea e solo il 5% con terapia immunomodulante. Dal punto di vista del supporto respiratorio, 29% dei pazienti è stato trattato con casco C-PAP, il 7% tramite NIMV e il 5.5% dei pazienti sono stati sottoposto a ventilazione

meccanica invasiva (IMV). All'analisi multivariata, fattori di rischio indipendenti per lo sviluppo di HA-BSI sono risultati essere l'uso di NIMV/CPAP (aOR 1.82, 95%CI 1.15–2.90, p=0.010), di IMV (aOR 4.75, 95%CI 2.32–9.72, p<0.001) e la terapia corticosteroidea (aOR 2.15, 95%CI 1.27–3.65, p=0.005). Per quanto concerne l'esito clinico, i pazienti con HA-BSI hanno avuto una degenza più lunga (28 contro 10 giorni, p<0.001) ed una maggiore ma non significativa mortalità (33% contro 25%, p=0.091). All'analisi competitiva del rischio, è stato osservato un aumento del rischio di morte nei pazienti con HA-BSI; tuttavia, all'analisi multivariata si è confermato un trend verso un aumento di mortalità ma non statisticamente significativo (aSHR 1.80, 95%CI 0.98-3.30, p=0.057). Mentre, è stato confermato l'aumento della lunghezza di degenza nei casi rispetto ai controlli (incidenza di dimissione ospedaliera 54% in HA-BSI vs 75% in non-BSI; aSHR 0.65, 95%CI 0.43-0.85, p=0.003).

L'outbreak di NDM-Kp descritto nel secondo studio è stato osservato da febbraio a marzo 2021 presso l'Ospedale San Carlo Borromeo di Milano. Ha coinvolto complessivamente 4 reparti COVID, compresa la terapia intensiva, per un totale di 12 pazienti. 5 di questi hanno sviluppato un'infezione sostenuta dal ceppo in questione mentre 7 sono risultati essere solamente colonizzati. 7 pazienti sono deceduti. L'analisi molecolare ha confermato che i casi erano dovuti al ceppo ST-945. L'outbreak è stato controllato grazie all'implementazione di misure aggiuntive di Infection Control.

Conclusioni. Nella nostra coorte l'incidenza di infezioni del circolo ematico ospedaliere è stata relativamente bassa. Tuttavia, è risultata essere associate ad un aumento dei tempi di degenza. I pazienti tratti con terapia steroidea hanno avuto un rischio aumentato di sviluppare tale infezione.

Per quanto riguarda l'outbreak di NDM-Kp, l'implementazione di misure di Infection Control aggiuntive, oltre a quelle già in atto, ha permesso il contenimento del cluster.

BACKGROUND AND AIM OF THE STUDIES

The pandemic Coronavirus Disease 2019 (COVID-19), caused by a novel coronavirus named *Severe Acute Respiratory Syndrome Coronavirus 2* (SARS-CoV-2), is an acute viral disease characterized by an unusual interstitial pneumonia which may lead to acute respiratory failure and death. Starting from a cluster of patients with interstitial pneumonia of unknown origin in Wuhan, China in December 2019, this novel viral disease has rapidly spread to the rest of the world representing a global threat to public health in every country.

Besides the high transmissibility, the ongoing COVID-19 pandemic is characterised by high rates of in-hospital complications and mortality, especially during the first wave of pandemic and in low- and middle-income countries.

The main risk factors for mortality are age and comorbidities. Bacterial and fungal hospital-acquired infections may complicate the clinical course of patients leading to longer durations of hospital stay and higher mortality, as commonly observed during influenza and other viral infections and non-infectious illness. However, while the mechanisms and the burden of bacterial infections during influenza are well described [1, 2], less is known to what extent COVID-19 is complicated by bacterial and fungal infections.

The unexpected diffusion and virulence of COVID-19, alongside the fear of bacterial super-infections in patients hospitalized for COVID-19 pneumonia and the lack of antimicrobial stewardship programs, has led to a large use of antibiotics worldwide probably contributing in the next years to a further spreading of multidrug resistant bacteria. As known, antimicrobial resistance (AMR) is the most important infectious and non-infectious disease public health threats causing 700,000 deaths annually worldwide [3]. Furthermore, clinical and Laboratoristic picture of COVID-19 pneumonia and bacterial infection, both characterized by fever, desaturation, worsening of general conditions with increase in inflammation markers, can overlap and therefore sometimes difficult to distinguish. As a consequence, many patients received antibiotics during the hospitalization often empirically and without microbiological findings.

Nevertheless, bacterial bloodstream infections (BSIs) as well as ventilator associated pneumonias (VAPs) are serious conditions that may negatively influence the patients outcome unless appropriate antibiotic treatment is promptly started [4–6]. To further complicate the scenario, an increment in the circulation of MDR bacteria during COVID-19 pandemic have been reported worldwide. This seems to be mainly due to the high intensity of care required for

COVID-19 patients, the extensive use of antimicrobials, and the lack of effective infection control measures and antimicrobial stewardship programs.
While a growing body of evidence on the overall incidence of bacterial co- and super-infections during COVID-19 is available [7–9], few studies focus exclusively on hospital-acquired BSI and respiratory infections especially outside intensive care (ICU) settings [10–12].

In the following dissertation, two original research studies will be included and discussed.
The aim of the first study is to estimate the real burden of bacterial bloodstream during hospitalization for COVID-19, exploring in particular the incidence and risk factors for these infections. Moreover, we aim to evaluate the impact of BSIs on the clinical outcomes of COVID-19 patients. In my opinion, there data may fill a current gap of knowledge and therefore be crucial to suspect a bacterial super-infection and to start an appropriate antibiotic treatment and eventually improve patients outcome.

The second study included in the dissertation aims to describe the epidemiological and clinical characteristics of an outbreak due to NDM-1-producing *Klebsiella pneumoniae* involving SARS-CoV-2 infected patients in ICU and non-ICU units. Molecular characterization of the strain will be discussed as well as the infection control measures implemented to control the outbreak.

LITERATURE REVIEW

Coronavirus Disease 2019 (COVID-19)

Origin and epidemiological features of Coronavirus Disease 2019 pandemic

In the last years, the world experienced three lethal zoonotic diseases caused by novel coronaviruses; in particular, *severe acute respiratory syndrome* (SARS) in 2002, *Middle East respiratory syndrome* (MERS) 2012 and *Coronavirus Disease 2019* (COVID-19) in 2019.
The ongoing COVID-19 pandemic is caused by a new coronavirus named *severe acute respiratory syndrome coronavirus 2* (SARS-CoV-2). The suffix -2 was added to distinguish this virus from the SARS-CoV-1, responsible in 2002 for the first outbreak caused by coronaviruses.

Severe acute respiratory syndrome (SARS)

The 2002–2004 outbreak of SARS, firstly reported on February 11, 2002, in Guangdong in China, overall infected 8,000 people and caused 800 deaths. This outbreak did not spread worldwide, becoming pandemic, but it has spread to 33 different countries [13]. Although the SARS epidemic was declared contained in 2003 by the World Health Organization (WHO) four cases were reported also in 2004 [14]. The etiologic agent responsible for the syndrome was recognized as a novel β-coronavirus subgroup B on April 10, 2003 using cell cultures and polymerase chain reaction (PCR)-based techniques also thanks to the contribution of Dr. Carlo Urbani, an Italian scientist who had died during the outbreak [15]. Based on epidemiological and molecular studies, the origin of the SARS-CoV-1 was discovered to be zoonotic and, in particular it is likely that the virus originated from bats and later crossed the xenographic barrier to humans using palm civets (*Paguma larvata*) as intermediate hosts [16]. The clinical picture of SARS was characterized by fever, cough, shortness of breath, or hypoxia and radiological evidence of atypical interstitial pneumonia. In the most severe cases, the disease can lead to acute respiratory distress syndrome (ARDS) and death. Noteworthy, a large proportion of cases were observed among health-care workers (from 20% to 40% of infections) [17]; this was later observed also during MERS and COVID-19 outbreaks [18, 19]. This can partly be explained by the fact that the highest peak of viral shedding is observed during the incubation period while patient is asymptomatic. The criteria for the definition of SARS were defined by the CDC and were composed by clinical, laboratory and epidemiological criteria [20]. Noteworthy, also for

SARS outbreak bacterial and fungal super-infections represented a common complication; Wang et al. reported, among a cohort of 76 SARS patients, 18 nosocomial infections of which 6 were bloodstream infections and 11 were lower respiratory infections. 9 episodes were caused by multidrug pathogens [21].

Middle East respiratory syndrome (MERS)

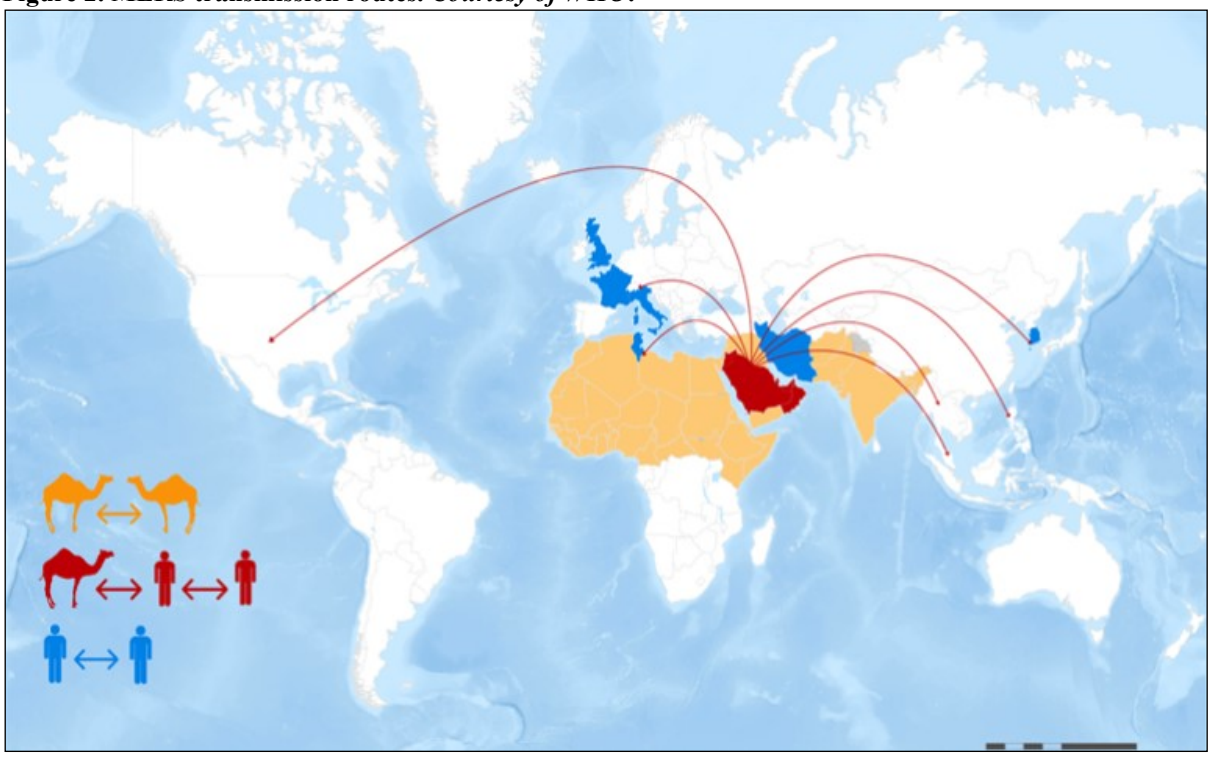
In 2012, another zoonotic pathogenic coronavirus, named *Middle East Respiratory Syndrome Coronavirus* (MERS-CoV), emerged among humans, and spread in the Middle East causing an outbreak which has become endemic in that geographic area with sporadic cases observed in other parts of the world (**figure 1**) or nosocomial outbreaks. The first case was described in Saudi Arabia in a patient admitted to the hospital for interstitial pneumonia and died for multiorgan failure; the virus was isolated from lung sample of the patient [22]. From 2012 to 2021, MERS has caused 2574 infections and 886 deaths with a fatality rate of 34.4% with the majority of cases reported from Saudi Arabia [23].

Figure 1. Global distribution of MERS cases till Dec 2019. From *Memish* et al.[24]

MERS-CoV is a member of the *Coronaviridae* family, β-Coronavirus subgroup C [25], which commonly infects dromedary camels (*Camelus dromedarius*) causing illness characterized by mild respiratory symptoms. Transmission to humans can happen through direct or indirect contact with infected animals or patients (**figure 2**) with inter-humans transmission happening

especially in hospital or household settings [24, 26]. In detail, healthcare-associated transmission accounted for approximately 50% of cases [27].

Figure 2. MERS transmission routes. ***Courtesy of WHO*.**



Clinical features of the disease are similar to the ones observed for SARS and COVID-19 ranging from asymptomatic to severe cases characterized by atypical pneumonia, respiratory failure, ARDS and death for multiorgan failure (**figure 3**). The typical radiological presentation of infected patients is bilateral extensive ground-glass area observed at chest CT scan or bilateral extensive opacities or consolidations at chest X-ray (**figure 4**). Nosocomial bacterial superinfections has been reported especially in aptients admitted to intensive care [28]. As for SARS, no specific therapies are available and therefore the mainstay of clinical management remains oxygen and supportive therapy [29].

Figure 3. Epidemiological, clinical, and laboratory features of MERS

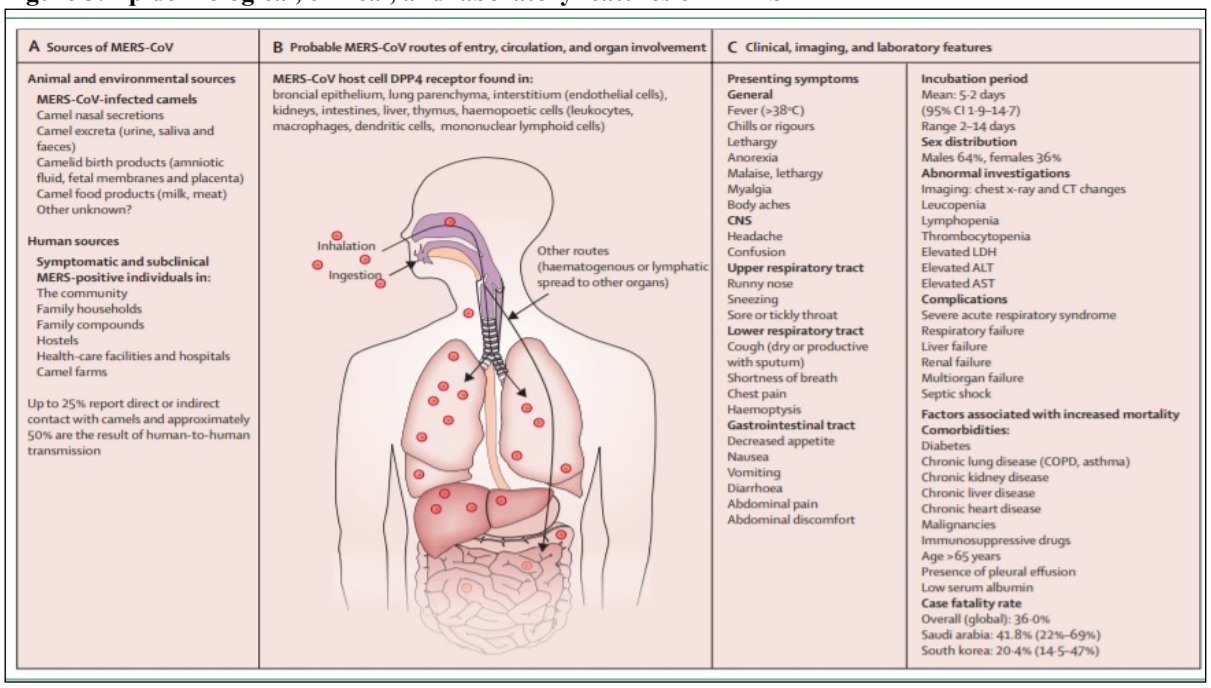
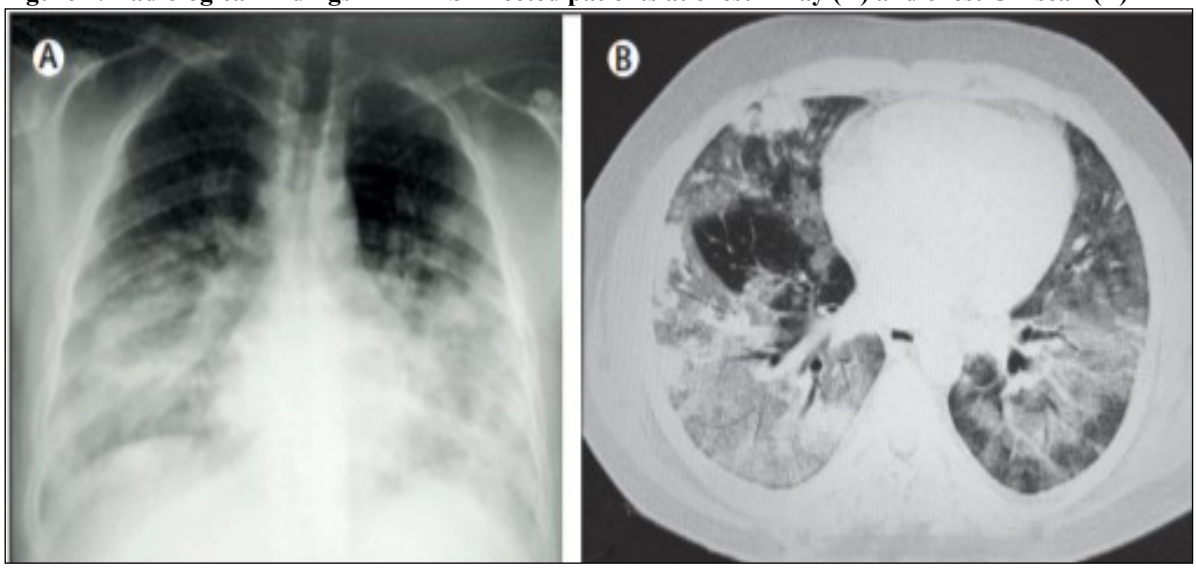


Figure 4. Radiological findings in MERS infected patients at chest X-ray (A) and chest CT-scan (B)

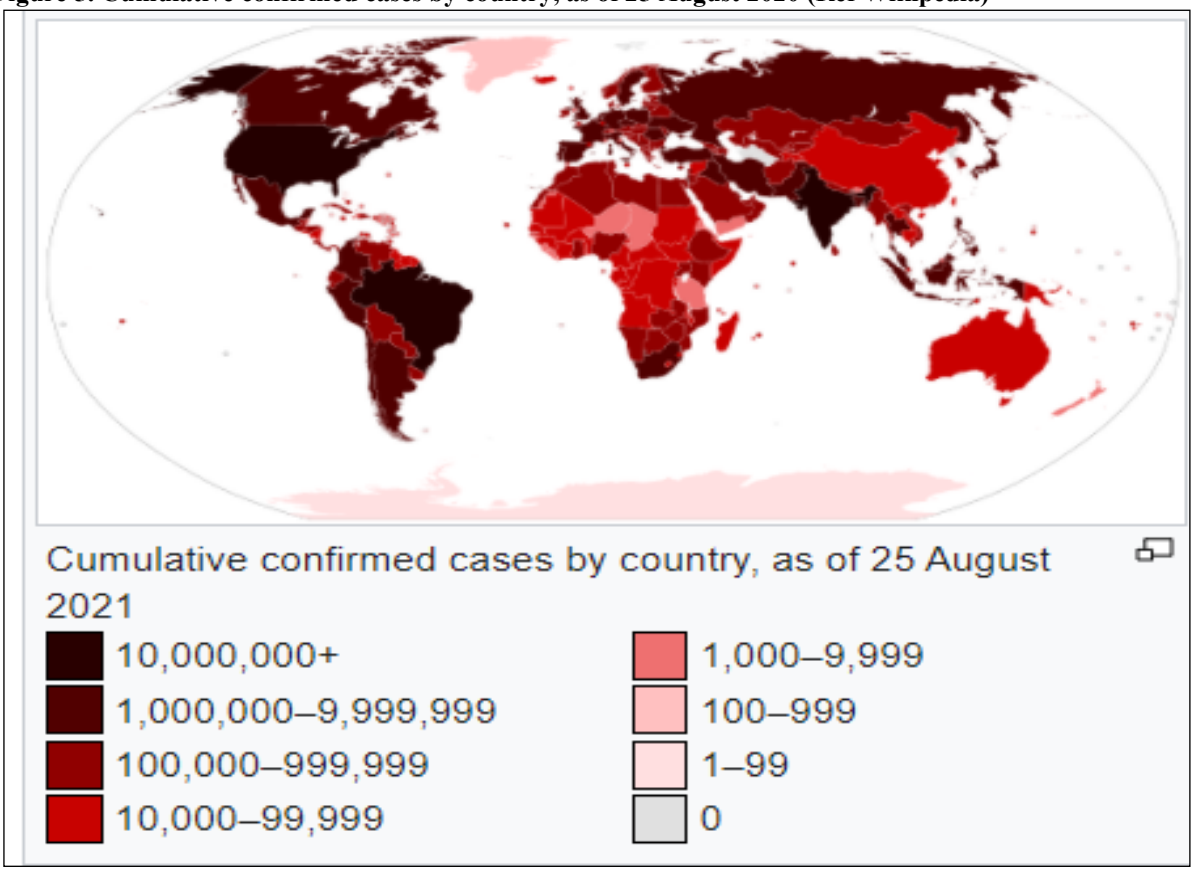


Coronavirus Disease 2019 (COVID-19)

In December 2019, a new coronavirus emerged in Wuhan, Hubei province of China, and rapidly spread all over the world overwhelming SARS and MERS. To date, 12 September 2021, the COVID-19 pandemic has caused more than 224,000,000 cases and 4,800,000 deaths[30].

Similarly to SARS and MERS, the first cases were characterized by fever, chough and shortening of breath with radiological evidence of interstitial pneumonia and they were all epidemiologically linked with the Huanan Seafood Wholesale Market in Wuhan [31, 32], a market located in Wuhan which sells also live animals. On 31 December, the World Health Organization (WHO) received the first report of 27 cases of pneumonia with unknown cause from China Health authorities [33]. Genetic evidence shows high sequence similarity between the genome of SARS-CoV-2 and other coronaviruses isolated from *Rhinolophus* bats in China suggesting that these animals may be the natural host of SARS-CoV-2. Likewise, SARS-CoV-2 was demonstrated to have high sequence similarity to pangolins coronavirus. Therefore, scientists suggest that pangolin (*Manis javanica*) may be the intermediate host of the virus[34]. From January, several new cases have been notified in the same Region with inter-human transmission including familiar clusters and nosocomial infection within hospital facilities. In one month, the outbreak reached all the provinces in China facilitated by the celebration for Chinese New Year. The peak of the epidemic in China was reached in February with sharply 3,000 cases per day. Within the country, infection control measures were implemented including the blockade of travels and transportations between the cities, closure of commercial activities and restriction of outside activities. Despite this in February, while the number of cases started to decline in China, the virus spread outside the country reaching neighbouring states. On 11 February, this novel disease was named by "COVID-19" by the WHO while the International Committee on Taxonomy of Viruses established the name of etiological agent as "SARS-CoV-2" [35]. Since late February, cases started to be reported in Europe and USA and eventually the outbreaks reached al the six continents (**figure 5**).

Figure 5. Cumulative confirmed cases by country, as of 25 August 2020 (Ref Wikipedia)



In Europe, the first country affected by the pandemic was Italy where the impact of the pandemic was dramatic; in fact, Italy experienced one of the highest number of cases and deaths in the World. As of 12 September 2021, in Italy COVID-19 pandemic has caused a total of 4,601,749 confirmed cases and 129,885 deaths since the pandemic began[36].

In Italy the first case of COVID-19 was diagnosed in Codogno (LO), in Lombardy on 20 February 2020 even though it seems likely that the virus was already circulating in our Country since before.

The first two regions involved by the pandemic in Italy have been Lombardy and Veneto that were declared under quarantine, the so-called “red zones”. Despite this, already in March the virus had spread to all the Italian territory. Therefore, on 4 March, all the schools and universities were closed and on 9 March the red zones were extended to all the regions and the first national lockdown was declared by the Italian Prime Minister. Commercial activities were closed, public events cancelled, and circulation of people restricted until 3 of June when the lockdown started in March was ended. The number of cases gradually decreased in summer but in September a new increment of cases was observed in Italy, as in many European country, outlining the so-called “second wave” of the pandemic. Therefore, the state of emergency was prolonged and further restrictions were introduced. The preparedness of healthcare system, with

upgrading of hospital facilities and increment in intensive care units capacity, helped to face and contain this second wave of pandemic limiting the number of new cases and deaths. Nevertheless, partly due to the appearance of new variants such as the Delta variants, the pandemic was never totally contained and Italy and the world in general had entered in the third wave of pandemic still ongoing.

On 21 December 2020, the first COVID-19 vaccine, the Pfizer–BioNTech COVID-19 vaccine, was approved and authorized by the European Medicines Agency (EMA) and the European Commission; on the 6 January 2020, a second vaccine was approved, the Moderna vaccine.

On 27 December 2020, the vaccination campaign started in Italy with the Pfizer-BioNTech vaccine. As 12 September 2021, Italy has administered at least 80,568,881 doses of COVID vaccines so far. Assuming every person needs 2 doses, that's enough to have vaccinated about 66.8% of the country's population [36].

The pandemic represented one of the major threats to the health-care system and to economy in Italian history. From an economical point of view, the damage caused by the pandemic was enormous with a predicted GPD decline of 6-7%. The tourism and accommodation sectors were the most damage sectors and many people lost their business activity.

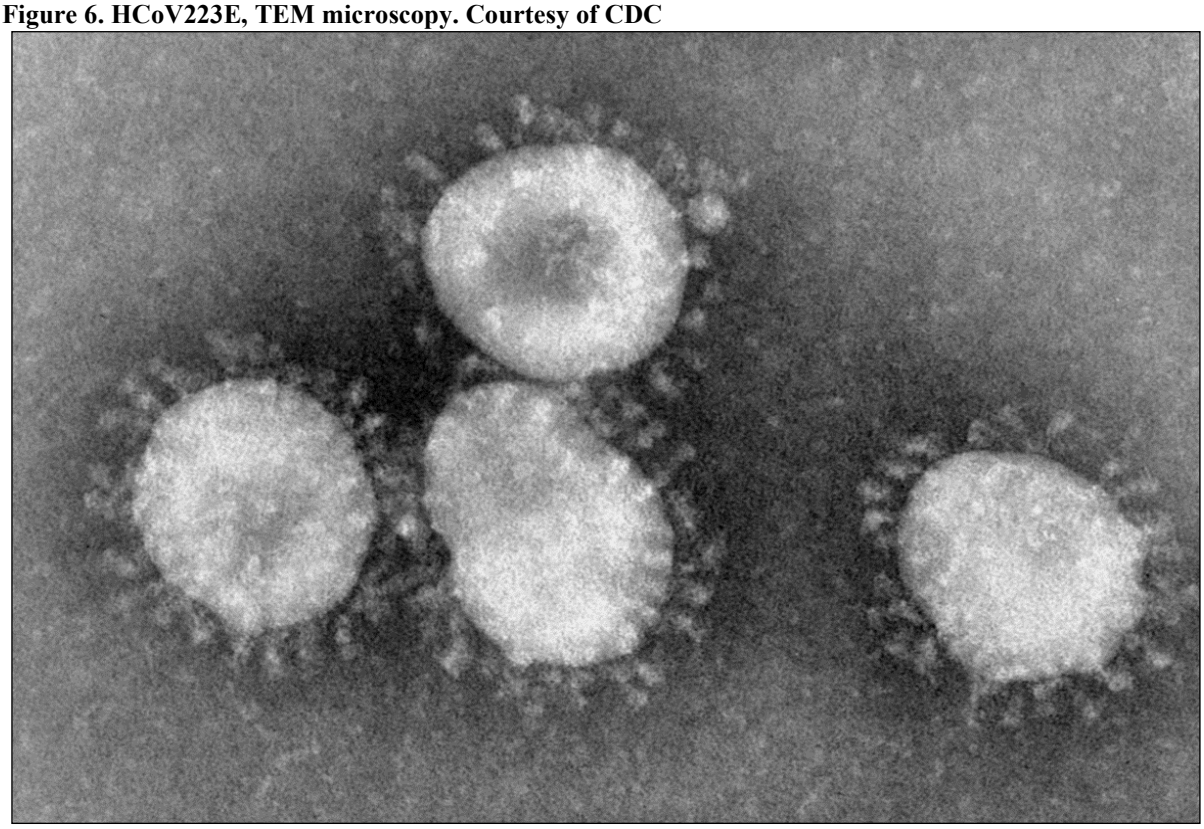
From a healthcare aspect, due to the rapid rise in coronavirus infections, emergency departments and hospitals in general were overcrowded; therefore, intensive care units were expanded, a large number of ventilators and other medical equipment were brought, and new hospitals were created in order the face the emergency. Healthcare workers overworked and experienced psychological and physical stress; moreover, high percentage of coronavirus infections were recorded among healthcare workers which has resulted in death in a considerable number of cases.

SARS-CoV-2: structure, transmission, pathogenesis, and virulence factors

SARS-CoV-2, formerly known as 2019 novel coronavirus (2019-nCoV) and human coronavirus 2019 (HCoV-19), is a novel coronavirus responsible for the novel pandemic disease COVID-19.

Coronaviruses, identified for the first time in the 1960s, are unsegmented single-stranded RNA viruses, belonging to the subfamily *Coronavirinae* of the family *Coronaviridae* of the order *Nidovirales*[37]. The presence of crown-like spikes on the surface of this viruses is the cause of the name giving at the family (**figure 6**).

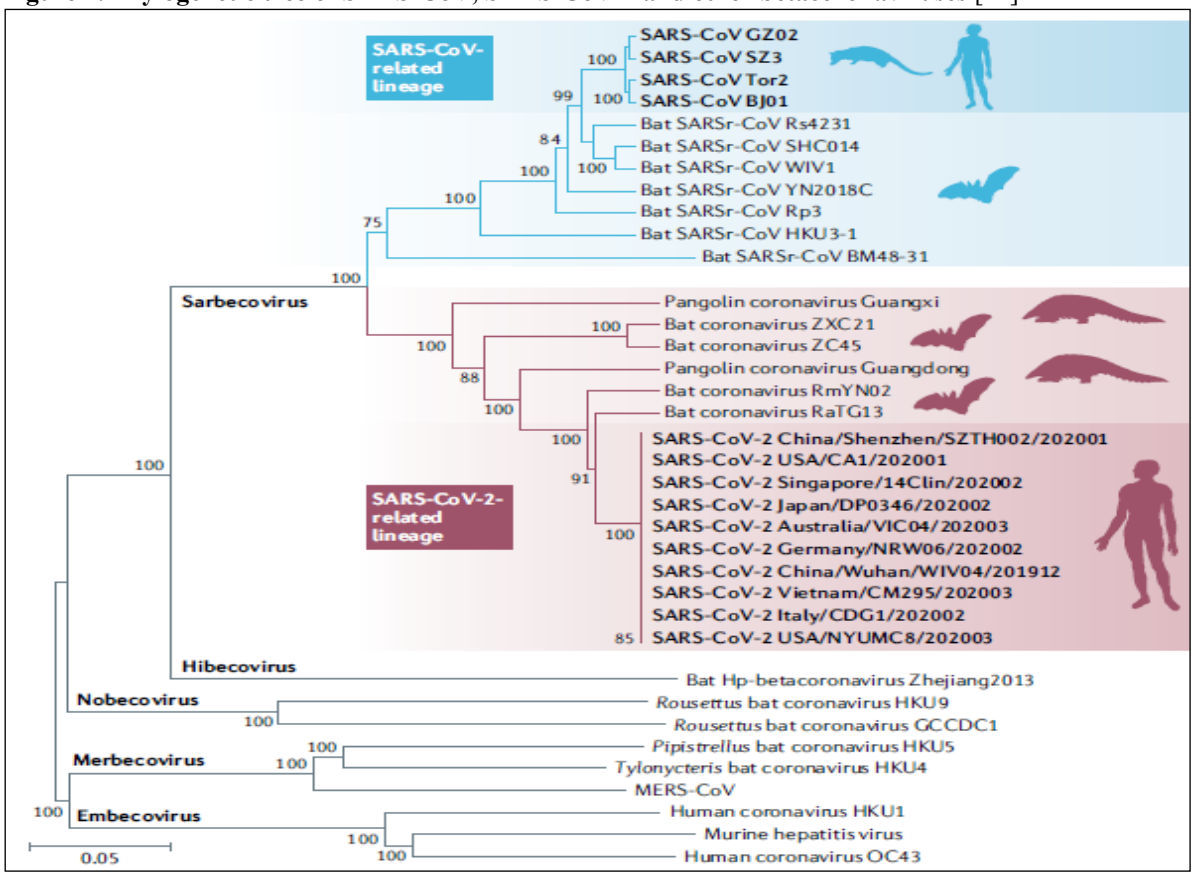
Figure 6. HCoV223E, TEM microscopy. Courtesy of CDC



Based on serotype, coronaviruses are divided into four major classes: Alpha-coronavirus, Beta-coronavirus, Gamma-coronavirus, and Delta-coronavirus [38]. Generally, alpha and beta-coronavirus infects mammals while Gamma and Delta infects birds. Four coronaviruses commonly infect humans, HCoVs-229E, OC43, NL63 and HKU1, causing respiratory and gastrointestinal tract infections[39] while the novel coronaviruses SARS-CoV, MERS-CoV, and SARS-CoV-2 have a different pathogenicity and lead to different clinical conditions with higher mortality rates.

Coronaviruses have four structural proteins: the S protein, responsible for receptor-binding and viral entry, the M and E proteins, involved in viral assembly, and the N protein which holds the genome. The transmission mechanisms of these viruses include across species barriers as well as person-to-person transmission. Generally, the virus is transmitted by respiratory droplets during coughing or via person-to-person contact by touch. CoV may undergo, within the animal host, genetic recombination leading to variants more pathogenetic to humans [40] (**figure 7**). Examples of this mechanism can be found in SARS [41] and MERS [42] outbreaks and recently in SARS-CoV-2 for which a zoonotic was recognized [43]. Indeed, SARS-CoV-2 has 79% genome sequence identity with SARS and 50% with MERS.

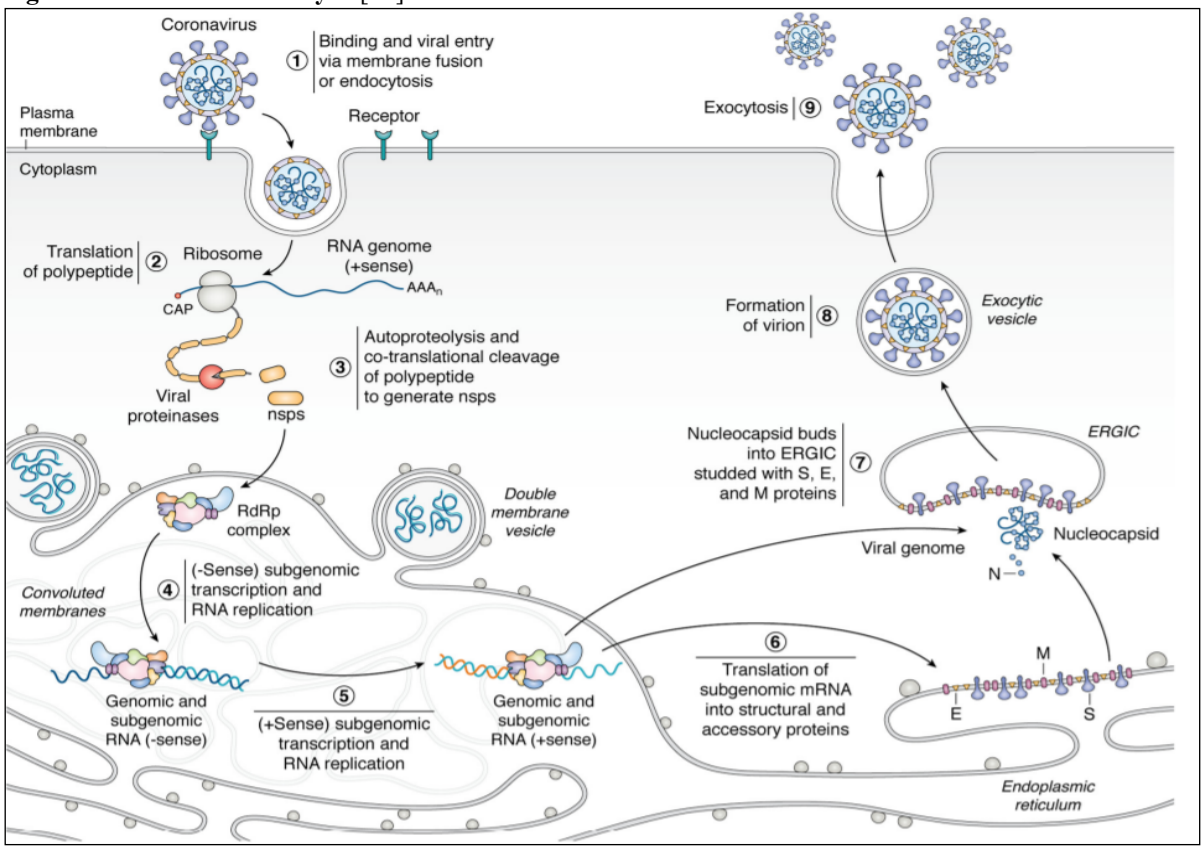
Figure 7. Phylogenetic tree of SARS-CoV, SARS-CoV-2 and other betacoronaviruses [44]



Structurally, SARS-CoV-2 has a diameter of 50-200 nanometres and has four main proteins named Spike, Envelope, Membrane and Nucleocapsid proteins. In particular, the glycoprotein S, responsible for the attachment and entry of the virus into the host cell, is divided in two subunits, S1 and S2 and contains a receptor-binding domain (RBD) that binds the host receptor [45]. The host receptor for SARS-CoV-2 is ACE2 (*Angiotensin-converting enzyme 2*), a cell-surface peptidase that hydrolyses angiotensin II, expressed in epithelia of lung and small intestine and other organs. After RBD-ACE2 binding, S proteins are cleaved and therefore

activated by a host cell-surface protease leading to membrane fusion [46]. After the entry of the virus, through endocytosis or direct membrane fusion, genome is released from the nucleocapsid and subsequent cleaved in smaller fragment of RNA. After that, transcription and replication start and the subgenomic mRNAs are included in nucleocapsid and structural proteins. The new virions are then excreted from the cell by exocytosis (**figure 8**).

Figure 8. Coronavirus life cycle[46]



The knowledge of SARS-CoV-2 is crucial to explore new therapeutic strategies. For instance, the viral entry represents the target of neutralizing antibodies against SARS-CoV-2 and convalescent plasma from patients with prior infection by SARS-CoV-2. Both strategies have been proven to effectively reduce viral load. Moreover, S protein is the target of SARS-CoV-2 vaccines.

Regarding pathogenesis and virulence factors, the specificity of S protein for ACE2 receptor is responsible for the signs and symptoms of COVID-19. In fact, ACE2 receptors are expressed especially in the lung tissue and in the small intestine; consequently, the main symptoms of the disease are respiratory and gastrointestinal.

The main function of ACE2 is to convert angiotensin 2 into angiotensin 1-7 which have anti-inflammatory activity with protecting effect for the lung. On the contrary, angiotensin 2

promotes cellular proliferation, vasoconstriction and fibrogenesis and therefore inflammation. The bond of S1 with ACE2 receptor causes a decrease in the expression of ACE2 receptors on cellular surface promoting inflammation and tissue damage.

After the bond of S1 to ACE2 receptors in the epithelial cells of the respiratory tract, the virus starts the replication in the lungs and migrates to the alveolar cells. This triggers a strong immune response with release of cytokine causing the so-called “cytokine storm syndrome” that causes ARDS and respiratory failure.

Epidemiology of COVID-19 and risk factors for mortality

The spectrum of clinical manifestations of SARS-CoV-2 can range from asymptomatic infections in a large number of cases to critical disease in a minority of cases. Among people who develop symptoms the majority will experience mild to moderate symptoms, up to 80%, while only 15% develop severe symptoms and 5% critical symptoms including ARDS, respiratory failure, multiorgan failure and death. Under investigation the long-term effects of organ damage during acute infection causing long lasting symptoms such as fatigue, breathing difficulties and headache ("long COVID").

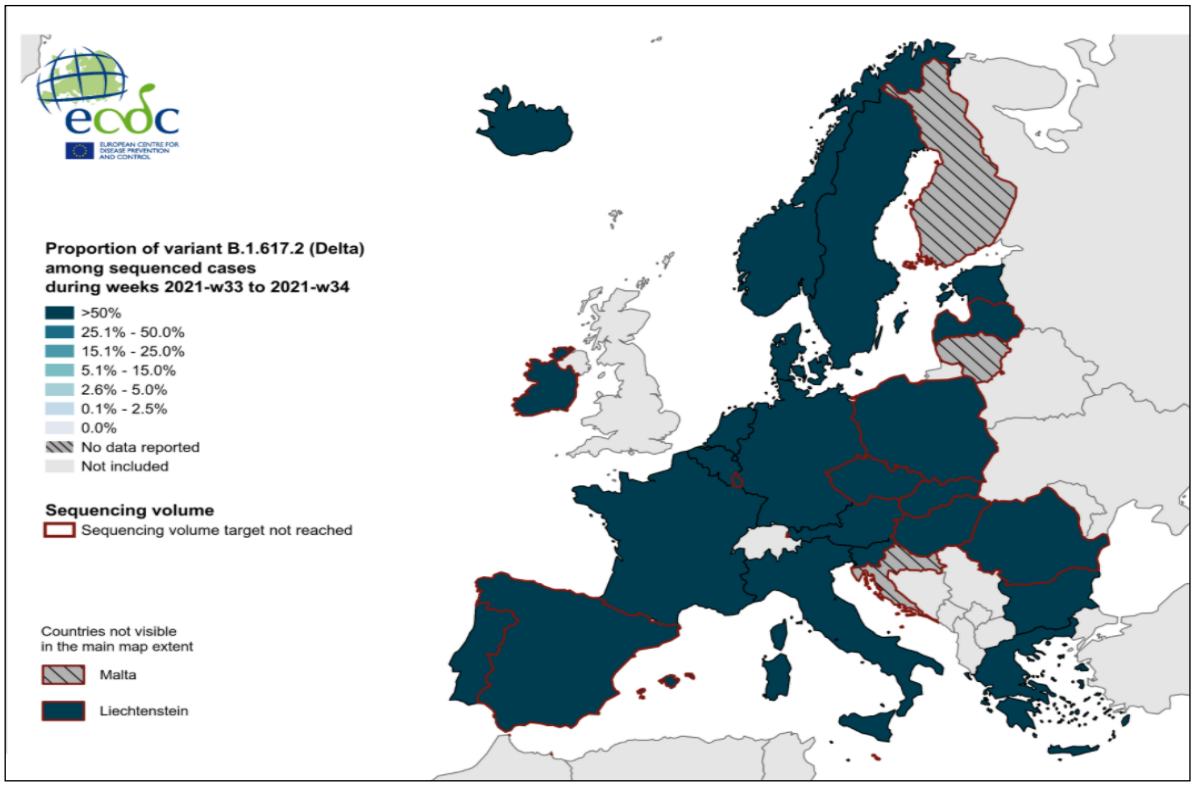
Asymptomatic carriers are of great importance since they can spread virus and be contagious up to 20 days after infection even though they will never experience symptoms. Therefore, preventive measures, including social distancing, hand washing and use of face masks, are crucial to minimize the risk of infections and to contain the pandemic. Transmission is possible for approximately 8 days after symptoms appear even though nasopharyngeal swab may continue to be positive for weeks after resolution of the disease. However, generally viable virus cannot be detected after about 8 days of disease.

Limiting in hospital transmission of COVID-19 crucial; firstly, to protect fragile patients already hospitalized for other reasons, secondly to avoid infections among healthcare providers that may become potential vehicle of further virus dissemination. Therefore, for the management of COVID-19 patients, infection control measures are recommended included single rooms or cohorting of patients, use of masks, gowns, eye protection and gloves.

Based on data provided in the last ECDC report on COVID-19 [47], as of 05 September 2021, the overall COVID-19 case notification rate for the EU/EEA was 187.0 per 100 000 population with a decreasing trend as compared to the previous weeks while the 14-day COVID-19 death rate was 14.5 deaths per million population compared with 12.7 deaths the previous week. In Italy, in the same week, as compared to EU/EEA lower case notification rates (145.6 per 100 000 population) and death rates (12.8 deaths per million population) were observed. Similarly, hospital admission rate due to COVID-19 in Italy is lower as compared to Europe (EU/EEA: 3.2 per 100 000 population; Italy: 2.2 per 100 000 population.

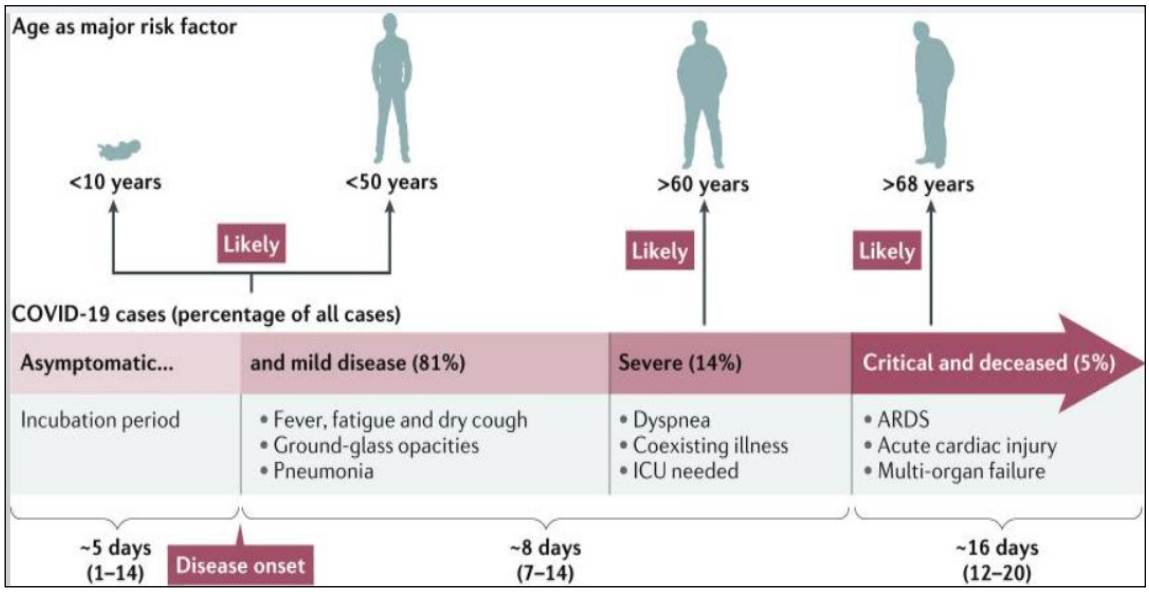
Currently, the SARS-CoV-2 variant of major concern is the delta variant (B.1.617.2). In fact, this variant accounts for 99.5% and 84.7% of all the circulating variants in EU/EEA and Italy respectively (**figure 9**).

Figure 9. Weekly variants distribution by country (ECDC).



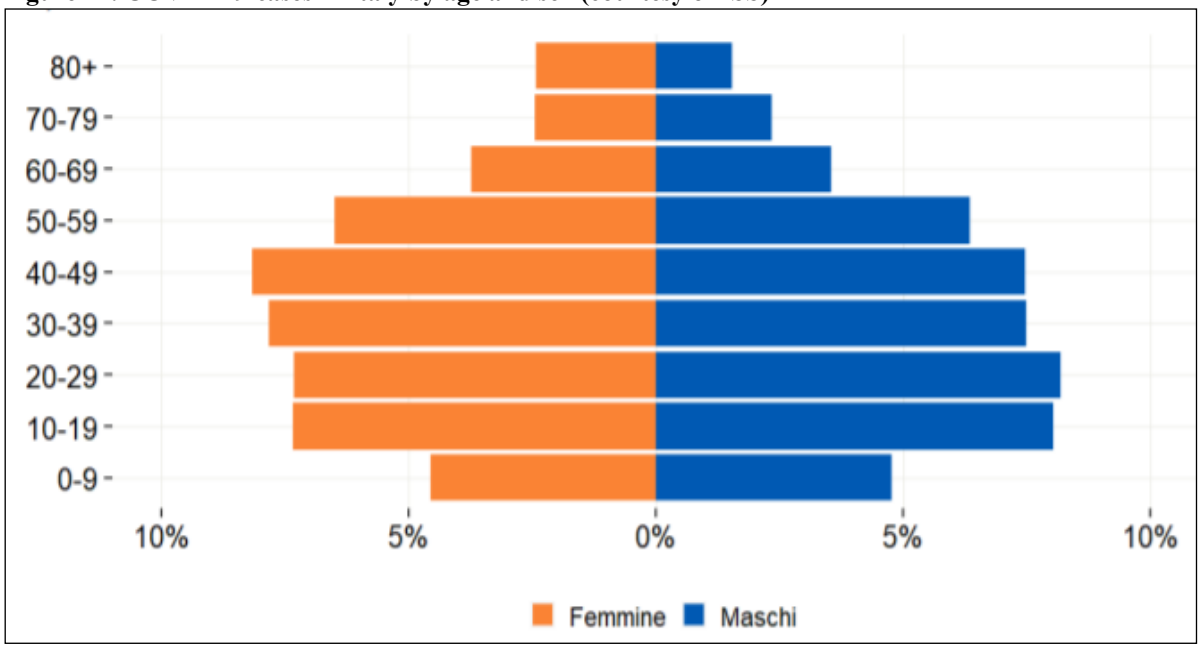
People of any age are susceptible to contagion; worldwide median age of infection is around 50 years. However, clinical manifestations and mortality rate vary with age with elderly people (>60 y/o) being more prone to severe disease and even death. On the other side, young people and children often remain asymptomatic or develop mild disease that does not require hospitalization [44] (**figure 10**).

Figure 10. Age distribution and clinical conditions of SARS-CoV-2 infection [44]



Based on data provided by the ISS (Istituto Superiore di Sanità), in Italy the median age of infection is 45 years whit a similar distribution of sex (49.1% males, 50.1% females). As above stated, also in Italy, critical disease and death were more common in elderly population [48]. The median age of infection in Italy has not been constant during the pandemic; in fact, it has passed from 60 years during the first months of pandemic to 30 years in August 2020. This may be due to several factors, including changes in criteria of execution of nasal swabs to identify infected people during time and different restrictive measures among the different periods of time (**figure 11**).

Figure 11. COVID-19 cases in Italy by age and sex (courtesy of ISS)

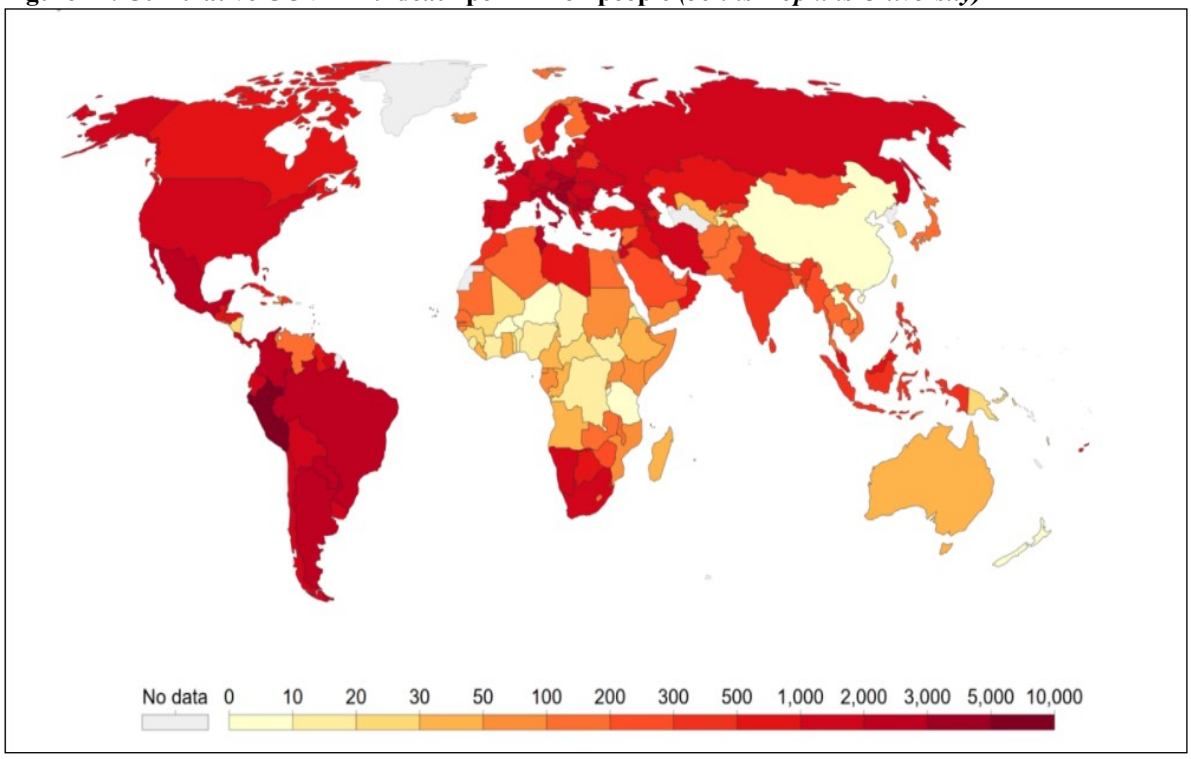


As expected, vaccination campaign has dramatically reduced the risk of infection and severe disease; in fact, fully vaccinated people have a 77% risk reduction of acquiring the infection, 93% risk reduction of hospitalization and 96% of death.

In EU/EEA, as of 05 September 2021, the median cumulative uptake of at least one vaccine dose among adults was 76.5% with a wide inter-country variation (range: 22.5–96.2%) and the median cumulative uptake of full vaccination among adults aged 18 years and older was 70.6%. In Italy, the percentage of fully vaccinated people is above 70% in adults aged > 50 years (age 50-59 76.4%; age 60-69 83.8%; age 70-79 88.4%; age >80 92.1%) while among population aged <50 years the percentage is around 60% (40-49 68,0%; 39-39 62,2%; 20-29 64,8%) [48]. Noteworthy, the majority of cases in the last month in Europe and in Italy has been diagnosed in unvaccinated people.

Regarding COVID-19 attribufigure mortality several measures have been used worldwide. However, the most used and reliable measure is the "case fatality rate" that is calculated as the number of confirmed COVID-19 deaths among the number of SARS-CoV-2-diagnosed cases. Worldwide, the highest CFR was reported in Yemen (CFR 19.49%) [49]. In Italy the CFR was calculated to be 2.8% with 215.46 death per 100,000 population (**figure 12**).

Figure 12. Cumulative COVID-19 death per million people *(Johns Hopkins University)*



Cumulative number of deaths and CFR are influenced by several factors such as healthcare system quality, treatment option, diagnostic test availability and patient characteristics such as age, sex, ethnicity, and comorbidities.

Concerning patient characteristics, mortality rates are lower for young people and relatively higher among the elderly and higher among men than women. In Italy, 56.5% of the deceased were male and almost 60% were elderly people (age>80 years)[48]. While the reasons for age differences in mortality are intuitable, gender differences may be attributable to lifestyle as well as sex-based immunological differences. As observed in other diseases, most of deceased patients of COVID-29 had underlying comorbidities including hypertension, diabetes mellitus, and cardiovascular disease. In Italy, according to data provided by ISS, 96% of dead patients had at least one comorbidity with the average person having 3.4 diseases. The most common comorbidities are hypertension (66% of deaths), type 2 diabetes (29.8), ischemic heart disease

(27.6%), atrial fibrillation (23.1%) and chronic renal failure (20.2%). Obesity and smoking habit are recognized risk factors for complication and mortality [50, 51].

Other recognized risk factors for mortality are COVID-19 severity at admission and laboratory signs of inflammation, such as increased C-reactive protein and D-dimer, and of organ damage including increased transaminases, creatinine, and lactate dehydrogenase [52].

Clinical conditions, diagnosis, and management of COVID-19

COVID-19 related clinical manifestations vary widely from asymptomatic or mild disease to severe pneumonia conditioning respiratory failure and eventually multiorgan failure and death. Risk factors for complications and mortality have been discussed in the previous paragraph. Among them, age, gender, and underlying comorbidities seem to be the most important factors. Following infection, the incubation period is generally 1 to 14 days with a median of 5 days. The first symptoms are non-specific and include fever, fatigue, sore throat, headache, diarrhoea, and anorexia. More specific symptoms such as taste and olfactory disorders may appear [53]. Pneumonia and dyspnoea usually develop within 8 days after the infection [54].
A minority of patients, about 10%, experiences serious symptoms characterized by fever, hypoxemia, and pneumonia with extended involvement of the lung parenchyma. During hospitalization, about 5% of the patients evolve into critical conditions characterized by respiratory failure, interstitial pneumonia, ARDS and shock with multiple organ failure that may require admission to ICU units to undergo mechanical ventilation.

Clinical manifestations of COVID-19 are mainly respiratory since the lung is the organ primarily affected. This is due to the receptor specificity of SARS-CoV2 for ACE2 receptors that are expressed mainly in the lung. However, other organs are able to express ACE2 receptors such as the intestine and vascular endothelium. The bond of the virus at these sites, alongside with a dysregulated immune response, may be responsible for the extrapulmonary manifestations of COVID-19 as diarrhoea and vascular thrombosis. Some authors hypothesize that SARS-CoV-2 could also have a neuroinvasive potential to entry in central nervous system, explaining the development of hyposmia and dysgeusia and contributing to the respiratory failure [55].
Extrapulmonary manifestations include:
- thrombotic manifestations including coronary thrombosis, pulmonary thromboembolism, cerebral ischemia, disseminated intravascular coagulation;
- cardiovascular events such as myocardial damage;
- acute kidney injury;
- neurological manifestations such as long-lasting headache, taste and olfactory disorder, dizziness;
- infectious complications as the recently recognized disease COVID-19-associated pulmonary Aspergillosis [56].

In children, a rare complication of COVID-19 is the Multisystem inflammatory syndrome in children (MIS-C), a systemic illness involving persistent fever and extreme inflammation following exposure to SARS-CoV-2 [57]. A similar syndrome has been recently recognized in adults and was named MIS-A (Multisystem inflammatory syndrome in adults) [58].

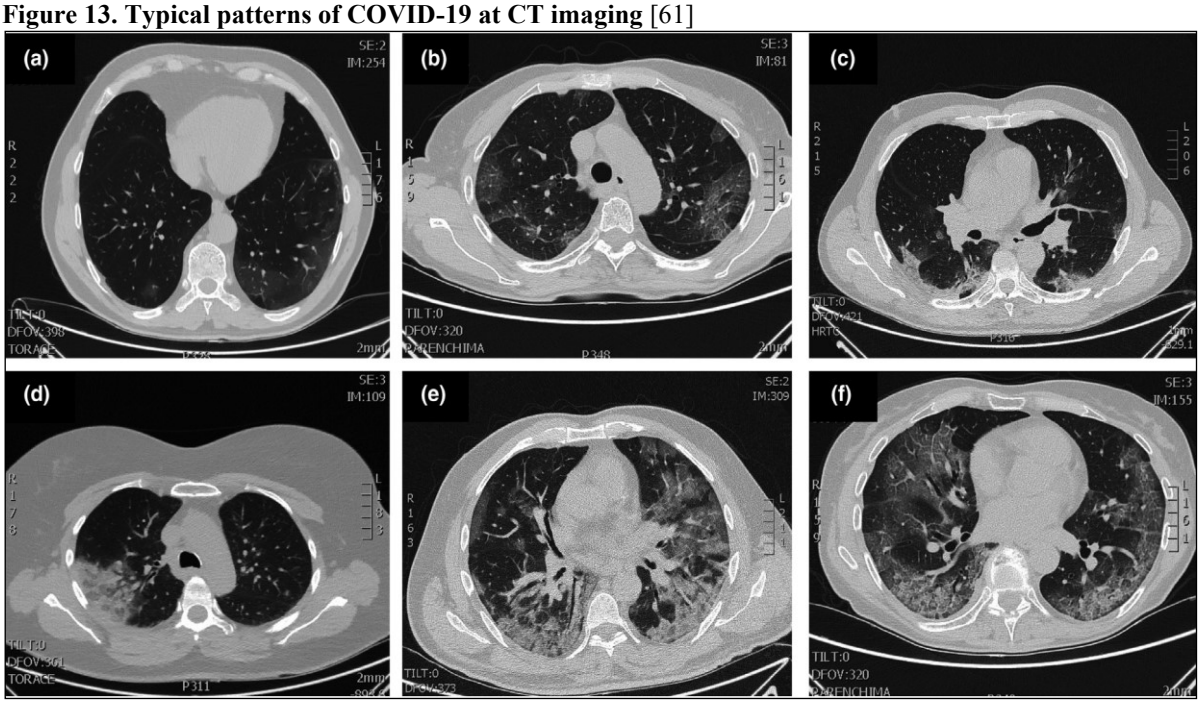
COVID-19 diagnosis is clinical, laboratory and radiological. Etiological diagnosis is achieved by demonstrated SARS-COV-2 nucleic acid in respiratory samples (nasal swab, tracheal aspirate or bronchoalveolar lavage) though molecular techniques.

Typical laboratory findings include marked lymphopenia, similar to what observed during SARS and MERS, thrombocytopenia and increase in inflammation markers including C-reactive Protein and D-dimer elevated levels of alanine aminotransferase and aspartate aminotransferase. An increment in neutrophils and procalcitonin may be suggestive of a bacterial superinfection and it is commonly observed in patients hospitalized to ICU.

From a radiological point of view, COVID-19 is an atypical interstitial pneumonia characterized at chest CT by ground glass opacities, typically multifocal and peripheral with crazy paving [59] (**figure 13**). In patients in critical conditions, at chest CT may be observed patchy bilateral consolidations and >50% of the lung parenchyma affected by the virus. Non-typical reliefs include pleural effusions, masses, and lymphadenopathies.

Histopathology analyses showed bilateral diffused alveolar damage, hyaline membrane formation, desquamation of pneumocytes and fibrin deposits in lungs [60].

Figure 13. Typical patterns of COVID-19 at CT imaging [61]



As above stated, molecular detection of SARS- CoV-2 nucleic acid is the gold standard for the diagnosis of COVID-19. Although SARS- CoV-2 has been detected from a variety of respiratory sources, the viral load is higher in lower respiratory tract samples. In patients with clinical symptoms compatible with COVID-19 and negative nasopharyngeal swab for SARS-COV-2, in which a false negative is suspected, the typical radiological findings can be helpful alongside with repeating swabs to increase sensitivity [62].
Lastly, SARS-CoV-2 serological tests are available and detect antibodies to N or S protein. These tests may can in handy for epidemiological studies or to guide vaccination policy even though it is unknown the neutralizing power of antibodies or the duration of immune response [63].

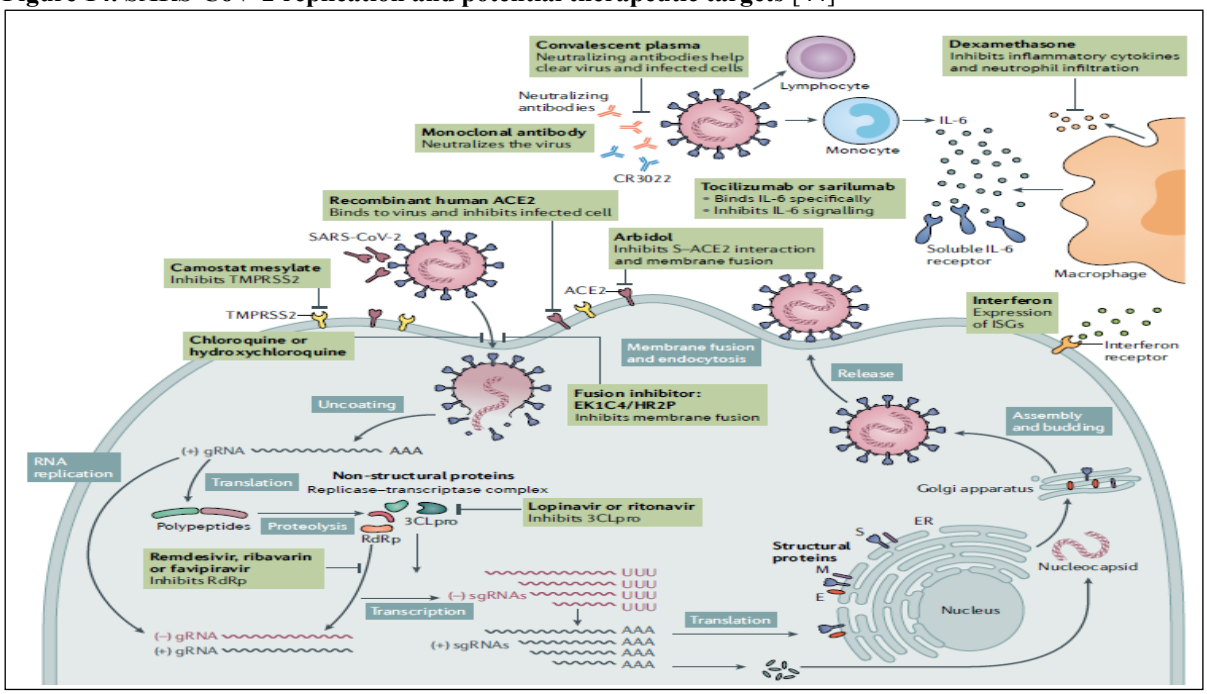
Regarding pharmacological treatment of COVID-19, currently there are no direct antiviral therapies against SARS-COV-2 of proven efficacy. Therefore, management is mainly based on supportive therapy to prevent disease progression, respiratory failure, and mortality. Since the beginning of the pandemic, several clinical trials of possible treatments for COVID-19 have been conducted or are ongoing, based on antiviral, anti-inflammatory and immunomodulatory drugs. Some of these agents have shown to have proven benefits in certain patients or a certain phase of the disease.
Based on a recent published review from *Xie* et al.[64], available guidelines on the management of COVID-19 are heterogeneous and only few recommendations are supported by strong evidence. Generally, COVID-19 management recommendations include respiratory support, acute respiratory distress syndrome management, and antiviral or immunomodulatory therapy. Regarding respiratory support, oxygen therapy is recommended when respiratory failure is present. Hypoxemia can be identified either analysing pO2 levels at arterial blood gas analysis or using SpO2 as a proxy for hypoxemia. The threshold levels when initiation of O2-therapy is recommended varies according to different guidelines; in fact, WHO, American Association for Respiratory Care (AARC) and Surviving Sepsis Campaign (SSC) guidelines use <90% as threshold while other guidelines have a threshold of <92% [65]. Oxygen therapy is generally administered through a nasal cannula, a face mask or non-invasive ventilation (continuous positive airway pressure helmet or full-face interface). Generally, patients respond positively to prone positioning [66]. If a sufficiently high arterial O2 level is not reached (ratio of arterial partial pressure of oxygen to fractional inspired oxygen ≤ 200 mmHg) admission to ICU for invasive mechanical ventilation and intubation is required. In mechanically ventilated adults with refractory hypoxemia despite optimizing ventilation and proning, extracorporeal membrane oxygenation (ECMO) is recommended by the majority of guidelines.

Regarding non-specific pharmacological therapies, anticoagulants are used as prophylaxis in order to avoid thrombotic complications at early stage of the disease while anticoagulation therapy is recommended when pulmonary thromboembolism is proven or suspected based on new worsening of respiratory state or increase in D-dimer. Antipyretics are widely used for temperature control and inflammation. The most recommend drug is paracetamol followed by nonsteroidal anti-inflammatory drugs (NSAIDs) and the choice should be guided by patient's comorbidities and clinical conditions.

Antibiotics have been extensively used, especially during the initial phases of the pandemic, in outpatient setting to prevent bacterial super-infections. This approach is not recommended since there is no evidence that antibiotic prophylaxis can prevent bacterial superinfections in COVID-19. Moreover, bacterial complications are more likely to happen during hospitalization rather than in community settings. Guidelines agree in recommending antibiotic therapy in patients who present reasonable evidence of bacterial infections. Antimicrobial stewardship principles should be always kept in mind in order to avoid inappropriate use of antibiotics which is proven to increase mortality and circulation of MDR bacteria [67].

As previously stated, there are no direct antiviral therapies against SARS-COV-2 of proven efficacy. Based on data so far available, dexamethasone and adjunctive tocilizumab or baricitinib may have a mortality benefit while some clinical improvements have been observed with remdesivir. Under investigation anti-COVID-19 drugs can be classified based on the mechanism of action. Therefore, anti-COVID drugs can be divided in 1) inhibitors of viral entry, 2) inhibitors of viral replication and 3) immunomodulatory agents (**figure 14**). While inhibitors of entry and replication are more likely to work early in the course of infection, immunomodulatory agents may have more impact later in the disease course.

Figure 14. SARS-CoV-2 replication and potential therapeutic targets [44]



Antivirals have been widely used during the initial phases of pandemic, when evidence was lacking, based on in vitro and in animal models and on previous experience with SARS and MERS outbreaks. Generally, they have been used during the initial phase of the disease in order to inhibit viral entry or replication. Some of these drugs have been totally abandoned since clinical studies have failed to prove efficacy; among them it can be mentioned hydroxychloroquine, in combination with azithromycin, and lopinavir/ritonavir. The first one is a drug commonly use for the of malaria, amoebiasis and some autoimmune diseases including Systemic Lupus Erythematosus (LES) and Rheumatoid Arthritis. The principle of the use of this drug in COVID-19 relies on his anti-inflammatory properties and proven in vitro efficacy in inhibiting viral entry. However, studies did not show any benefit in the treatment of COVID, instead an increased risk of adverse events was observed[68].

Replication inhibitors include remdesivir and lopinavir/ritonavir. Lopinavir/ritonavir are antiretroviral protease inhibitors used for the treatment of HIV infection; their efficacy of lopinavir/ritonavir against SARS-CoV has been proven[69] therefore they have been used for SARS-COV-2. However, efficacy has not been confirmed by clinical studies[70].

Remdesivir is a nucleotide analogue that has in vitro activity against SARS-CoV-2 [71] and it is currently recommended by some guidelines for hospitalized patients with severe COVID-19 who require low-flow oxygen because it may reduce time to recovery[72, 73]. The suggested adult dose is 200 mg intravenously on day 1 followed by 100 mg daily for 5 days total, with extension to 10 days if there is no clinical improvement. Clinical evidence on remdesivir is based mainly on two randomized control trials. The first, the WHO-sponsored SOLIDARITY

trial, did not show a reduction in mortality in patients treated with remdesivir[74]. On the other hand, the ACTT-1 RCT showed faster recovery time in patients treated with remdesivir as compared to standard of care (median 10 versus 15 days) [75].

Dexamethasone, a glucocorticoid, is recommended for severely ill COVID-19 patients who are on supplemental oxygen or ventilatory support. The recommended dosage is 6 mg daily for 10 days. Data on the basis of the recommendation on the use of steroids are robust and comes from randomized control trials. A recent meta-analysis of seven RCT showed a reduction in 28-day mortality in severe patients treated with dexamethasone versus placebo (32% versus 40%, OR 0.66, 95% CI 0.53-0.82) [76, 77]. In contrast, a benefit was not seen among patients who did not require either oxygen or ventilatory support. Moreover, glucocorticoids are well known for their toxicity therefore patients should be monitored for adverse effects such as hyperglycaemia and increased risk of bacterial and fungal infections.

Immunomodulator agents are drugs able to mitigate the immune response to infection and to block the “cytokine storm syndrome” responsible for the development of ARDS and shock in patients with severe COVID-19. Immunomodulators with proven efficacy for the treatment of COVID-19 are tocilizumab and baricitinib.

Tocilizumab is an interleukin-6 (IL-6) inhibitor that target the IL-6 pathway blocking inflammation and therefore disease progression[78]. Tocilizumab is used as a single intravenous dose of 8 mg/kg in association with dexamethasone. It is indicated in patients who require high-flow oxygen or more intensive respiratory support with laboratory evidence of inflammation. Tocilizumab was proven to reduce mortality in a recent meta-analysis of 27 randomized trials including more than 10,000 patients (odds ratio 0.83, 95% CI 0.74-0.92) [79].

Baricitinib is a Janus kinase (JAK) inhibitor used for treatment of rheumatoid arthritis. In addition to immunomodulatory effects, it is thought to have potential antiviral effects through interference with viral entry therefore is used for patients in any oxygen support. Data from two RCTs show that baricitinib use, in association with remdesivir, is effective in reducing time to recovery and mortality [80, 81].

Another class of therapy that may have a role in the management of COVID-19 is the antibody-based therapy which includes anti-SARS-CoV-2 monoclonal antibodies and convalescent plasma. A combination of anti-SARS-CoV-2 monoclonal antibodies, casirivimab and imdevimab, have been proven to have a mortality benefit and can be used out-patiently or in hospitalized patients if they are hospitalized for a reason other than COVID-19[82]. Convalescent plasma from individuals who have recovered from COVID-19 has been hypothesized to have clinical benefit for COVID-19 but clinical studies have failed to demonstrate any efficacy [83].

Bacterial co- and super-infections during COVID-19 pandemic

In the last year, more researchers have focused their efforts on the investigation of the relationship between bacterial infections and COVID-19.

Theoretically, COVID-19 patients are at increased risk of bacterial complications for several reasons. Firstly, SARS-CoV2 infection itself is associated with a dysregulation of both innate and acquired immunity [78]; moreover, some of the drugs, used for the treatment of COVID-19, as for instance tocilizumab or baricitinib, act by modulating the cytokine storm and the aberrant immune response to the infection while corticosteroid therapy is known to increase the risk of infections as adverse effect. Secondly, historically bacterial co- and super-infections, especially affecting the lungs, have been described during influenza [1] and other viral pandemics (including SARS [84] and MERS [85]). Thirdly, critically ill COVID-19 patients will require during their hospitalization admission to ICU endotracheal intubation and invasive mechanical ventilation. Patients in ICU are at greater risk of nosocomial infections related to the use of ventilators or intravascular devices in a setting where MDR circulation is higher as compared to other units.

Yet, despite these premises, studies so far have demonstrated that the incidence of community- and hospital-acquired bacterial infections in COVID-19 seems to be lower as expected and compared to other viral diseases. Nevertheless, antibiotics have been extensively used for the management of COVID-19 patients.

Bacterial infections during influenza and other viral infections

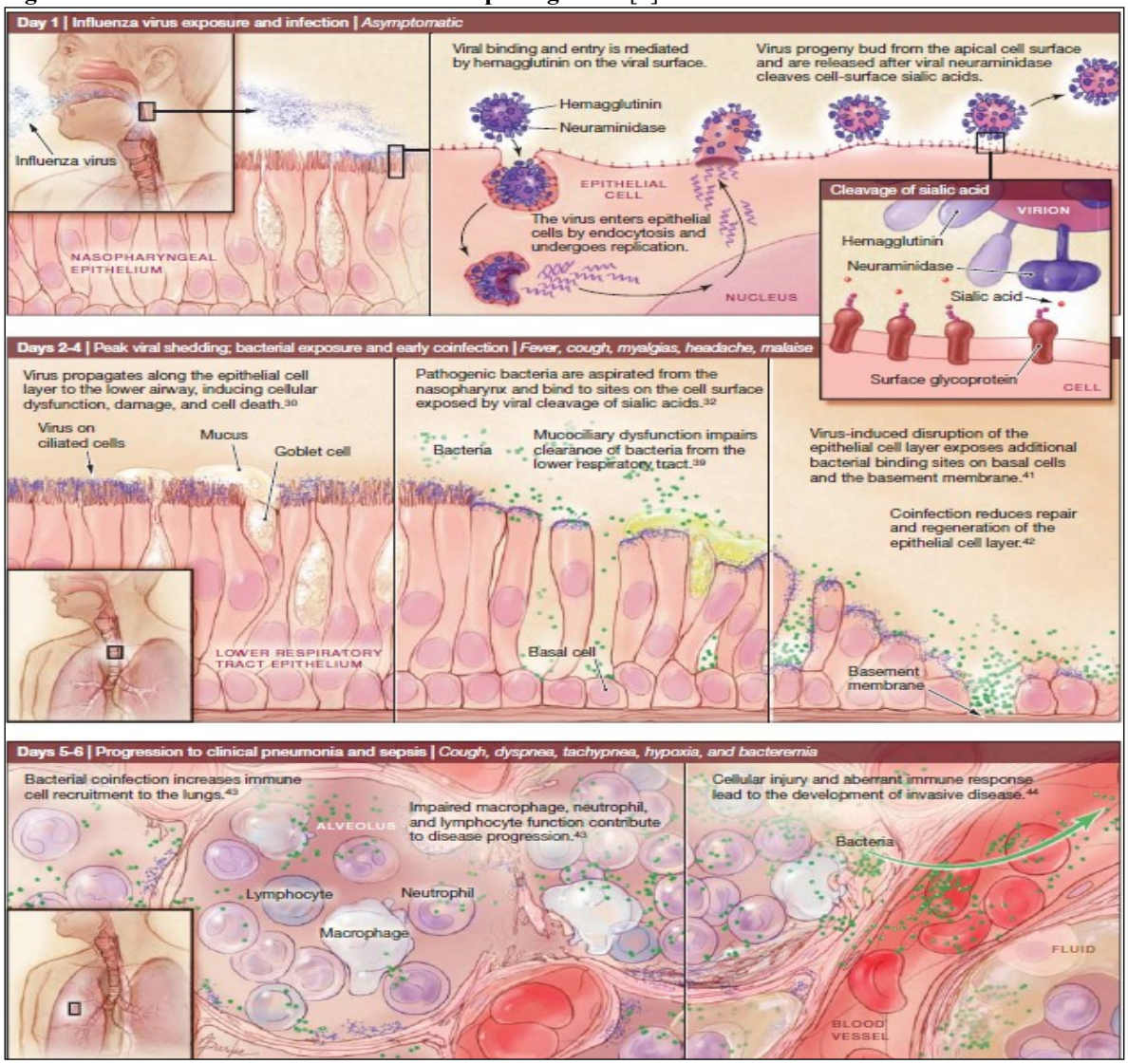
During 2009 pandemic H1N1 influenza, the incidence of bacterial infections was 18% and 34% and was associated with increased duration of mechanical ventilation, prolonged ICU stay and increased mortality. Even higher incidence of bacterial co-infections was recorded, based on autopsy studies, during 1918 pandemic influenza[2].

Generally, the pathogens most commonly isolated from respiratory cultures are *Staphylococcus aureus*, *Pseudomonas species*, *Streptococcus pneumoniae* and *Haemophilus influenzae*.

Viral, bacterial, and host factors contribute to the pathogenesis of coinfection. In fact, the entry of influenza virus into respiratory airway causes dysfunction and death of epithelial cells contributing to increased bacterial adhesion; Influenza produces neuraminidases which increases adhesion of some bacterial species by removing sialic acid to expose host cell receptors. The upper respiratory tract has been shown to host a diverse microbiota, within which a number of bacterial species may be found. Colonization of the nasopharynx with pathogenic bacteria may predispose to coinfection. Migration of the virus along the respiratory tree impairs

clearance of bacteria from the lower respiratory tract. Inflammation causes further tissue damage, revealing more attachment sites for bacteria. Therefore, bacteria replicate and invade the underlying tissues causing invasive infections (**figure 15**).

Figure 15. Model Influenza and Bacterial Copathogenesis [2]



Nowadays, bacterial super-infections are a major concern in critically ill patients with influenza admitted to ICU. In this population, in-hospital mortality is around 30%. In cases where the infection is acquired in nosocomial environment MDR play an important role; the most common MDR pathogens responsible for VAP in ICU are methicillin-resistant *S. aureus* (MRSA), *Enterobacterales* ESBL-producers and carbapenem-resistant and non-fermenting gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter spp.* Risk factors for bacterial complications during flu include extreme ages, obesity and comorbidities such as

chronic pulmonary, cardiovascular, renal, hepatic and neurological diseases and immunosuppression.

In the setting of influenza, especially in critical ill patients, antibiotics are crucial to reduce the impact on mortality of secondary bacterial infections. However, the worldwide threat of increasing antibiotic resistance imposes a judicious use of these molecules. Therefore, vaccination against flu becomes essential in the fight against influenza and associated bacterial infections.

Incidence and aetiology of bacterial infections in COVID-19

As previously stated, incidence of bacterial co- and super-infections in COVID-19 is lower than expected and observed for influenza, SARS, and MERS.

Before proceeding, a distinction has to be made between co-infections and super-infections. In fact, the term co-infection refers to infections onset in the community while super-infection, or secondary infection, refers to infections that onset in hospital setting and diagnosed after 48 hours from hospital admission. As it will be discussed later, these two kinds of infections have different incidence, risk factors and impact on clinical outcomes and are caused by different pathogens with different susceptibility to antibiotics.

From the beginning of COVID-19, several studies have been published on this topic. Regarding co-infections, the most robust data come from two meta-analyses. The first one, from Lansbury et al, includes 30 studies of which only one RCT and overall includes 3834 patients [8]. Aim of the review was to review the prevalence of bacterial co-infections in COVID-19. Overall, only 7% of hospitalised COVID-19 patients had a bacterial co-infection. The most commonly reported bacterial

pathogen was *Mycoplasma pneumoniae*, followed by *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Klebsiella pneumoniae*.

The second meta-analysis was conducted by Langford et al. and included 24 retrospective studies for a total of 3506 patients [7]. In this study, the prevalence of both co- and super-infections were evaluated. The global prevalence of bacterial infections was 6.9%; among them prevalence of co- and superinfections was 3.5% and 14.3% respectively. Even in this study, the most common organisms causing co-infections were *Mycoplasma species*, *Haemophilus influenzae* and *Pseudomonas aeruginosa.*

Findings from prospective and retrospective studies are concordant whit this prevalence [9, 86–88].

In a Spanish cohort of COVID-19 patients, described by Garcia-Vidal et al., community-acquired infections were observed in 3.1% of patients whit the most common pathogens being *Streptococcus pneumoniae* and *Staphylococcus aureus* including 2 MRSA [9]. Some incidence and pathogens were reported in UK by Hughes et al.[87].

A recent prospective cohort study from Russel et al. have included more than 48,000 patients from 260 hospitals in England, Scotland, and Wales with the aim of describing microbiologically confirmed co-infections and secondary infections, and antimicrobial use, in patients admitted to hospital with COVID-19 [89]. Among 48 902 patients included in the study only 1107 patients had a microbiologically confirmed infection, the majority of them were

super-infections. *S. aureus* and *H. influenzae* were the most common pathogens causing respiratory co-infections.

Hospital-acquired infections, as compared to community infections, are generally associated with worse clinical outcomes, and usually are sustained by MDR bacteria. The greater mortality is driven, among other factors, by the underlining conditions of patients that are generally more frailty. Circulation of MDR is sustained by the extensive use of antibiotics and by the lack of infection control measures and antimicrobial stewardship programs. Recognized risk factors for super-infections are: use of vascular devices and bladder catheters, orotracheal intubation, parental nutrition, and admission to ICU. The most common and serious super-infections are bloodstream infections (BSIs) and ventilator-associated pneumonia (VAPs). In patients hospitalized for COVID-19 the clinical suspicion of a bacterial superinfection arises in the presence of:

- new onset of fever with chills;
- hypotension;
- Worsening of respiratory exchange after initial improvement;
- dysuria;
- signs of infection at CVC exit-site.

Laboratory signs includes Neutrophilic leucocytosis and elevation of procalcitonin and CRP levels. From a radiological point of view, new lung consolidations may be indicative of bacterial pneumonia. Collection of blood, urine, and respiratory samples to perform cultures are essential for diagnosis and to drive antibiotic therapy.

In COVID-19, several studies have shown that the incidence of super-infections is higher than co-infections and it is even higher for critically ill patients in ICU. In the study from Garcia-Vidal et al. the incidence of super-infections was 4.7%, of which 56.8% in ICU. The most common hospital-acquired superinfections were pneumonia and bacteraemia. The infections are mainly due to Pseudomonas spp. and Escherichia coli; the mean time from hospital admission to superinfection diagnosis was 10.6 days [9]. In the study from Langford et al, bacterial super-infections were detected in 14.3% of patients [7].

The most common and fearsome bacterial complications of COVID-19 are VAP and BSI. The presence of ARDS, duration of mechanical ventilation and the ICU lengths of stay are the main risk factors for VAP. A study conducted in UK showed that COVID-19 patients were significantly more likely to develop VAP than patients without COVID-19 [90]. Another important multicenter trial from the COVID-ICU Group of the REVA Network was conducted in several European countries and included more than 4000 critically ill COVID-19 patients; the incidence of VAP was 58% [91]. In both cohorts, the most common bacteria were Gram-

negative bacilli, mainly *P. aeruginosa* and *Enterobacterales* spp., followed by Gram-positive cocci, mainly *S. aureus*.

The second most common secondary infections in COVID-19 patients are BSIs. BSI is defined by the presence of symptoms and signs of infection plus positive blood cultures. Giacobbe et al. recently described the incidence of BSI in an Italian cohort of COVID-19 patients in ICU. The incidence was 40% and the cumulative risk of developing an episode of BSI was of nearly 25% after 15 days of ICU stay. BSIs were mainly due to Coagulase-negative staphylococci, *E. faecalis* and *S. aureus* [12]. Ripa et. al., in a recent study settled in ICU and non-ICU wards, found an overall incidence of secondary infections of 9%. 85 episodes were BSI, 70% of which due to Coagulase-negative staphylococci, and 32 were lower respiratory tract infections, 30% of which sustained by *Acinetobacter baumannii* [86].

Risk factors for the development of bacterial infection in COVID-19

Risk factors for community-acquired infections and hospital-acquired infections are different. Moreover, risk factors can be divided in COVID-19 non-specific factors, that are common also for bacterial infections in general population, and COVID-19 specific factors. The latter have been the main objective of several studies since the beginning of COVID-19 pandemic.

Regarding community acquired infections, recognized COVID-19 non-specific risk factors include age, smoking, malnutrition, previous infections, chronic bronchitis/chronic obstructive pulmonary disease, poor dental health, immunosuppressive therapy, oral steroids, dialysis, diabetes, and transplant patients [92]. In addition, a long course of SARS-CoV-2 infection treated out-patiently, and the use of steroids have been recognized to be a risk factor for a concomitant bacterial infection especially involving the respiratory tract.

Among COVID-19 specific risk factors for the development of BSI data are scarce, especially in non-ICU settings. The role of immunomodulatory agents and corticosteroids on the adjunctive risk of BSI is not clear. Regarding immunomodulators, especially Tocilizumab, Anakinra and Baricitinib, it seems reasonable that these agents targeting the dysregulated immune response to the SASR-CoV-2 infection may interfere as well with the immune response to bacterial infections. Data so far available are conflicting. In fact, while an increased risk of secondary infections with the use of these agents was observed in a recent systematic review and meta-analysis [93] and in a RCT [94], two other RCTs [95, 96] did not showed an increased risk.

In a recent published Spanish multicenter study from Abelenda-Alonso et. al, risk factors for the development of BSI in COVID-19 patients were analysed. At multivariate analysis, neither use of steroids or Tocilizumab resulted associated with an increased risk of BSI while the only risk factor recognized was D-dimer > 700 ug/L [97]. Buetti et al., in a case control study comparing BSIs in COVID-19 and non-COVID 19 patients in ICU in France, described a significant increase in the risk in patients treated with tocilizumab or anakinra [98].

Concerning steroids, similarly to what observed for immunomodulatory therapy, data are discordant. Giacobbe et al. recently described, in an Italian cohort of COVID patients in ICU, an increased risk of developing ICU-acquired BSI in patients receiving anti-inflammatory therapy (tociluzumab, methylprednisolone or methylprednisolone plus tocilizumab) [12]. Conversely, in another Italian cohort of COVID patients in ICU and non-ICU wards, steroids use did not result associated with the development of BSI. In this study factors associated with an increased risk of secondary infections resulted to be severe hypoxaemia, severe lymphopenia, and ICU admission [86].

Impact on clinical outcomes

In general, hospital-acquired infections are known to have a negative impact on the clinical outcomes of patients admitted to the hospital for other reasons. Similarly, data so far available showed that co- and super-infections in COVID-19 patients negatively influence patients outcome increasing length of hospital stay, and mortality in non-ICU patients and prolonging the duration of ventilation in ICU patients. As expected, mortality is higher in patients with super-infections as compared to co-infections and in patients in ICU as compared in patients in non-ICU Units.

In regard to community-acquired infections, in the Spanish cohort from Garcia-Vidal et. mortality in patients with co- and super-infections was 16.1% and 18.6% respectively compared to 9.4% in patients without infections. In addition, patients with co-infections were more likely be admitted to the ICU and had an increased length of stay [9]. In the meta-analysis from Lansbury, COVID-19 patients with a co-infection were more likely to die than patients who did not have a co-infection [8]. In the study from Hughes et al. bloodstream infections increase the risk of in-hospital death while a similar finding was not observed for respiratory tract infections [87]. In contrast to other studies, in the study from Russel et al. which includes more than 48,000 COVID-19 critical patients in UK, co-infection and super-infection were not associated with inpatient mortality [89].

Concerning hospital-acquired infections, available data are more robust. In the Spanish cohort of 2,000 patients from Abelenda et. al the development of BSI was associated with a higher in-hospital mortality risk [97]. A recent study conducted in Israel, aimed to define the impact of secondary bloodstream and respiratory tracts infections on the clinical course and mortality in COVID-19 patients as compared to influenza patients, has found that COVID-19 patients had more secondary infections than influenza patients, and these infections were associated with death in COVID-19 [99].

Besides BSIs, the impact of VAPs has been evaluated by several studies. In particular, authors are concordant in the observation of an increment in the duration of ventilation and in ICU stay in COVID-19 patients with VAP as compared to patients without [100, 101].

Antibiotic consumption and antimicrobial stewardship

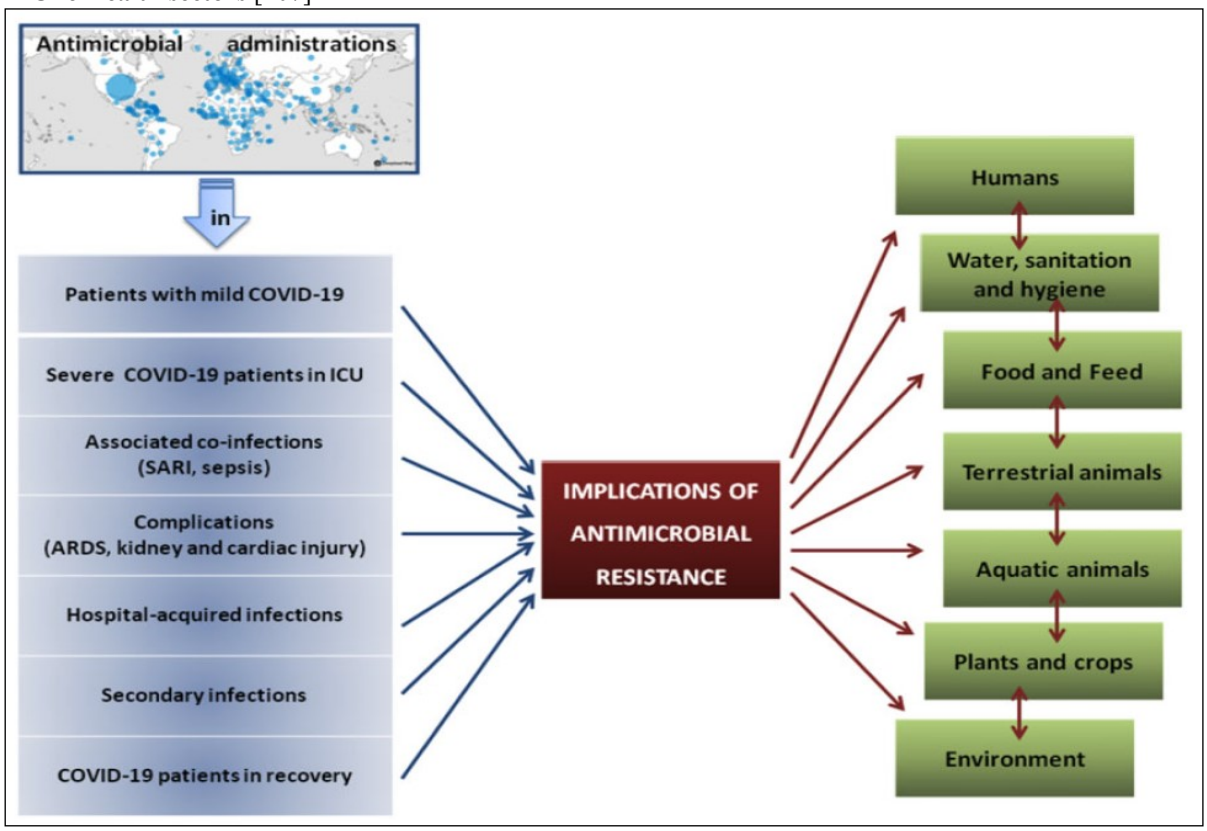
Worldwide, antibiotics for the management of COVID-19 patients have been extensively used in the hospital setting while it seems decreased in the community setting [102]. Moreover, in a large percentage of cases broad-spectrum antibiotics have been used empirically without collection of microbiological samples and therefore without the chance to be de-escalated during the treatment course. Some antibiotics, such as azithromycin and teicoplanin, have been also investigated for the treatments of COVID-19 due to supposed antiviral or anti-inflammatory activity but evidence failed to prove any *in vivo* efficacy [103, 104].

Even though antibiotic consumption was higher during the initial phase of the pandemic, it is still unacceptably high considering the available data show that the incidence of bacterial co- and super-infections is lower than expected and observed for other viral diseases. In fact, as previously stated, bacterial infections complicate only the 10% of COVID-19 cases.

Unintended consequences of antimicrobial overuse are, among others, the increment of bacterial resistance and circulation of MDR, incidence of *Clostridioides difficile* infection, and adverse events such as hypersensitivity and renal impairment.

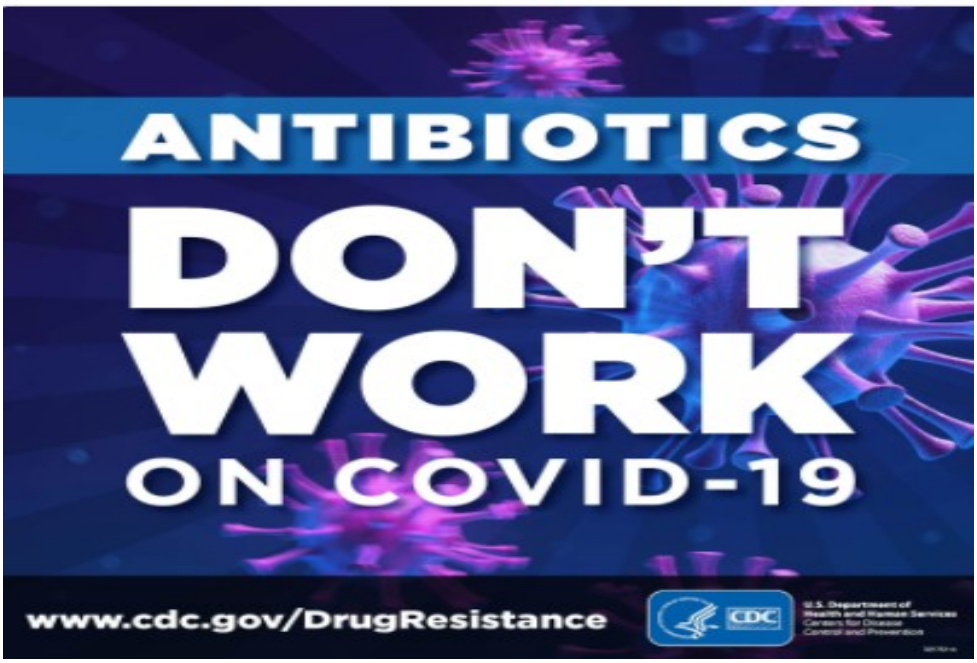
Antimicrobial resistance (AMR) still represents a major threat for public health since it currently causes 700, 000 deaths worldwide yearly and this number is estimated to increase in the very next future [3]. On the individual level, antimicrobial resistance limits the list of effective therapeutical agents to treat infections, increasing therefore the patients risk of receiving an inappropriate empirical antibiotic therapy. Inappropriate empirical antibiotic therapy is associated with an increased mortality in patients with serious bacterial infection [105, 106]. At population level, AMR have serious repercussions not only for human health but also for animal health and for the global economy. As underlined by the WHO, prudent antimicrobial use and comprehensive infection prevention and control strategies targeting all healthcare sectors ("One Health approach") are the cornerstones of effective interventions aiming to prevent selection and transmission of bacteria resistant to antimicrobial agents. Therefore, unless urgent measures will be taken, it seems reasonable to expect that antimicrobial overuse and misuse in COVID-19 pandemic will have negative consequences for all the healthcare and economic sectors in the future (**figure 16**).

Figure 16. Administration of antimicrobials during the COVID-19 pandemic and the implications for AMR in One Health sectors [107]



CDC has recently published a report in which advocated as COVID-19 represents "the perfect storm" for healthcare-associated infections and antimicrobial resistance due to increased length of stay, increased number of patients, staffing shortages, sick patients, antibiotic use, challenges implementing infection prevention and control and interruption of antimicrobial stewardship programs[108]. Therefore, CDC is implementing several measures to fight against antibiotic resistance during the ongoing pandemic. These measures include investments in infection prevention and control programs, implementation of COVID-19 testing and identification antibiotic-resistant outbreaks through CDC's Antibiotic Resistance Laboratory Network, and informative campaigns for the wise use of antibiotics for the population (**figure 17**).

Figure 17. CDC campaign against the misuse of antibiotics in COVID-19 patients



In regard to antibiotic consumption in COVID-19, several studies have been published. In the study from Russel et al., which includes 49,000 COVID-19 patients from 260 hospitals in UK, 37% and 85% of patients received antibiotics respectively before and during hospitalization despite only 1,000 of them had a microbiologically confirmed bacterial infection [89].
In the meta-analysis from Langford et al., only 7% of the patients had a bacterial infection but 72% of patients received antibiotics[7].
Of great interest in the topic is a recent review from Chedid et al. Authors aimed to evaluate and describe the available data in the literature regarding frequency, indications, and efficacy of antibiotics in COVID-19 patients [109]. In the study 19 studies reporting data from 2834 patients were included. Antibiotics were used in 74% of cases despite proven infection was documented only in 17% of patients. Regarding antibiotic classes, only four studies reported detailed data. Among them, fluoroquinolones were the most prescribed antibiotics followed by Ceftriaxone and azithromycin.

In order to fight inappropriate use of antimicrobials in COVID-19 patients, infection precaution and control measures should be implemented. These measures should be aimed not exclusively at limiting SARS-CoV-2 in hospital transmission but also to avoid horizontal transmission of MDR, avoiding nosocomial bacterial infections. In addition, antimicrobial stewardship (AMS) programs should not be interrupted or should be implemented where not already introduced.

AMS programs can be defined, based on NICE definition, as “organizational or healthcare system-wide approach to promoting and monitoring judicious use of antimicrobials to preserve their future effectiveness” [110].

As advocated by many infectious diseases experts, AMS should be implemented also during the COVID-19 pandemic. In order to assess the best AMS program, some research gaps have to be filled. In particular, as suggested by Huttner et al. in a recent commentary on AMS and COVID [67], it has be established (i) the exact incidence of bacterial co-infection and superinfection at the different phases of the disease, (ii) the diagnostic performance of biomarkers to identify a bacterial superinfection, (iii) the impact of the COVID pandemic on antibiotic use and antimicrobial resistance in community and hospital settings. Authors of the paper have provided some recommendations to limit to inappropriate use of antibiotics in SARS-CoV2 infected patients. Among others, they suggest reserving antibiotics for patients with severe disease, to obtain microbiological tests before the prescription of antibiotics, to re-evaluate therapy every day and stop when bacterial infection has been ruled out, to limit the use of broad-spectrum antibiotics and the duration of the treatment course. In addition, the use of azithromycin for the treatment of COVID should be discouraged based on available evidence.

In conclusion, even during this challenging time of pandemic, antibiotic stewardship principles should not be forgotten and should be continued to be applied and promoted.

STUDY 1: EPIDEMIOLOGY, RISK FACTORS AND IMPACT ON MORTALITY OF HOSPITAL-ACQUIRED BLOODSTREAM INFECTIONS IN COVID-19 PATIENTS

Rationale and aims of the study

Hospital-acquired infections by definition are infections not present at hospital admission. Among all the nosocomial infections, hospital-acquired bloodstream infection is the most fearsome complication. In fact, it is known to increase the length of stay, costs, and mortality. Appropriate of initial antibiotic therapy is one of the main protective factors for survival. However, the overuse of antibiotics and the lack of effective infection control and antimicrobial stewardship programs have led to increased rates of MDR bacteria circulation and, as consequence, to higher rates of inappropriate empirical antibiotic therapy and mortality [111].
Coronavirus disease 2019 (COVID-19) is an interstitial pneumonia caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which might lead to acute respiratory failure, multiorgan failure, and death. The already complicated course of the disease, characterized by high rates of ICU admission and mortality, can be further complicated by the onset of a bacterial infection including VAP and BSI. Moreover, an increment in the circulation of MDR during COVID-19 has been already described.
The unexpected diffusion and virulence of COVID-19, alongside the fear of bacterial super-infections in patients hospitalized for COVID-19 pneumonia and the disruption of antimicrobial stewardship programs, has led to a large use of antibiotics worldwide probably contributing in the next years to a further spreading of multidrug resistant bacteria.
However, contrarily to what observed during influenza, SARS and MERS pandemics, available data shows that the incidence of bacterial superinfection in COVID-19 is low. Therefore, the extensive use of empirical and directed broad-spectrum antibiotics is no longer justified.

While the incidence of bloodstream infections (BSIs) and their impact on patients outcome has been extensively described in the intensive care unit (ICU) setting, data on the epidemiology of BSIs in non-critical wards are scarce. Moreover, risk factors for the development of BSIs during hospitalization for COVID-19 have not been clearly identified yet and findings from available studies are discordant.

Recently our research team published a study on the incidence and risk factors for BSIs in COVID-19 patients[112]. In the study, carried out at San Paolo Hospital in Milan from 24 February to 30 November 2020, 1,351 COVID-19 patients were included; of which 51 patients had an hospital-acquired BSI (HA-BSI) with an incidence of 3.3/1000 patient-days. Corticosteroids treatment during the course of the hospitalization resulted associated with an increased risk of developing an HA-BSI (aOR 2.11, 95% CI 1.06–4.19, p = 0.032). The study design was not adequate to evaluate the impact of BSI on clinical outcomes.

The present study represents a prosecution of the previous one, in which we have enlarged our cohort by increasing the study period and in which we have conducted a nested study in order to evaluate the impact of BSI on mortality.

Hence, the aim of the study is to evaluate the burden and epidemiology of hospital-acquired bloodstream infections in patients admitted to San Paolo Hospital in Milan for COVID-19 pneumonia, exploring in particular risk factors associated with HA-BSI and impact on mortality and length of hospital stay.

These data may fill a current gap of knowledge on this topic and therefore be crucial to recognize bacterial super-infections in COVID-19, to start an appropriate antibiotic treatment and eventually improve patients survival.

Material and methods

Design and objectives of the study

Prospective observational cohort study conducted at San Paolo Hospital in Milan Italy. All patients admitted to hospital for symptomatic SARS-CoV-2 infection from 24 February 2020 to 31 March 2021 were included in the study. The main objectives of the study were: (i) description of microbiological and clinical characteristics of HA-BSIs; (ii) assessment of the incidence and prevalence of HA-BSIs; (iii) evaluation of risk factors for the development of HA-BSIs; (iv) evaluation of the impact of HA-BSIs on length of stay and in-hospital mortality.

Setting of the study, inclusion/exclusion criteria

San Paolo Hospital is a university hospital located in Milan, Northern Italy. The hospital has 426 beds and about 20,000 admissions/year; there are Infectious Diseases, Pneumology and Intensive Care Departments while cardiac and neurosurgery Department are missing; in our institution, solid organ and bone marrow transplants are not performed.

As of February 2020, with the increase in cases of COVID-19 in Italy, several departments have been converted into COVID departments, including the Intensive Care, and multidisciplinary teams have been created formed by specialists in Infectious Diseases, Internal Medicine, Pneumology and Resuscitation to face the current health emergency. Distribution of COVID and non-COVID Units is constantly changing within the hospital according to the progress of COVID-19 pandemic.

Inclusion criteria of this study were: (i) confirmed diagnosis of symptomatic SARS-CoV-2 infection by real-time reverse transcription polymerase chain reaction (RT-PCR) on nasopharyngeal or oropharyngeal or broncho-alveolar swab specimens; (ii) age>18 years; (iii) hospitalisation at San Paolo Hospital from 24 February 2020 to 31 March 2021.

Exclusion criteria: age < 18 years; death or discharge from the emergency room within 24 hours from the admission.

Definitions and data collection

Diagnosis of COVID-19 was performed on the basis of a clinical and radiological conditions suggestive for COVID-19 plus a positive RT-PCR for SARS-CoV-2 performed on nasopharyngeal or oropharyngeal or broncho-alveolar swab specimens.

COVID-19 disease severity on admission was classified as: mild (no radiological or clinical evidence of pneumonia); moderate (radiological evidence of pneumonia; PaO2/FiO2 > 300 mmHg; respiratory rate (RR) ≥ 24/min; SO2 ≥ 92% in room air); severe (radiological evidence of pneumonia; PaO2/FiO2 100–300 mmHg; RR < 24/min; SO2 < 92%); critical (radiological evidence of pneumonia and PaO2/FiO2 < 100 mmHg).
COVID-19 disease severity during hospitalisation was defined by the highest level of respiratory support required and was classified as:
- no need for oxygen-therapy;
- low/high flow supplemental oxygen with nasal cannula, face mask, venturi mask or non-rebreather mask (with a flow of up to 15 L/min);
- continuous positive airway pressure (CPAP) via a helmet device;
- non-invasive mechanical ventilation (NIMV), mainly bi-level positive airway pressure (BiPAP) via facemask;
- invasive mechanical ventilation (IMV).

Diagnosis of BSI was performed on the basis of clinical findings suggestive for infection plus evidence of bacterial growth from a single blood culture. In case of coagulase-negative *Staphylococcus spp.* (CONS) or other skin flora commensals (for instance, *Actinomyces spp*., *Aerococcus spp*., *Bacillus spp*., *Corynebacterium spp*., *Cutibacterium acnes*, *Micrococcus spp*., *Propionibacterium spp*, and *Rhodococcus spp)*, at least two positive blood cultures for the same bacterial species, or a single blood culture from a central venous catheter (CVC) plus additional blood cultures from a peripheral vein, in symptomatic patients were needed to define BSI[113]. Polymicrobial BSIs, defined as the growth of at least two species from the same blood culture, were considered as a single clinical episode. Hospital-acquired bloodstream infections (HA-BSIs) were defined as infections arising at least 48 h after hospital admission [114].

Data were collected and entered in a dedicated electronic database (RedCap); Electronic medical records were reviewed to include the following variables: age, sex, ethnicity, comorbidities (obesity, hypertension, diabetes, cardiovascular diseases, cerebrovascular diseases, chronic obstructive pulmonary diseases (COPD), chronic liver diseases, solid or haematological malignancies, chronic kidney diseases, HIV infection, rheumatic diseases), age-unadjusted Charlson comorbidity index [115], risk factors for SARS-CoV-2 infection (close contact/household, healthcare worker, hospitalisation last 30 days/ long-term care facility residency, unknown/other), calendar period of hospital admission, symptoms and signs at presentation and during disease course, laboratory findings, radiological findings, PiO2/FiO2

ratio on admission, severity of COVID-19 on admission and during hospitalization. Administered COVID-19 treatments, including remdesivir or other antivirals, immunomodulatory agents (tocilizumab, sarilumab, ruxolitinib, baricitinib) and high-dose corticosteroids (dexamethasone and methylprednisolone), were also collected. For BSI episodes the following data were collected: pathogen and antimicrobial resistance profile, origin of sepsis (respiratory, urinary, CVC, intra-abdominal, other, unknown), signs and symptoms at onset (fever, hypotension), laboratory data (White Blood Cell Count, Neutrophils, C-Reactive Protein, Procalcitonin), days from hospitalization to the onset of BSI, assessment of the appropriateness of empirical and targeted antibiotic therapy and number of BSI with onset in ICU. Data on microbiological investigations conducted at onset or during hospital stay were collected; these included blood cultures, respiratory cultures (sputum/bronchoalveolar aspirate/bronchoalveolar lavage), urine cultures, pneumococcal and legionella urinary antigen test, PCR for influenza, serology for atypical pulmonary pathogens, multi-drug resistant bacteria (MDR) colonisation. Concerning clinical outcomes were collected data on length of hospital stay, ICU admission and in-hospital mortality.

Identification of microorganisms and susceptibility test were performed using standard microbiologic procedures (BACT/ALERT VIRTUO BioMerieux, as blood culture detection system, VITEK 2 BioMeriux automated system to perform antibiotic susceptibility, MALDI-TOF-MS BioMeriux for microbial identification, STANDARD F analyzer SD Biosensor used to perform qualitative analysis by detecting Legionella pneumophila and Streptococcus pneumoniae antigens in the urine samples and, GeneXpert a real-time RT-PCR-based assay for the detection and differentiation of influenza A and B viral RNA, LIAISON Diasorin for quantitative serology tests).

Statistical analysis

Continuous variables are presented as median and interquartile ranges (IQR), categorical variables are presented as frequency and percentage. Chi-square and Kruskal–Wallis test were used when appropriate to compare characteristics of patients who had HA-BSI and those who did not.

The incidence rate of HA-BSI was estimated as the numbers of HA-BSI episodes over 1000 patients-day of hospitalization with 95% confidence interval calculated using a Poisson distribution. Factors associated with the development of HA-BSI were analysed using an

unadjusted and adjusted logistic regression model. Covariates included in the model were chosen a priori based on variables described in literature.

The mortality of patients with and without HA-BSI was evaluated by an unadjusted and adjusted logistic regression model. To better understand the impact on mortality of the incident HA-BSIs during a hospitalization for COVID-19 we also performed a nested study in the cohort: all the COVID-19 subjects with an HA-BSI during hospitalization has been included, in case of multiple BSI per patients, the date of the first event has been considered in the analysis. This group represents the exposed group of interest (cases). A group of non HA-BSI (unexposed/controls) was randomly selected among subjects of the study if after the same duration from admission (index date), were free from HA-BSI. Not exposed were further matched for age (over or under 70 years), gender and ICU admission at index date. A 1:1 matching of exposed and unexposed to HA-BSI, was used for this sub-study. In-hospital mortality and hospital discharge have been evaluated by competing risks analysis, using cumulative incidence function (CIF).

In this study, discharge from hospital after recovery is considered a competing risk event, which precludes the occurrence of the events of interest (hospital death), whereas in standard survival analyses (i.e., Kaplan Meier curves), patients who recover are right-censored. This censoring violates the assumption of non-informative censoring, as the recovered and discharge patients are not representative of those who are still admitted to the hospital in terms of their risk of dying. Censoring patients induce bias and overestimating the incidence of death. *Vice versa* in-hospital death has been used as competing event for the hospital discharge event

Using the competing risk analysis, probabilities of interest are the cumulative incidence functions (CIF) for each event type. CIF estimate the probability of occurring a specific event in a given time, allowing for the possibility of occurring for other events. Pepe-Mori test has been used to compare equality of CIFs across subgroups (HA-BSI vs non HA-BSI). A proportional sub-distribution hazard (SHR) model by Fine and Gray has been fitted to estimate the effect of having a HA-BSI (first incident HA-BSI event) on-hospital death and hospital-discharge. The follow up in the survival analysis accrued from the date of HA-BSI in the exposed group, or index date for the matched non-exposed group to the date of discharge or death. The following confounders has been included in the Fine-Gray model: intensity of respiratory support at index date as proxy of COVID-19 severity, use of corticosteroids, age-unadjusted Charlson Comorbidity Index, and age as continuous variable.

A p-value < 0.05 was considered as statistically significant. All analyses were per-formed using Stata (v14, StataCorp, TX, USA).

Ethical consideration and funding

The study was approved by the Ethic Committee Area 1, Milan (2020/ST/049 and 2020/ST/049_BIS, 3 November 2020) and was conducted according to the guidelines of the Declaration of Helsinki. All patients gave informed consent for the use of their anonymised data for research purposes.

No fundings was received for the study.

Results

From 24 February 2020 to 31 March 2021, a total of 1,950 consecutive hospitalized SARS-CoV-2 infected patients were included in the study. A total of 129 patients had a concomitant or a subsequent BSI (6.6%) during the hospitalization. In 28 patients (1.4%) BSI was already present at hospital admission (community-acquired BSI (CA-BSI)) while 101 patients (5.2%) developed a BSI during the hospital stay (hospital-acquired BSI (HA-BSI)). A total of 151 episodes of BSIs was observed, 30 (19.1%) CA-BSI and 121 (80.1%) HA-BSI.
Overall prevalence of BSI 6.6% (95%CI 5.6-7.8), while the prevalence of HA-BSI was 5.2% (95%CI 4.2-6.3). The incidence rate of HA-BSI was 3.5/1000patient-days (95%CI 2.3–4.3).
92 microorganisms isolated from blood cultures, coagulase-negative *staphylococcus spp*. or other skin commensals, were considered as contaminants and excluded from the study.

Demographic and clinical characteristics of the population

Of the 1,950 COVID-19 patients included in the study, 32% (621/1,950) were hospitalized between February and August 2020 and 68% (1,329/1,950) between September 2020 and March 2021.
Demographic and baseline characteristics of the patients are described in **table 1**.
Median age was 70 year (IQR 56-81) and 61.5% (1,200/1,950) of the patients were male. The most frequent comorbidities recorded were hypertension (48.7%), cardiovascular diseases (29.5%), diabetes (20.7%) and COPD (12.6%). The median Age unadjusted Charlson score was 1 (0-2).
In the majority of patients, risk factor for SARS-CoV-2 infection was unknown. Among patients with recognized risk factors, 18.4% were recently hospitalized or had residency in long-term care facilities, 7.3% were household o close contact of other SARS-COV-2 infected patients and only 3% were healthcare workers.

Comparing patients with and without HA-BSI, no differences were observed in demographic and baseline characteristics. Patients with HA-BSI were slightly older (median age 74 years) but with a similar distribution of comorbidities. More patients in this group had, as known risk factor for SARS-CoV-2 infection the recent hospitalization/long-term care facility residency (HA-BSI: 33.7%, non-BSI: vs 17.7%).

Table 1. Demographics and clinical characteristics of 1,950 patients hospitalised with COVID-19

	Patients without HA-BSI n= 1,849 (94.8%)	Patients with HA-BSI n= 101 (5.8%)	P value	Overall n= 1,950
Gender, Male, n (%)	1,130 (61.1%)	70 (69.3%)	0.099	1,200 (61.5%)
Age, years, median (IQR)	70 (55-81)	74 (60-81)	0.094	70 (56-81)
Age strata, years, n (%)			0.120	
18-49	285 (15.4%)	8 (7.9%)		293 (15.0%)
50-69	626 (33.9%)	38 (37.6%)		664 (34.1%)
>70	938 (50.7%)	55 (54.5%)		993 (50.1%)
Ethnicity, n (%)			0.603	
Caucasian	1,539 (83.6%)	89 (88.2%)		1,628 (83.9%)
Latin/Hispanic	119 (6.5%)	6 (5.9%)		125 (6.4%)
Black	12 (0.7%)	1 (1.0%)		13 (0.6%)
Asian	72 (3.9%)	3 (3.0%)		75 (3.9%)
Other	98 (5.3%)	2 (1.9%)		100 (5.2%)
Age unadjus. Charlson score, median (IQR)	1 (0-2)	1 (0-2)	0.013	1 (0-2)
Comorbidities, n (%)				
Hypertension	895 (48.4%)	54 (53.5%)	0.322	949 (48.7%)
Diabetes	375 (20.3%)	28 (27.7%)	0.072	403 (20.7%)
Cardiovascular diseases	538 (29.1%)	37 (36.6%)	0.106	575 (29.5%)
Cerebrovascular diseases	162 (8.8%)	6 (5.9%)	0.325	168 (8.6%)
COPD/asthma	232 (12.6%)	14 (13.9%)	0.699	246 (12.6%)
Chronic liver diseases	71 (3.8%)	4 (4.0%)	0.951	75 (3.9%)
Solid or haematological malignancy	157 (8.5%)	7 (6.9%)	0.582	164 (8.4%)
Chronic kidney disease	142 (7.7%)	13 (12.9%)	0.066	155 (8.0%)
HIV infection /AIDS	16 (0.9%)	1 (1.0%)	0.896	17 (0.9%)
Rheumatic Diseases	26 (1.4%)	3 (3.0%)	0.206	29 (1.5%)
Obesity, n (%)			0.779	
No	386 (20.9%)	20 (19.8%)		406 (20.8%)
Yes	231 (12.5%)	15 (14.9%)		246 (12.6%)
Unknown	1,232 (66.6%)	66 (65.3%)		1,298 (66.6%)
Calendar period of admission, n (%)			0.855	
Feb-Aug 2020	588 (31.8%)	33 (32.7%)		621 (31.9%)
Sep-Mar 2020-21	1,261 (68.2%)	68 (67.3%)		1,329 (68.2%)
Risk factors for SARS-CoV-2, n (%)			<.0,001	
Close contact/household	133 (7.2%)	9 (8.9%)		142 (7.3%)
Healthcare worker	57 (3.1%)	1 (1.0%)		58 (3.0%)
Hospitalisation last 30 days/ long-term care facility	267 (17.7%)	33 (33.7%)		360 (18.4%)
Unknown/other	1,332 (72,0%)	58 (57.4%)		1,390 (71.3%)

HA-BSI = hospital-acquired bloodstream infection; COPD = Chronic obstructive pulmonary disease; HIV = Human immunodeficiency virus; AIDS = acquired immunodeficiency syndrome; IQR= Interquartile Range.

Regarding signs and symptoms of SARS-COV-2 infection at hospital admission, fever was observed in the majority of patients (72%). Dyspnoea and cough were reported by 48% and 36% of the patients.

Concerning COVID-19 severity at admission, based on respiratory rate, PiO2/FiO2 ratio on admission and radiological findings, 11.5% of the cases were classified as mild, 41% moderate, 45% severe and 2.4% critical. Radiologically confirmed pneumonia was present in 82% of the patients at admission **(table 2**).

More patients in the HA-BSI group, as compared to non-BSI group, were classified as severe at hospital admission (HA-BSI: 63.4% vs non-BSI: 44.9%, p<0.001) and had a radiological confirmed pneumonia (HA-BSI: 89% vs non-BSI: 81.5%, p=0.053). No other major differences were observed in clinical characteristics at admission, including signs and symptoms and laboratory findings with the exception of higher levels of CRP and D-dimer in the HA-BSI group (**table 2**).

Table 2. Clinical, laboratory, and radiological characteristics of 1,950 COVID-19 patients at hospital admission

	Patients without HA-BSI n= 1,849 (94.8%)	**Patients with HA-BSI n= 101 (5.8%)**	**P value**	**Overall n= 1,950**
Signs and symptoms at admission, n (%)				
Fever	1,340 (72.5%)	64 (63.4%)	0.047	1,404 (72.0%)
Dyspnoea	883 (47.8%)	54 (53.5%)	0.263	937 (48.1%)
Cough	669 (36.2%)	33 (32.7%)	0.474	702 (36.0%)
Syncope/Pre-syncope	63 (3.4%)	4 (4.0%)	0.766	67 (3.4%)
Fatigue	233 (12.6%)	20 (19.8%)	0.036	252 (13.0%)
GI symptoms	247 (13.4%)	11 (10.9%)	0.476	258 (13.2%)
Arthralgia	106 (5.7%)	8 (7.9%)	0.361	114 (5.9%)
Chest pain	70 (3.8%)	1 (1.0%)	0.144	71 (3.6%)
Anosmia/dysgeusia	86 (4.7%)	3 (3%)	0.431	89 (4.6%)
COVID-19 Severity at admission, n (%)			<0.001	
Mild	219 (11.8%)	5 (5%)		224 (11.5%)
Moderate	775 (41.9%)	30 (29.7%)		805 (41.3%)
Severe	811 (44.9%)	64 (63.4%)		875 (44.9%)
Critical	44 (2.4%)	2 (2%)		46 (2.4%)
Pneumonia at X-ray or CT scan, n (%)	1,507 (81.5%)	90 (89.1%)	0.053	1,597 (81.9%)
Laboratory findings at admission, median (IQR)				

Hb gr/dL	13.3 (11.8-14.6)	13.3 (11.5-15)	0.841	13.3 (11.8-14.6)
PLT 10^3/uL	208 (161-266)	194 (160-251)	0.386	208 (161-266)
WBC, 10^3/uL	7.1 (5.1-9.7)	6.9 (4.9-10-2)	0.917	7.1 (5.1-9.8)
N 10^3/uL	5.2 (3.5-7.9)	5.6 (3.4-8.6)	0.642	5.2 (3.5-7.6)
L, 10^3/uL	1 (0.7-1.4)	0.9 (0.6-1.3)	0.112	1 (0.7-1.4)
CRP, mg/L	53.2 (22.1-94.6)	66.7 (31.6-94)	0.020	54 (22.8-95.3)
LDH, U/L	287 (223-378)	300 (238-412)	0.129	288 (224-381)
D-Dimer, ng/mL	360 (218-715)	430 (310-869)	0.026	363 (222-719)

HA-BSI = hospital-acquired bloodstream infection; CT = Computed Tomography; Hb = Haemoglobin; PLT = platelets; WBC = white blood cells count; N = neutrophils; L = Lymphocyte count; CRP = C-reactive protein; LDH= Lactate dehydrogenase; IQR= Interquartile Range.

A list of other microbiological finding can be found in **table 3**. Respectively, 52 and 38 patients had a pulmonary co-infection due to *Streptococcus pneumoniae* and *Mycoplasma spp.* Overall, positive respiratory and urine samples were found in 4% and 8% of the population, respectively. Patients who developed HA-BSI during the course of hospitalization were more prone to have other microbiological positivity or MDROs colonization.

Table 3. Microbiological findings, other from BSI, in the population

	Patients without HA-BSI n= 1,849	Patients with HA-BSI n= 101	P value	Overall n= 1,950
Respiratory samples (sputum/tracheal aspirate/BAL)	68 (3.9%)	15 (14.9%)	<0.0001	83 (4.3%)
Urine cultures	130 (7.0%)	28 (27.7%)	<0.0001	158 (8.1%)
pneumococcal urine antigen test [a]	51 (6.2%)	1 (2.2%)	0.262	52 (6.0%)
Legionella urine antigen test [b]	5 (0.6%)	0 (0.0%)	0.586	5 (0.6%)
Influenza [c]	0 (0.0%)	0 (0.0%)		0 (0.0%)
Atypical pulmonary bacteria serology				
Chlamydia [d]	2 (0.3%)	0 (0.0%)	0.732	2 (0.3%)
Mycoplasma [e]	37 (6.4%)	1 (2.9%)	0.400	38 (6.2%)
MDROs colonization	16 (0.9%)	3 (3.0%)	0.036	19 (1%)
Other	20 (1.1%)	8 (7.9%)	<0.0001	28 (1,4%)

For Pneumococcal and Legionella Urinary Antigen, influenza, and serology for atypical pulmonary bacteria, the percentage of positive patients is related to the number of patients tested. [a] Pneumococcal Urinary Antigen: 867 patients tested (821 non HA-BSI group, 46 HA-BSI group); [b] Legionella Urinary Antigen: 843 patients tested (796 non HA-BSI group, 47 HA-BSI group); [c] Influenza: 365 patients tested (344 non HA-BSI group, 21 HA-BSI group); [d] *Chlamydia*: 708 patients tested (669 non HA-BSI group, 39 HA-BSI group); [e] *Mycoplasma*: 614 patients tested (579 non HA-BSI group, 35 HA-BSI group). HA-BSI = hospital-acquired bloodstream infection; BAL = Bronchoalveolar lavage; MDROs = Multidrug resistant organisms.

Clinical course, treatment and outcome of patients are described in **table 4**. As inhibitors of viral entry and replication, Remdesivir was used in 16% of the patients while hydroxychloroquine +/- azithromycin and antiretroviral protease inhibitors were used in 32.5% and 7% of cases respectively. Heparin prophylaxis was used in 72% of the patients. Regarding anti-inflammatory treatment, corticosteroids were used in half of the cases while only 5% of patients received immunomodulator agents. No difference in the distribution of therapies was noted between the HA-BSI and non-BSI groups except for a higher consumption of steroids in the HA-BSI group (73% vs 54%, p=0.001). Concerning the highest grade of oxygen support received during the hospitalization, the majority of patients (43%) was treated with low/high flow of oxygen (up to 15 l/min), via nasal cannula, face mask, venturi mask or non-rebreather mask, while 16% of patients did not need oxygen support. 29% and 7% received C-PAP or NIMV while 5.5% of patients received IOT and IMV in ICU. Overall, 6% of the patients were transferred of ICU for worsening of their clinical conditions. More patients in the HA-BSI group received NIMV/IMV as respiratory support (NIMV/IMV: 34% in HA-BSI vs 12%, p<0.001) and was admitted to ICU (16% vs 6%, p<0.001).

Table 4. Treatment and clinical outcome of 1,950 COVID-19 patients.

	Patients without HA-BSI n = 1,849 (94.8%)	Patients with HA-BSI n= 101 (5.8%)	P value	Overall n= 1,950
COVID-19 treatment, n (%)				
lopinavir/r or darunavir/c	128 (6.9%)	9 (8.9%)	0.444	137 (7.0%)
Remdesivir	292 (15.8%)	15 (14.9%)	0.800	307 (15.7%)
hydroxychloroquine +/- azithromycin	414 (32.3%)	20 (39.2%)	0.534	439 (32.5%)
heparin prophylaxis	1,325 (71.7%)	81 (80.2%)	0.062	1,406 (72.1%)
corticosteroids	1,003 (54.3%)	74 (73.27%)	0.001	1,077 (55.2%)
immunomodulators	86 (4.7%)	7 (6.9%)	0.295	93 (4.8%)
Highest grade of O2 therapy, n (%)			<0.001	
IMV	92 (5.0%)	15 (14.9%)		107 (5.5%)
NIMV	122 (6.6%)	19 (18.8%)		141 (7.2%)
CPAP	528 (28.6%)	30 (29.7%)		558 (28.6%)
O2 low/high flow	803 (43.4%)	30 (29.7%)		833 (42.7%)
No O2 therapy	304 (16.4%)	7 (6.9%)		311 (16.0%)
Length of stay, Median days (IQR)	10 (6-18)	28 (21-35)	<0.001	10 (6-20)
ICU admission, n (%)	103 (5.6%)	16 (15.8%)	<0.001	119 (6.1%)
Death, n (%)	465 (25.2%)	33 (32.7%)	0.091	498 (25.5%)

HA-BSI = hospital-acquired bloodstream infection; lopinavir/r = lopinavir + ritonavir; darunavir/c = darunavir + cobicistat; ICU = Intensive Care Unit.

Clinical and microbiological characteristics of HA-BSI episodes

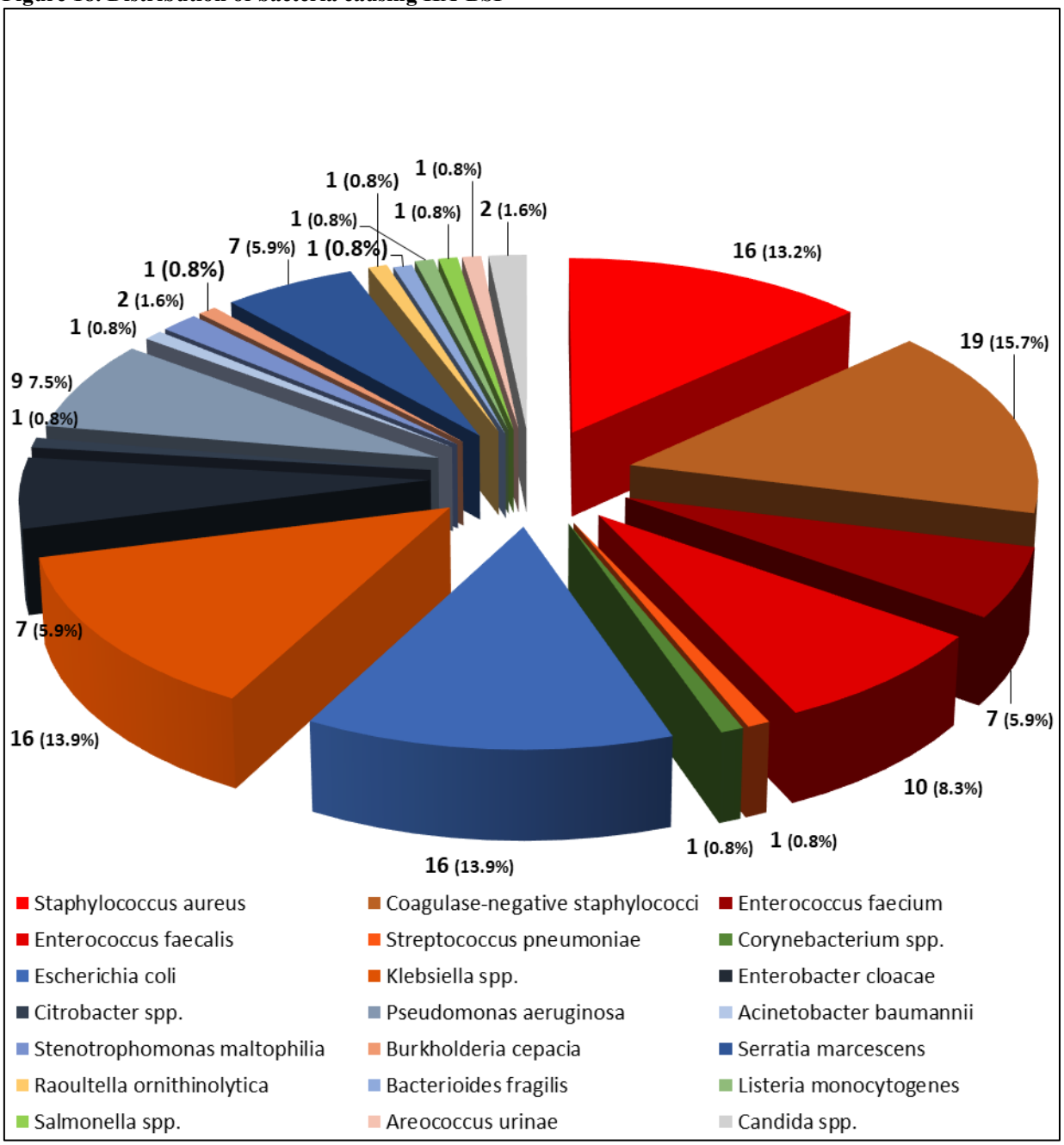
A total 121 episodes of BSI were observed in 101 patients. Prevalence of HA-BSI was 5.2% (95%CI 4.2-6.3) with an incidence rate of was 3.5/1000patient-days (95%CI 2.3–4.3). Clinical and microbiological characteristics of BSI episodes are detailed in **table 5**.

52% of the episodes were caused by gram-negatives and 46% by gram-positive strains. Two BSIs due to *Candida spp.* were observed. Among gram-positives, the most represented species were coagulase-negative staphylococci (16%) and *S. aureus* (13%), followed by *Enterococcus spp.* (*E. faecalis* 8%, *E. faecium* 6%).

Methicillin-resistance was observed in 84% and 19% of coagulase-negative staphylococci (MRSE) and *S. aureus* (MRSA) isolated while 29% of the E. faecium were Vancomycin-resistant (VRE).

Among Gram-negative strains, *Klebsiella spp.* (13%) and *Escherichia coli* (13%) were the bacteria most frequently isolated. *Pseudomonas spp.* cause 7% of the BSI episodes. Surprisingly, 6% of the HA-BSIs were caused by *Serratia marcescens* (**figure 18**). Antibiotic resistance in gram-negatives was distributed as follow: 13/49 (27%) ESBL-producing *Enterobacterales spp.*; 3/16 (19%) KPC-producing carbapenem-resistant *Klebsiella spp.*

Figure 18. Distribution of bacteria causing HA-BSI



The source of the BSI was identified in 72% of cases. Among BSI episodes with a recognized source, urosepsis were the most common type of BSI (32%), followed by Central Line-associated Bloodstream Infection (CLA-BSI; 18%) and bacteremic pneumonias (7%). Clinical and laboratory characteristics at BSIs onset are described in **table 5**. At BSI onset, the majority of patients experienced fever (74%). Hypotension was recorded in 30% of cases and 10% of the episodes required vasoactive agents for the treatment of sepsis-related hypotension. Common laboratory findings at BSI onset were modest leucocytosis with neutrophilia and marked increase in CRP levels. The median duration from hospital admission to BSI onset was 14 days. Median time from BSI to discharge or death was 12 and 6 days respectively. Regarding

therapy, empirical antibiotic therapy resulted appropriate, based on the susceptibility profile of the bacteria, in 66% of cases while targeted antibiotic therapy was appropriate in 90% of the episodes.

Table 5. Clinical and microbiological characteristics of 121 episodes of HA-BSI

	Episodes of HA-BSI n = 121
Origin of sepsis, n (%)	
Respiratory	9 (7.4%)
Urinary	39 (32.2%)
Catheter-related	22 (18.2%)
Intra-abdominal	8 (6.6%)
Cutaneous	9 (7.4%)
Other/unknown	34 (28.1%)
Gram, n (%)	
Positive	56 (46.3%)
Negative	63 (52.1%)
Fungi	2 (1.7%)
Causative agents, n (%)	
Staphylococcus aureus [a]	16 (13.2%)
Coagulase-negative staphylococci [b]	19 (15.7%)
Enterococcus faecium [c]	7 (5.9%)
Enterococcus faecalis	10 (8.3%)
Streptococcus pneumoniae	1 (0.8%)
Corynebacterium spp. [d]	1 (0.8%)
Escherichia coli [e]	16 (13.2%)
Klebsiella spp. [f]	16 (13.2%)
Enterobacter cloacae [g]	7 (5.9%)
Citrobacter spp.	1 (0.8%)
Pseudomonas aeruginosa [h]	9 (7.5%)
Acinetobacter baumannii	1 (0.8%)
Stenotrophomonas maltophilia	2 (1.6%)
Burkholderia cepacia	1 (0.8%)
Serratia marcescens	7 (5.9%)
Raoultella ornithinolytica	1 (0.8%)
Bacterioides fragilis	1 (0.8%)
Listeria monocytogenes	1 (0.8%)
Salmonella spp.	1 (0.8%)
Areococcus urinae	1 (0.8%)
Candida spp. [i]	2 (1.6%)
Fever at onset of BSI (TC > 37.5), n (%)	89 (73.56%)
Hypotension at onset of BSI, n (%)	37 (30.6%)
Laboratory findings at onset, median (IQR)	
WBC, 10^3/Ul	10.5 (6.5–14.6)
N, 10^3/Ul	8.71 (4.68–13.02)
CRP, mg/L	84.3 (46.8–111.1)
PCT, ug/L	0.37 (0.11–2.39)
Onset in ICU, n (%)	17 (14.1%)
Days from admission to BSI, median (IQR)	14 (9–21)
Days from BSI to discharge, median (IQR)	12 (7-22)
Days from BSI to death, median (IQR)	6 (3-9)
Appropriate empiric ATB, n (%)	80 (66.1%)
Appropriate targeted ATB, n (%)	109 (90.1%)
Vasoactive agents use, n (%)	12 (9.9%)

[a] 3/16 *Staphylococcus aureus* were methicillin-resistant (19%); [b] 16/19 Clinically significant Coagulase-negative staphylococci were methicillin-resistant (84%); [c] 2/7 *Enterococcus faecium* were vancomycin-resistant (29%); [d] *Corynebacterium jeikeium* (1/2), *Corynebacterium striatum* (1/2); [e] 4/16 *Escherichia coli* were third-generation cephalosporin–resistant (25%); [f] *Klebsiella pneumoniae* (14/16), *Klebsiella oxytoca* (2/16). 5/16 *Klebsiella spp.* were third-generation cephalosporin–resistant (31%), 3/16 were KPC-producing carbapenem-resistant (19%); [g] 4/7 *Enterobacter cloacae* were third-generation cephalosporin–resistant (57%); [h] *Pseudomonas aeruginosa* (9/9), *Pseudomonas putida* (1/9); [i] *Candida albicans* (1/2), *Candida tropicalis* (1/2).
HA-BSI = hospital-acquired bloodstream infection; WBC = White Blood Cells; N = Neutrophils; CRP = C-reactive protein; PCT = Procalcitonin; ICU = Intensive Care Unit; ATB = Antibiotic.

Risk factors associated with the development of HA-BSI

Factors associated with the development of HA-BSI BSI during hospitalization for COVID-19 were analysed using an unadjusted and adjusted logistic regression model.

At univariate analysis, factors associated with an increased risk of HA-BSI were age, NIMV/CPAP, IMV and corticosteroid treatment. At multivariate analysis, these variables were confirmed as independent factors associated with the onset of HA-BSI (**Table 6**).

Table 6. Uni- and multi-variable analysis of risk factors for the development of HA-BSI

	OR	95% CI	*p* value	aOR *	95% CI	*p* value
Age, per 10 years older	1.14	1.01–1.29	0.041	1.20	1.03–1.40	0.018
Gender, male (vs. female)	1.43	0.93–2.21	0.106	1.30	0.83–2.04	0.251
Charlson age unadjusted, per one-point raise index	1.06	0.99–1.14	0.115	1.06	0.96–1.15	0.165
Max O_2-tp (vs. no O_2-tp or high/low flow O_2)						
NIMV/C-PAP	2.23	1.44–3.45	<0.001	1.82	1.15–2.90	0.010
IMV	4.78	2.53–9.03	<0.001	4.75	2.32–9.72	<0.001
Calendar Period of Admission, Sep-Mar 20/21 (vs. Feb-Aug 2020)	1.04	0.68–1.59	0.854	0.79	0.47–1.33	0.375
Anti-inflammatory treatment						
Corticosteroids	2.30	1.46–3.60	<0.001	2.15	1.27–3.65	0.005
Immunomodulators	1.50	0.68–3.33	0.317	1.11	0.49–2.51	0.811

*Adjusted for all the factors showed in table. OR= Odd Ratio; aOR = adjusted Odd Ratio; IMV = Invasive Mechanical Ventilation; NIMV = Non-Invasive Mechanical ventilation; CPAP= Continuous positive airway pressure therapy.

Impact of HA-BSI on length of stay and in-hospital mortality

Overall, the median length of stay was 10 days (IQR 6-20). Of all patients, 6% (119/1,950) were admitted to ICU (**Table 4**). Comparing patients with HA-BSI and patients without, a longer hospital stay was observed (28 vs 10 days, p<0.001) and more patients were admitted in ICU (HA-BSI 16% vs non-BSI 6%, p<0.001). In-hospital mortality was 25.5%; no significant difference was observed among the two groups (HA-BSI 33% vs non-BSI 25%, p=0.091).
Even after adjusting for confounders (age, sex, Charlson Index, CRP and D-dimer levels on admission, severity of disease at admission, calendar period of admission, immunomodulatory agents), patients with HA-BSI did not have an increased probability of death (HA-BSI vs. non-BSI: aOR 1.01, 95%CI 0.62–1.64, p = 0.980).

However, a difference in the median time from admission to death was observed among the two groups (non-BSI group: 8 days (95%CI 5-15) vs HA-BSI group: 22 days (95%CI 11-28), p<0.001). This may represent a bias in the evaluation of the association between BSI and mortality underestimating the real impact of the event on clinical outcomes. Therefore, a nested study was performed within the cohort using as controls a group of non-BSI patients randomly selected and matched for age and gender, and ICU admission at index date. A 1:1 matching was used; for 100 HA-BSI patients the matched control was identified while for one HA-BSI patient no control was found.
Demographic and clinical characteristics of matched patients are described in **table 7**. No differences were observed except for a higher, although non-significant, Charlson score and COVID-19 severity at index date in the HA-BSI group.

Table 7. demographic and clinical characteristics of matched patients

	Matched non-BSI patients n= 100	Matched HA-BSI patients n= 100	P value
Gender, Male, n	69	69	1.000
Age, years, median (IQR)	72 (57-81)	74 (60-82)	0.433
Age unadjusted Charlson score, median (IQR)	1 (0-2)	1 (0-2)	0.057
Calendar period of admission, n			0.764
Feb-Aug 2020	34	32	
Sep-Mar 2020-21	66	68	
COVID-19 Severity at admission, n			0.065
Mild	11	5	
Moderate	42	30	
Severe	46	63	

Critical	1	2	
Respiratory support at index date, n			0.058
IMV	9	9	
NIMV	2	9	
CPAP	12	18	
O2 low/high flow	43	47	
No O2 therapy	32	17	
COVID-19 treatment, n			
Corticosteroids	60	73	0.051
Immunomodulators	8	7	0.788
ICU admission at index date, n	9	9	1.000
Days from index date to outcome, median (IQR)	8 (2.5-15.5)	12.5 (7-22)	0.002
Days from index date to death, median (IQR)	5 (2-11)	6 (3-9)	0.984
Death, n	18	33	0.015

HA-BSI= hospital-acquired bloodstream infection; IMV = Invasive Mechanical Ventilation; NIMV = Non-Invasive Mechanical ventilation; C-PAP= Continuous positive airway pressure therapy; ICU= Intensive Care Unit; IQR= Interquartile range.

In the nested study, over a median follow-up time of 9 days from index date, 18 and 33 patients died in the HA-BSI group and non-BSI group respectively. In the **figure 19** is shown cumulative incidence of death according to HA-BSI in the two group of patients. An increased risk of death in patients with HA-BSI was observed (30-days cumulative incidence of death HA-BSI 33% vs 18% non-BSI; Pepe-Mori test p=0.030).

After fitting a multivariable competing-risk regression model, adjusted for age, unadjusted Charlson score, intensity of respiratory support, corticosteroid therapy, a trend toward an increased risk of death in patients with HA-BSI was observed even tough statistical significance was not reached (SHR 1.99, 95%CI 1.13-3.53, p=0.017; aSHR 1.80, 95%CI 0.98-3.30, p=0.057) (**table 8A**).

Similarly, the impact of BSI on length of stay was evaluated by competing-risk analysis. The median time from index date to outcome (death or discharge) was 8 and 12 days respectively in patients without and with HA-BSI. Patients with HA-BSI had a longer time to discharge; in detail, the 30 days cumulative incidence of hospital discharge was 54% and 75% in patients with and without HA-BSI, respectively (Pepe-Mori test p=0.019) (**figure 20**); this finding was confirmed after multivariable competing-risk regression model adjusted for the same confounders (SHR 0.54, 95%CI 0.39-0.74, p<0.001; aSHR 0.65, 95%CI 0.43-0.85, p=0.003) (**table 8B**).

Figure 19. Cumulative incidence of in-hospital death according to HA-BSI by competitive risk analysis

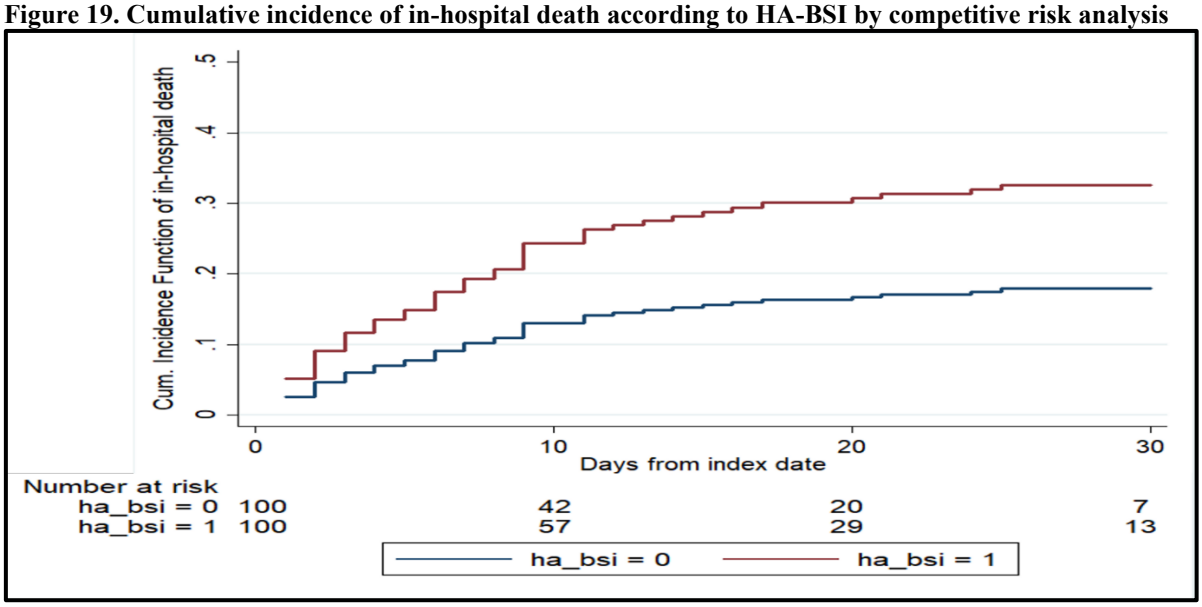


Figure 20. Cumulative incidence of hospital discharge according to HA-BSI by competitive risk analysis

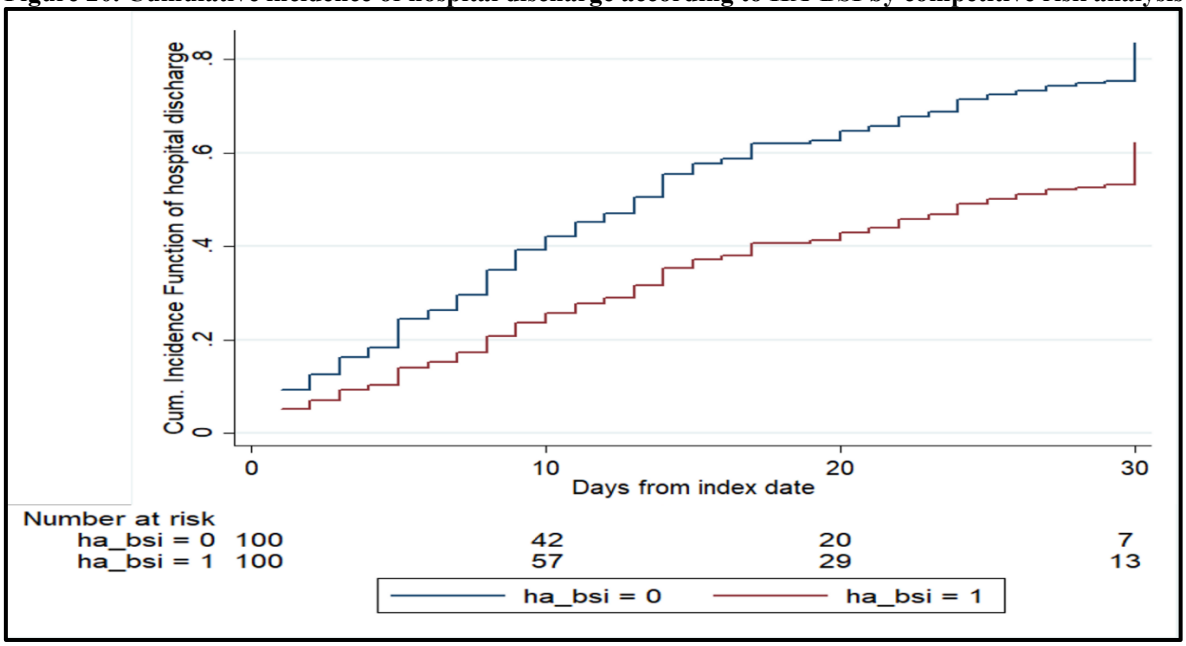


Table 8. uni- and multi-variable competing-risk regression model on the impact of HA-BSI on (A) in-hospital mortality and (B) length of stay.

	SHR	95% CI	*p* value	aSHR *	95% CI	*p* value
A) **in-hospital mortality**						
HA-BSI (vs non-BSI)	1.99	1.13-3.53	0.017	1.80	0.98-3.30	0.057
B) length of stay						
HA-BSI (vs non-BSI)	0.54	0-39-0.74	<0.001	0.60	0.43-0.85	0.003

*Adjusted for age, unadjusted Charlson score, intensity of respiratory support, corticosteroid therapy. SHR= Subdistribution Hazard Ratio; aSHR = adjusted Subdistribution Hazard Ratio; HA-BSI= hospital-acquired bloodstream infection.

Discussion

In our study population of 1,950 patients hospitalized for COVID-19, we found an incidence of HA-BSI of 3.5/1000patient-days. In detailed, 101 patients developed 121 episodes of HA-BSI. In our cohort, clinical characteristics of patients with HA-BSI did not differ significantly from those of patients who did not have a BSI except for a slightly higher COVID-19 severity at admission and a higher use of corticosteroids therapy and oxygen support via C-PAP or NIMV. Notably, a trend toward increased mortality in patients who developed a BSI was observed without reaching statistical significance while a significantly longer hospital stay was observed. Main risk factors for BSI resulted to be age, intensive respiratory support and use of corticosteroid therapy.

To date, this is one of the largest monocentric cohort studies focused on bloodstream infections in COVID-19 including both ICU and non-ICU so far described in literature. In fact, solid data coming from systematic reviews and meta-analysis are available on the incidence of general co- and super-infections in COVID-19 [7, 8] and several authors, in observational studies, have described characteristics of BSI in critically ill COVID-19 patients in ICU[10–12]. However, fewer authors have focused on HA-BSI outside the ICUs with a particular regard to the risk factors associated.

The main finding of our study is the association between the use of corticosteroids and the risk of HA-BSI. In fact, patients treated with these agents resulted to be at higher of developing a BSI during the course of hospitalization. The findings from this study confirm and reinforce what was observed in a recent published study from our research team on HA-BSI in a smaller cohort of COVID-19 patients[112].

Risk factors for the development of BSI in COVID-19 patients have been investigated by several authors; while the role of certain risk factors, including age, comorbidities, hyper-inflammation, lymphocytopenia, ICU admission, and COVID-19 severity, has been adequality clarified, the role of immunomodulatory agents and corticosteroids on the adjunctive risk of BSI has to be further investigated since available data are scarce and conflicting. Regarding corticosteroids, Giacobbe et al.[12] described an increased risk of developing ICU-acquired BSI in patients receiving methylprednisolone, in line to what observed in our study. A similar association was not found in the other studies [86, 97]. In our study, patients treated with corticosteroid therapy had double the risk of developing BSI. As known, corticosteroid agents are widely used in medicine for their immunosuppressive effects; however, as side effects, these agents are able to impair to host response to pathogens and increase the risk of bacterial and opportunistic infections[116–118]. Nevertheless, the beneficial effects of steroids are well

known and, in regard to SARS-CoV-2, the favourable impact of steroids on patients outcome in severe COVID-19 cases has been proven by the RECOVERY-trial[76]; indeed, corticosteroids were introduced in international guidelines for COVID-19 management and are currently considered one of the cornerstones of severe COVID-19 management.
Concerning immunomodulatory agents, in our study an increased risk with the use of these drugs was not observed. In my opinion, the small number of patients who received these agents in our cohort did not allow to draw any conclusion on this possible association.
Another important variable associated with BSI emerged from our analysis was the use of intensive respiratory support such as C-PAP, NIMV and IMV. Even though this association has been already described in literature[119, 120], in our opinion, this association should not be interpreted as a direct consequence of ventilation itself; ventilation should rather be seen as an approximation of disease se-verity, requiring a higher intensity of care and therefore exposing patients to a higher risk of hospital-acquired infections.

While baseline and demographic characteristics were comparable among patients with HA-BSI and those without, COVID-19 disease severity on admission differs significantly between the two groups. As a matter of fact, patients who later developed HA-BSI had more frequently a radiologically documented pneumonia on admission and a more severe clinical presentation. This finding can be partly explained by the fact that a more severe disease requires a higher intensity of care (implying, for example, the use of vascular and urinary devices), and this exposes individuals to a higher risk of nosocomial infections.
Concerning clinical outcomes, in-hospital mortality was comparable in the two groups; however, a longer hospital stay was observed and more patients in the HA-BSI group required admission to ICU.
At competing-risk analysis and after adjusting for confounders, a trend toward an increased probability of death in patients with HA-BSI was observed, although statistically significant was not reached. The main confounder resulted to be intensity of respiratory support, considered a proxy of COVID-19 severity during hospitalization. It can be speculated that these results may partly be explained by the low number of events observed in our cohort. Therefore, multicenter studies including larger cohorts of patients are needed. On the other hand, the increased length of hospital stay in patients who developed a BSI event was confirmed at the competing-risk analysis, revealing a direct correlation between the two events.
Hospital-acquired infections are known to have a negative impact on the clinical outcomes of patients admitted to the hospital for other reasons. Similarly, data so far available show that super-infections in COVID-19 patients negatively influence patients outcome; however, data

on mortality are conflicting. Two recent studies have evaluated this point. In the study from Hughes et al. [87] bloodstream infections increase the risk of in-hospital death while in the study from Russel et al. [89] co-infections and super-infections were not associated with higher mortality.

In our study the prevalence and the incidence of HA-BSI were relatively low as compared to historical data on influenza, SARS, and MERS. However, it is concordant to the incidence of bacterial infections in COVID-19 reported by several authors. In the meta-analysis from Lansbury et al[8]. the prevalence of co-infections, therefore not exclusively BSI, was 7% while a second meta-analysis, from Langford et al., reported a global prevalence of bacterial infections was 6.9%; among them prevalence of co- and superinfections was 3.5% and 14.3% respectively [7]. Regarding the incidence of BSI outside ICUs, data from observational studies are discordant. Engsbro et al. [120] reported a prevalence of 5.3%, which is in line to ours, while Ripa et. [86] al found an incidence rate of secondary BSI of 6.7/1000/person-days, which is higher than the incidence reported in our study.

Therefore, our data reinforce the current knowledge showing that bacterial infections in SARS-COV2 patients are less common than expected. Based on our data on the incidence, the use of empirical antibiotic therapy does not seem reasonable in most cases unless in presence of a strong suspicion of bacterial superinfection. Nonetheless, in case of confirmed or strongly suspected super-infection, appropriate antibiotic therapy is mandatory to improve patients' outcome.

In regard to the micro-organisms responsible for the HA-BSIs, an equal distribution of gram-positive and gram-negative strains have been observed. In fact, the most isolated bacteria were CONS, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia*. This is concordant with the distribution of source of BSI observed; in fact, the majority of the BSI episodes were vascular catheter related, mainly due to gram-positives, or with an onset from the urinary tract due to gram-negatives.

Our results do not differ significantly from those reported by other authors[9, 86, 89, 121, 122].

Concerning the prevalence of multi-drug resistant bacteria, in our cohort 28% of pathogens isolated from blood samples were MDR. In details, 84% of CONS and 19% of *Staphylococcus aureus* were methicillin-resistant, 29% of *Enterococcus faecium* were vancomycin-resistant. 27% of E*nterobacterales spp.* were ESBL and 19% of the *Klebsiella spp*. isolated were KPC-producing carbapenem-resistant.

This study has several limitations that should be acknowledged. Firstly, the monocentric nature of the study may limit the generalizability of our findings and conclusions to other settings. Secondly, being an observation study may have some selection and information bias. Lastly, some data were not collected; for instance, the presence of intravascular and urinary catheters were not collected and therefore not included in our multivariate analysis even though it represents a known risk factor for bacterial infections. Furthermore, data on antimicrobial therapy were not collected in all patients but only in patients with BSI. In my opinion, data analysis of the consumption of antibiotic therapy in correlation with the incidence of proven bacterial infections and circulation of MDR, is crucial in order to fill a current gap of knowledge and to guide clinicians in the prescription of these agents for the management of prove or suspected super-infection. We aim to include these data in a forthcoming study. On the other hand, the major strength of this study is the large cohort size; it is in fact one of the largest monocentric studies in literature with an exclusive focus on bloodstream infections inside and outside ICUs, which is one of the most fearsome complication among healthcare-associated infections. The association between the use of steroids and the increased risk of BSI represents one main finding. In fact, after the results from the RECOVERY trial and other studies including some meta-analysis, these agents are widely used worldwide. However, our findings are meant to suggest a wise use rather than discourage it and clinicians should be aware of this possible complication.

STUDY 2: DESCRIPTION AND MOLECULAR CHARACTERIZATION OF AN OUTBREAK OF NEW DEHLI METALLO-BETA-LACTAMASE PRODUCING KLEBSIELLA PNEUMONIAE IN COVID-19 UNITS

Rationale and aims of the study

An increment in the circulation of MDR bacteria during COVID-19 pandemic have been reported by several authors [123–126]. This seems to be mainly due to the high intensity of care required for COVID-19 patients, the extensive use of antimicrobials, and the lack of effective infection control measures and antimicrobial stewardship programs.

MDR organisms (MDROs) includes extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae*, carbapenem-resistant *Enterobacterales* (CRE), *Acinetobacter baumannii* MDR, methicillin-resistant *Staphylococcus aureus* (MRSA). For all of them an increase in colonization and infection rate has been described recently.

Tiri et al. have recently described, in an Italian cohort of ICU COVID-19 patients, an increase in the incidence of CRE acquisition from 6.7% in 2019 to 50% in 2020 despite the implementation of infection control measures. The authors speculate that the high intensity of care, the prone positioning of patients and the prolonged contact with the patients may be the reasons for the spread of this MDRO [125].

Several outbreak due to MDROs have been reported so far in COVID-19, especially in ICU. Amarsy et al. reported an outbreak due to NDM-1-producing *Klebsiella pneumoniae*, in a COVID ICU in Paris, which have involved 12 patients. Among them, 5 developed an infection sustained by this micro-organism [127]. Similarly, Farfour et al. described an outbreak of NDM-5-producing *Escherichia coli* involving six COVID-19 patients in a COVID-19 ICU in France [128].

The main mechanism of carbapenem resistance in *Enterobacterales* is the production of carbapenemases, enzymes able to hydrolyse carbapenems. The three main carbapenemases are KPC, NDM, and OXA-48. These carbapenemases have different geographical distribution and different efficacy in hydrolysing carbapenems. Moreover, their action is inhibited by different antibiotics.

One of the most clinically significant carbapenemase is the New Delhi-metallo-beta-lactamase (NDM), a Class B Metallo-beta-lactamase. The New Delhi-metallo-beta-lactamase (NDM) is a

beta-lactamase belonging to the class B of Ambler and it utilizes zinc as an essential cofactor in cleaving the beta-lactam ring. The class B hydrolyse all beta-lactams, except the monobactams and they cannot be hydrolysed by the available beta-lactamase inhibitors.
New Delhi-metallo-beta-lactamase was detected for the first time in 2008 in a Swedish patient who travelled to New Delhi, India, where he was hospitalized [129]. The Indian region has been identified as a reservoir for NDM [130]. After the first description, several cases have been described and the strain has spread worldwide.
In Italy KPC are the most widespread carbapenemase in *Klebsiella spp*; from 2018 was observed an increase in reports of isolation of NDM-producing strains while, until then, NDM were found rarely in our country. Starting from November 2018 we assisted to a considerable increase of cases: from November 2018 to January 2021 in Tuscany have been registered 353 patients with blood culture positive for NDM [131]. The infection prevention measures implemented in the region have proved effective in containing the NDM outbreak even if fewer cases continue to be reported in all the country.

Hereafter, we describe the epidemiological characteristics of an outbreak due to NDM-1-producing *Klebsiella pneumoniae* involving SARS-CoV-2 infected patients in ICU and non-ICU Units. Molecular characterization of the micro-organism will be discussed as well as the infection control measures implemented to control the outbreak.

Outbreak description

From February 2021 to March 2021 at the San Carlo Hospital (ASST Santi Paolo e Carlo), Milan, was registered a nosocomial outbreak of NDM-producing *Klebsiella pneumoniae* (NDM-Kp). This strain, characterized by limited therapeutic options, has been sporadically isolated among Italian hospitals.

The outbreak involved three COVID units: Intensive Care Unit (ICU), Pneumo-COVID Unit and Internal Medicine Department, involving 10 patients overall. Inside the hospital two additional patients in COVID free Units presented an NDM-Kp colonization: one in the Emergency Room and the other in the Gastroenterology Unit.

Overall, the NDM-Kp identifications from the end of January to the end of March 2021 were 12. Five patients developed an infection (2 VAP, 2 pulmonary sepsis and 1 urinary sepsis). The non-attributable mortality rate among patients with an NDM-Kp isolation was 58.3% (7 patients of 12 died) while mortality in patients with NDM-Kp infection was 80% (4 out of 5). It's important to notice that this data represents the overall intra-hospital mortality, not only the mortality due to infection. Most patients were already compromised before the NDM-Kp isolation, going through an advanced phase of the illness that required hospitalization and ICU admission, with several other microbiological identifications or unstable conditions. However, it was impossible to establish the exact impact of the *Klebsiella pneumoniae* NDM on adverse outcome for these patients. Regarding the therapy, in most cases a specific antibiotic therapy was not set because the presence of NDM-Kp was considered as a colonizer, not as a pathogen. Four therapies have been started with different regimes considering the isolation of other microorganisms.

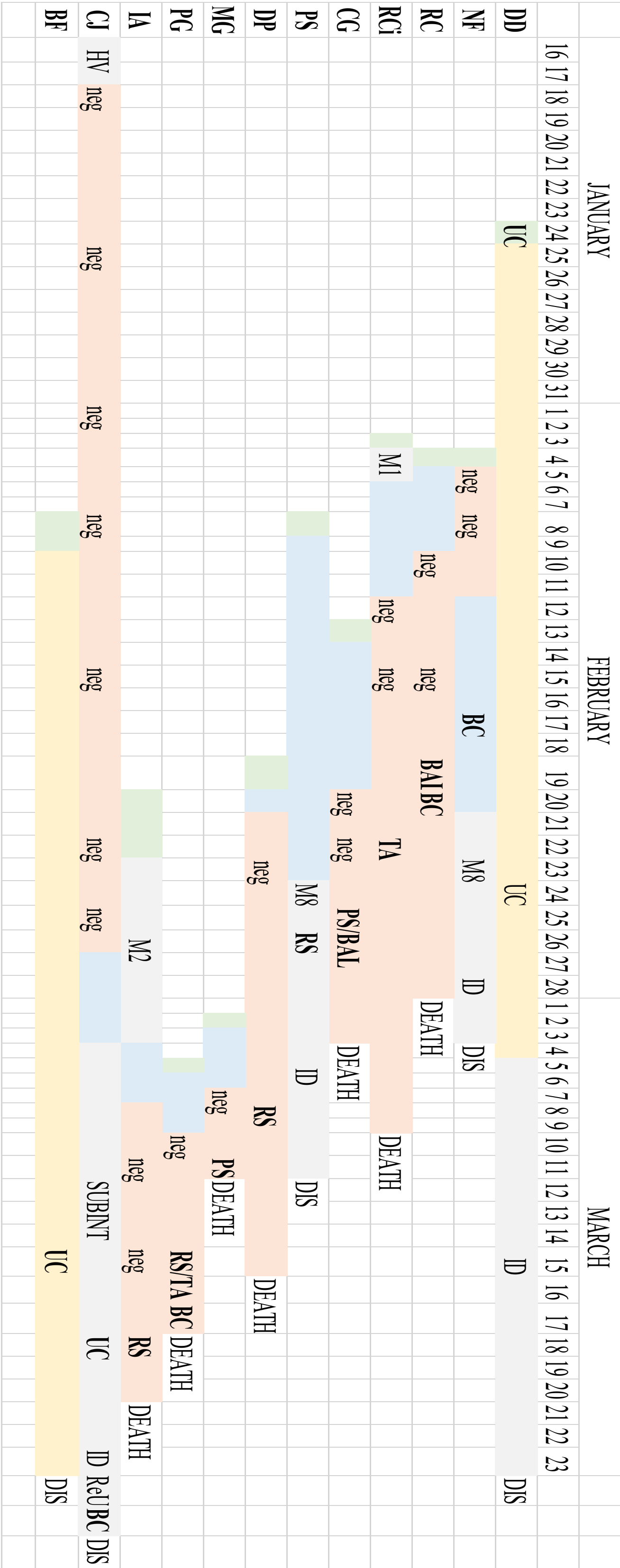
Contact tracing and surveillance swabs have permit to recognize the outbreak while the implementation of Infection Precaution and Control measures has limited the further spread of the strain. Indeed, no more cases of NDM-Kp were detected until July 2021. Unfortunately, starting from July 2021 few other cases of NDM-Kp colonization have been observed within the hospital. Epidemiological and molecular studies are currently ongoing.

Contact tracing and patient characteristics

Through the reconstruction of the contacts and patients' movements, an epidemiological link has been identified between the patients which has outlined a framework of outbreak, subsequently confirmed by genetic analysis. Although the patient with the first isolation of NDM was identified, it was not possible to exactly identify the primary case from which the outbreak started. As part of the routine infection control measures normally provided in ICU, all patients admitted to ICU undergo to surveillance swabs; notably, all the patients involved in the outbreak had a negative swab for MDR at admission in ICU. Unfortunately, in our institution, routine surveillance swabs are not performed in regular wards. Therefore, identification of NDM colonized patients was not possible outside ICU.

To be acknowledged, A case prior to the outbreak in description was detected in late January 2021. The patient (DD, man, 69 years), hospitalized for colitis from C. difficile, aspiration pneumonia and UTI, had a positive urine culture for NDM-Kp at hospital admission. In this case, however, no epidemiological link was identified with the other cases; genetic sequencing of the strain confirmed that this case wasn't related to the outbreak since the NDM-Kp in question belonged to a different subtype (**figure 21**).

Figure 21. Contact tracing of patients with NDM-Kp.



Gastroenterology
ICU
PneumoCOVID
ER
Others

HV = Vimercate Hospital
M1 = Internal Medicine 1
M2 = Internal Medicine 2
M8 = Internal Medicine 8
ID = Clinic of Infectious Disease
SUBINT = Subintensive Care Unit
ReU = Rehabilitation Unit
DIS = discharge

neg = negative screening for NDM-Kp
UC = first urine culture positive for NDM-Kp
BC = first blood culture positive for NDM-Kp
RS = first rectal swab positive for NDM-Kp
PS = first pharingeal swab positive for NDM-Kp
BAL = first bronchoalveolar lavage positive for NDM-Kp
TA = first tracheal aspirate positive for NDM-Kp

The first patient with NDM-Kp isolation, the index case, was a man (NF, 54 y/o) admitted to the hospital for respiratory failure due to SARS-CoV-2 pneumonia. He was initially hospitalized in COVID ICU and subsequently transferred to Pneumo-COVID, a pneumology department. In a context of sepsis from the urinary tract, NDM-Kp has been isolated in urine culture and blood culture on February 17th. At the beginning of the septic event an empiric antibiotic therapy with meropenem iv and oral fosfomycin has been set. For the continuation of treatment, the patient has been transferred to Internal Medicine 8D COVID unit. Once the identification of bacteria was available, the patient was moved to Infectious Disease Unit at San Paolo Hospital for isolation in single room. From a clinical point of view, after the identification of the strain the antibiotic therapy has not been changed for clinical response and resolution of the infection. The patient was later discharged home.

By running surveillance microbiological cultures (rectal swab, tracheal aspirate, and pharyngeal swab), provided by the regular infection control procedure in place at ICU, *Klebsiella pneumoniae* NDM have been detected in 7 patients. They were hospitalized for SARS-CoV-2 respiratory failure at the COVID-ICU and, after an initial asymptomatic phase of NDM-Kp, colonization some of them developed an infection. All of them died, in none of the cases it was possible to establish the role played by the NDM-Kp infection in defining the outcome, due to the clinical course complicated by the development of several concomitant infections. Here a short description of cases.

The second patient of the outbreak was a woman (RC, 74 y/o) with a surveillance tracheal aspirate and rectal swab positive for NDM-Kp (19/02), without clinical symptoms. Considering the intestinal and respiratory colonization no specific antibiotic therapy has been set. Following a febrile episode, NDM-Kp has also been isolated in blood culture (20/02), also positive for coagulase negative staphylococci. In a setting of concomitant *Acinetobacter spp.* MDR VAP an antibiotic therapy was started with fosfomycin iv, colistin and ampicillin/sulbactam. The patient died for multiple organ failure due to septic shock (01/03).

The third detection of NDM-Kp was, on 22/02, a man (RC, 64 y/o) with positive tracheal aspirate. He had concomitant *Serratia spp.* and *Enterococcus faecalis* sepsis and MRSA VAP. Even in this case, antibiotic therapy was started with meropenem, fosfomycin, colistin and ampicillin/sulbactam without success. The patient died on 11/03 for respiratory failure unresponsive to maximal medical therapy.

On 25th of February another NDM-Kp isolation emerged in a woman (CG, 67 y/o) on tracheal aspirate surveillance swab. Initially the introduction of therapy was postponed in consideration of the state of colonization. During hospitalization the patient developed several nosocomial

infections caused by other bacterial strains (sepsis due to CoNS and then due to *Corynebacterium spp*). After respiratory worsening in known NDM airways colonization therapy was set with fosfomycin and colistin. Nevertheless, the patient died for respiratory failure few days later (04/03).

The fifth case was a man (DP, 60 y/o) in COVID-ICU with positive surveillance rectal swab (08/03). The patient already known for a VAP due to *Klebsiella pneumoniae* KPC and *Enterobacter aerogenes,* received initially a treatment with ceftazidime/avibactam, then with meropenem and colistin. Because of severe persistent hypoxemia and reduced pulmonary compliance he passed away on 17/03.

Another patient in COVID-ICU was involved (IA, man, 68 y/o), hospitalized for respiratory failure due to *Legionella spp.* with overlapping SARS-CoV-2 infection. During his stay several microorganisms were detected and a combination therapy become necessary (ampicillin/sulbactam + daptomycin + fosfomycin iv + colistin iv and inhalator + voriconazole). The NDM-Kp positivity was at the rectal swab test (18/03) and despite the therapy ongoing the patient died on 21/03.

The patient number seven implicated in the outbreak was a man (MG, 73 y/o) hospitalized in the COVID-ICU for ARDS due to SARS-CoV-2 complicated by a polymicrobial VAP. The NDM-Kp was find at the throat swab the same day he died (11/03).

The last patient of the COVID-ICU was a man (PG, 64 y/o) with blood cultures positive for NDM-Kp (17/03) in an already compromised context of SARS-CoV-2 pneumonia complicated by fungal super-infection. Even in this case the patient died on the same day of the blood sample.

Following the recurring isolation of NDM-Kp, on a recommendation of the Hospital Infection Control Committee (HICC), infection prevention and containment measures have been implemented and standard procedures, provided to control the MDROs spread, have been strengthened. From the surveillance swabs emerged a new case in Internal Medicine 8D, a man (PS, 82 y/o) with an intestinal colonization for NDM-KP (positive rectal swab on 26/02). He was placed in isolation in a single room, remained asymptomatic for the entire hospital stay and was transferred to rehabilitation without the need for specific antibiotic treatment. He was a co-resident of the index case, but the genetic analysis highlighted a different subtype, excluding the horizontal transmission.

Other two NDM colonized patients were considered initially part of the outbreak. One of the two patients was hospitalized in Pneumo-COVID Unit, where the index case passed, and the other patient in Gastroenterology non-COVID Unit.

The patient of the Gastroenterology (BF, 54 y/o, man) was hospitalized for AKI with metabolic alkalosis and duodenal ulcer. The finding of NDM-Kp occurs at the surveillance rectal swab. In this case a specific therapy has not been set and the patient was discharge home without any complication.

The patient from the Pneumo-COVID was a man (CJ, 44 y/o) with detection of NDM-Kp at urine culture and rectal swab (18/03). He was previously hospitalized at COVID-ICU in the same period as the other patients involved in the outbreak. The patient, known to be immunosuppressed due to haematological neoplasia, during the ICU stay developed KPC-Kp intestinal colonization, *Acinetobacter baumannii* MDR VAP, CMV pneumonia and disseminated strongyloidiasis. For the NDM-Kp he did not receive specific treatment and he was subsequently transferred to rehabilitation facility and then discharged home.

Table 9 shows the demographic and clinical characteristics of the 12 patients who were involved in the outbreak of NDM-1-producing Klebsiella pneumoniae.

Table 9. Demographic and clinical characteristics of the 12 patients involved in the outbreak

Case	Major comorbidities	Other colonization/ infection	Site of NDM isolation	Date of first isolation	Unit	NDM colonization/ infection	Clinical outcome	Sequence type
DD, 69, m	Hypertension COPD, stroke	- *Clostridium difficile* - *Escherichia coli* (UC)	UC	26/01	Gastro	Colonization	Hospital discharge	ST-307
NF, 54, m	intrauterine toxoplasmosis	- KPC-Kp (UC)	BC, UC	17/02	Pneumo COVID	Sepsis	Hospital discharge	ST-945
RC, 74, f	Hypertension, post traumatic subdural hematoma	- *A. baumannii* (BAS) - CoNS (EC)	Rectal swab, tracheal aspirate, BC	19/02	COVID ICU	Sepsis	Death for septic shock and MOF (03/03)	ST-945
RC, 64, m	Diabetes, hypertension, obesity	- *Serratia spp*. (BAL, BC) - *Enterococcus faecalis* (BC) - MRSA (BAL)	Tracheal aspirate	22/02	COVID ICU	VAP	Death for respiratory failure (10/03)	ST-945
CG, 67, f	Obesity, anxious syndrome	- CoNS (BC) - *Corynebacterium spp* (BC)	Tracheal aspirate	25/02	COVID ICU	VAP	Death for respiratory failure (04/03)	ST-945
PS, 82, m	hypertension, atrial fibrillation, diabetes, meningeal neoplasm		Rectal swab	26/02	Internal Med 8D COVID	Colonization	Hospital discharge	ST-147
DP, 60, m	Werlhof disease with splenectomy	- KPC-Kp (BAL) - *Enterobacter spp.* (BAL)	Rectal swab	08/03	COVID ICU	Colonization	Death for respiratory failure (16/03)	ST-945
MG, 73, m	Hypertension, obesity	- *Serratia spp* (BAL) - MSSA (BAL) - *P. aeruginosa* MDR (BAL)	Pharyngeal swab	11/03	COVID ICU	colonization	Death for respiratory failure (11/03)	ST-945
PG, 64, m	COPD	*Aspergillus fumigatus* (BAL)	BC	17/03	COVID ICU	Sepsis	Death for respiratory failure and MOF (17/03)	ST-945
CJ, 44, m	Diabetes, chronic myeloid leukemia	- *KCP-Kp* (Rectal swab) - *A. baumannii* (BAL) - *CMV* (BAL) *strongyloidiasis*	UC, rectal swab	18/03	Pneumo COVID	Colonization	Hospital discharge	ST-945

IA, 68, m	Diabetes, obesity, hypertension	- *A. fumigatus*, - *A. baumannii* XDR - VRE *E. faecium*	Rectal swabs	18/03	COVID ICU	Colonization	Death for respiratory failure and MOF (21/03)	ST-945
BF, 54, m		- *Proteus mirabilis* (BC) - *K. pneumoniae* (BC)	Rectal swab	23/03	Gastro	Colonization	Hospital discharge	ST-945

At the COVID-ICU, after the discharge of all patients, on 23/03 all objects and furniture potentially contaminated were removed from the ward. The next day a sanitization intervention was carried out and, after negative environmental swabs, items and materials have been rearranged. On 25/03 the ICU was reopened for admissions again and till it was in function no more cases of NDM-Kp were detected. COVID-Units were subsequently closed as a result of COVID cases reduction.

As previously stated, no more cases of NDM-Kp were detected until July 2021. Unfortunately, starting from July 2021 few other cases of NDM-Kp colonization have been observed within the hospital.

Infection Prevention and Control

The almost simultaneous identification of the first two cases of NDM-1-producing *Klebsiella pneumonia* in two different departments, respectively in PneumoCOVID and COVID ICU raised the suspicion and the concern of an outbreak at initial phase. The uncontrolled diffusion of this strain, currently sporadically isolated in our country, eventually with an endemic rather than epidemic diffusion, could have dramatic consequences for our institution and for the entire National Healthcare System due to the limited therapeutical options and high mortality related to this pathogen. Therefore, standard Infection Precaution and Control (IPC) measures were strengthened, and new IPC were implemented during the course of the outbreak. Moreover, COVID-related IPC measures were already in place to avoid in-hospital transmission of SARS-CoV-2.

The first IPC measure implemented was "contact tracing" on recommendation of the IOC that made possible the recognition of outbreak.

Routine IPC measures in ICU, already in place before the outbreak and before COVID-19 pandemic with the aim of limiting MDR diffusion inside ICU, include universal active surveillance screening; the protocol provide for the execution of nasal, and rectal swabs and tracheal aspirates to detect MDR colonized patients at the entrance to the ICU and on a weekly basis. Recognition of colonized patient implies the implementation of contact precautions and isolation of the patient in single room, if available, or cohorting of the patients colonized by the same strain.

Instead, at our institution in non-ICU departments universal screening is not routinely performed and the standard IPC measures include contact precautions and isolation of colonized patients transferred from ICU throughout the entire duration of the hospitalization.

On 25th of February, the first IOC meeting was convened during which additional IPC measured were decided and therefore implemented in the departments involved by the outbreak. Extraordinary measures have included: isolation of patients and contacts with transfer of patients to Infectious Diseases Unit, when necessary, universal surveillance swabs, environmental screening and disinfection, suspension of new entries in the departments involved.

As previously reported, universal screening of the contacts did not reveal any horizontal transmission in Internal Medicine Units with the exception of a single case of patient that turned positive of NDM colonization on the 26 of February and the next day transferred to the Infectious Diseases Unit of San Paolo Hospital to isolation in single room.

On the contrary, surveillance screening in ICU has led to identification of several secondary cases that were cohorted with dedicated medical and nursing staff. On the 23rd of March new patients admission in ICU were suspended for few days in order to sanitize the Unit removing of potentially contaminated materials and furnishings, even though environmental screening did not show any contaminated equipment. On the 25th of March the Unit was reopened.
The additional IPC measures set, in addition to those already in force at our institution, resulted effective in the containment of the outbreak limiting the number of cross-transmissions despite the high risk of transmission of MDR related to the COVID-19 pandemic and to ICU setting.

Microbiological and molecular characterization of the strain

Microbiological analyses of samples were performed. Identification and susceptibility tests were performed using standard microbiologic procedures (BACT/ALERT VIRTUO BioMerieux, as blood culture detection system, VITEK 2 Biomeriux automated system to perform antibiotic susceptibility, MALDI-TOF-MS Biomeriux for microbial identification at the species level). Carbapenemases were determined by molecular testing of bacterial isolates using PCR-based platforms (Xpert-CarbaR). Next-generation sequencing (NGS) of samples, using NGS Illumina-Miseq Nextera DNA Flex was performed at the Infectious Diseases laboratory of Sacco Hospital, was performed to obtain information regarding resistance genes and sequence types.

Based on the NGS data on the *Klebsiella pneumoniae* isolates, all the strains had sequence type 945 (ST-945) confirming the horizontal transmission of the same bacteria that caused the outbreak. Two samples, collected from two patients (DD, 69 y/o and PS, 82 y/o), showed a different ST, respectively ST-307 and ST-147. Therefore, these two cases were not involved in the outbreak. To confirm this, contact tracing did not revealed any epidemiological link with the other cases. **Table 10** shows the complete molecular characterization of NDM-Kp strains.

Table 10. Molecular characterization of NDM-Kp strains

Case	gapA	infB	mdh	pgi	phoE	rpoB	tonB	ST	scg-ST	wzi
DD, 69, m	4	1	2	52	1	1	7	307	753	262
NF, 54, m	2	1	1	1	9	1	31	945	3973	262
RC, 74, f	2	1	1	1	9	1	31	945	3973	262
RC, 64, m	2	1	1	1	9	1	31	945	3973	262
CG, 67, f	2	1	1	1	9	1	31	945	3973	262
PS, 82, m	3	4	6	1	7	4	38	147	738	/
DP, 60, m	2	1	1	1	9	1	31	945	3973	262
MG, 73, m	2	1	1	1	9	1	31	945	3973	262
PG, 64, m	2	1	1	1	9	1	31	945	3973	262
CJ, 44, m	2	1	1	1	9	1	31	945	3973	262
IA, 68, m	2	1	1	1	9	1	31	945	3973	173
BF, 54, m	2	1	1	1	9	1	31	945	3973	262

Phenotypically, the three strains had an identical susceptibility profile to antibiotics and thus indistinguishable without the use of molecular techniques. As expected, all the isolates were resistant to aminoglycosides, quinolones, TMP/SMX, and beta-lactams including carbapenems. ST-147 was susceptible to colistin (**table 11**).

Table 11. Antimicrobial susceptibility profiles of NDM-Kp isolates

	DD, 69, m	NF, 54, m	RC, 74, f	RC, 64, m	CG, 67, f	PS, 82, m	DP, 60, m	MG, 73, m	PG, 64, m	IA, 68, m	CJ, 44, m	BF, 54, m
Sequence type	**307**	**945**	**945**	**945**	**945**	**147**	**945**	**945**	**945**	**945**	**945**	**945**
Amikacin	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)	32 (R)
Amoxicilin/ Clavulanate	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Cefepime	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Cefotaxime	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)
Ceftazidime	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)
Ciprofloxacin	>2 (R)	1 (R)	1 (R)	1 (R)	1 (R)	>2 (R)	1 (R)	1 (R)	1 (R)	1 (R)	1 (R)	1 (R)
Colistin	>8 (R)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)	<0.5 (S)
Ertapenem	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)
Gentamicin	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)
Meropenem	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)
Piperacillin/ Tazobactam	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)
TMP/SMX	>160 R)	>160 (R)	>160 (R)	>160 (R)	>160 (R)	>160 (R)	>160 (R)	>160 (R)	>160 (R)	>160 (R)	>160 (R)	>160 (R)
Imipenem							>8 (R)	>8 (R)				
Fosfomycin	64 (R)											

Discussion

From February 2021 to March 2021 a nosocomial outbreak due to NDM-1-producing *Klebsiella pneumonia* was registered at the San Carlo Hospital. It involved 4 Units, including COVID-ICU, for a total of 12 patients. Five of these patients developed an infection caused by NDM-Kp while the rest of them had an asymptomatic colonization. Genomic sequencing has confirmed that all the cases were due to the same strain. The outbreak was controlled thanks to the implementation of additional infection control measures.

Multiple outbreaks of NDM-producing *Klebsiella pneumoniae* have recently been described both before and during COVID-19 pandemic. One of the largest European Outbreak of NDM-Kp was recently described in Greece. The outbreak was due to ST11 NDM-Kp and involved 300 patients in several hospitals [132]. In Italy an outbreak of NDM-producing CRE affected seven hospitals in the Tuscany between November 2018 and 23 May 2019; 350 cases were reported[133]. The majority of the cases were caused by the ST-147 lineage. Noteworthy, one of the cases of our cohort was caused by this strain. Recently, during COVID-19 a new increment of NDM- Kp cases in Tuscany has been described [131].

NDM-Kp outbreaks have already been reported in COVID-19 Units. Amarsy et al. reported an outbreak due to NDM-1-producing *Klebsiella pneumoniae*, in a COVID ICU in Paris, which have involved 12 patients. All the isolates belonged to the sequence type ST15 [127]. Farfour et al. described an outbreak of NDM-5-producing *Escherichia coli* involving six COVID-19 patients in a COVID-19 ICU in France [128].

As reported by the ECDC, the spread of NDM is generally less clonal than KPC and mediated by plasmids; as a consequence, a single outbreak can be due to different species and STs.

At our institution three different strains were isolated, ST-307 and ST-147 and ST-945; the latter responsible for 12 out of 10 cases. Noteworthy, while the first two strains are widespread and well described in literature, the ST-945 strain has been rarely reported worldwide. As previously reported ST-147 is the lineage isolated during the Tuscany outbreak; sporadic cases are reported in Italy nowadays. ST-307 is one the most common isolated reported in Europe and worldwide. Recently an outbreak due to this clone was described in Germany [134]. Notably, in literature data on ST-945 are scarce. This lineage has been previously described as an hypervirulence and multi-drug resistance invasive *Klebsiella pneumoniae* from South and Southeast Asia [135]. Analysis to further characterize this rare strain are currently ongoing.

The overall in-hospital mortality has been 58.3%, while mortality in patients with NDM-Kp infection was 80%, slightly higher to the ones reported in literature. However, this data represents the hospital mortality attributable to all causes, not the mortality attributable to NDM infection. Moreover, the outbreak was settled in an ICU Units where patients are compromised and the circulation of MDR is higher as compared to other Units. However, it cannot be excluded that the NDM-Kp infection contributed to these patients' adverse outcome.

Regarding the procedures adopted, since the first opening of the COVID-ICU in March 2020, an infection surveillance protocol was implemented, considering the high risk of acquisition and selection of antibiotic-resistant pathogens. The protocol was similar to that already in place at ICU before the COVID-19 pandemic: at the entrance to the unit and weekly (every Monday) were collected tracheal aspirate and urine samples and nasal, pharyngeal and rectal swabs were performed for multidrug-resistant pathogens research. The additional infection control measures, in association with those already in force, made it possible to contain the NDM-Kp outbreak by limiting the number of cross-transmissions.

The three COVID-19 Units involved in the outbreak were later closed due to reduction of COVID-19 cases in Italy. Unfortunately, in July 2021 few more cases of NDM-Kp have been identified in non-COVID-19 patients; epidemiological and molecular analysis are currently ongoing.

CONCLUSION

The studies included in this dissertation aim to establish in what extent bacterial super-infections complicate an already complicated disease as COVID-19. The first study is focused exclusively on hospital-acquired bloodstream infection, one of most dangerous health-care associated infection. In our cohort, we found the BSIs were uncommon and not associate with an increased mortality. However, an increased length of stay was observed. Main risk factor for BSI were age, the use of intensive respiratory support and corticosteroids use. In the second study I have described an outbreak caused by NDM-producing carbapenem-resistant *Klebsiella pneumoniae*, one of the most fearsome MDR, due to the limited therapeutic options available. The outbreak involved twelve patients in three COVID units, including an ICU, and was associated with a notably high mortality. Although in which extend the pathogen was responsible for the observed mortality is not clear due to the already severe condition of the patients involved. Infection control measures were implemented, and molecular analysis confirm the monoclonal nature of the cluster.

In conclusion, despite the burden of secondary infections in COVID-19 seems low and with small impact on mortality it should not be neglected since clinical outcomes other than mortality may be affected by this complication. Instead, it should guide clinicians for the prescription of antibiotics limiting their use when bacterial infection is proven or strongly support. On the other hand, in-hospital outbreaks due to MDR, especially in ICU, are difficult to control and burdened by high morbidity and mortality.

Therefore, infection control measures and antimicrobial stewardship principles should be promoted even in these dark times.

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