

**UNIVERSITY OF MEDICINE AND PHARMACY
"VICTOR BABEȘ" FROM TIMIȘOARA
FACULTY OF GENERAL MEDICINE
DEPARTMENT XIII – INFECTIOUS DISEASES**

CĂPRARU IONUȚ-DRAGOȘ



**THE IMPACT OF THE SARS-COV-2 PANDEMIC ON
PATIENTS FROM VULNERABLE GROUPS IN WESTERN
ROMANIA**

**ABSTRACT
DOCTORAL THESIS**

**Scientific coordinator
PROF. UNIV. DR. CĂTĂLIN MARIAN**

**Timișoara
2024**

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GENERAL PART

1. CURRENT STATE OF KNOWLEDGE OF SARS-COV-2 INFECTION

1.1. General impact and structure

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a viral variant that causes the respiratory condition known as COVID-19. Originally identified as "novel coronavirus 2019" (2019-nCoV) or "human coronavirus 2019" (HCoV-19 or hCoV-19), it was originally identified in Wuhan City in Hubei province, China. The global pandemic was recognized by the World Health Organization (WHO) as a 'public health emergency of international concern' on 30 January 2020 and subsequently as a pandemic on 11 March 2020, given the speed with which it spread and its significant impact on global public health [1,2].

The rapid and unexpected spread of the SARS-CoV-2 virus had devastating consequences that exceeded all anticipation. In addition to essential medical factors, such as patient susceptibility and virus virulence, there were additional elements related to organizational, logistical and administrative aspects that amplified the impact of the pandemic. Among them, insufficient budget allocations in the health sector have led to critical shortages in the supply of essential protective equipment such as masks, gloves, gowns and testing pads. High population density in certain areas has also facilitated the spread of the virus through frequent physical contact, creating an environment conducive to rapid transmission of infection. General panic and distrust exacerbated the difficulties, affecting the proper management of the situation and contributing to significant changes in the way patients are organized and treated. These issues, together with other contextual factors, created a complex and dynamic framework that demanded rapid and adaptable responses from the health system and responsible authorities [3,4].

Minority groups, whether defined along ethnic, economic or social lines, have been severely affected by the profound reverberations of the global health crisis generated by COVID-19. Next, we aim to investigate the devastating impact of the pandemic on diverse minority communities globally. We focus on the disproportions and inequalities highlighted or amplified by COVID-19, highlighting the vulnerabilities and specific needs of these marginalized groups [5].

SARS-CoV-2, a member of the coronavirus family of viruses, is a positive, single-stranded RNA virus, which allows it to be directly translated into viral proteins after infection of the host cell. Similar to other coronaviruses such as SARS-CoV-1, which caused the SARS outbreak in 2002-2004, and MERS-CoV, responsible for the 2012-2015 MERS outbreak, SARS-CoV-2 can cause severe respiratory infections in humans. Structurally, SARS-CoV-2 viral particles have a diameter between 60 and 140 nanometers, being classified as medium-sized viruses. Its structure is composed of four main types of proteins: Protein S (spike), which allows the virus to attach to human cells and enter inside them; Protein E (envelope), essential for assembling and releasing virions from the host cell; Protein M (membranes), which contributes to the shape and structure of the viral particle; Protein N (nucleocapsid), responsible for packaging and protecting the genetic material of the virus [6,7].

This protein complex consisting of proteins S, E and M constitutes the outer shell of the virus, providing it with protection and recognition at the cellular level. At the same time, the single-stranded RNA genome of the virus, which is linear and positive, contains about 30,000 nitrogenous bases. This genetic sequence serves as an instruction plan for replication and production of new viral particles in the infected host cell. The spike protein is essential in infecting human cells by the SARS-CoV-2 virus. This protein facilitates virus entry into the cell

by interacting with specific receptors on the surface of host cells and initiates viral replication. Glycoproteins and membrane I proteins, known as S proteins, are key components of the virion [8].

Type I proteins have a single transmembrane domain, facing the outside of the cell. The S1 subunit binds to specific receptors on the surface of human cells, and the S2 subunit facilitates membrane fusion, allowing viral material to enter the cell. The spike protein contains a receptor-binding domain (RBD) with a high affinity for angiotensin-converting enzyme receptor 2 (ACE2), essential for virus infectivity. The structure of the RBD domain, stabilized by a disulfide bond, is crucial for recognition and binding to the ACE2 receptor [9,10].

Serine 2 (TMPRSS2) transmembrane protease is essential in infection of the host cell by SARS-CoV-2, activating the virus's S protein through the priming process. The interaction of the SARS-CoV-2 virion with the target cell's TMPRSS2 leads to cleavage of the S protein, thereby facilitating the fusion of virus and host cell membranes. The ACE2 receiver serves as the primary anchor point for SARS-CoV-2. By binding the spike protein to the ACE2 enzyme domain on the cell surface, an endocytosis process is initiated, whereby the virus and receptor are internalized into the endosomes. In an acidic environment or under the action of cellular enzymes, the virion is released from the endosome, thereby allowing the release of viral RNA into the cytoplasm. Viral RNA serves as a matrix for the synthesis of new viral particles, which are subsequently released from the cell, propagating the infection into adjacent or distant tissues. Understanding these molecular and cellular mechanisms is crucial for developing effective therapeutic strategies and understanding the pathogenesis of COVID-19 disease [11].

Coronaviruses, including SARS-CoV-2, encode a number of nonstructural proteins (NSPs) essential for viral replication and transcription, influencing cellular responses and promoting survival and spread of the virus in host cells. Among them, Nsp1 inhibits host RNA translation, Nsp2 is involved in endoplasmic reticulum remodeling and can suppress the immune response, and Nsp3 participates in viral polyprotein cleavage and replicative complex formation. Nsp4 contributes to the alteration of cell membranes, and Nsp5, also known as the main protease (Mpro or 3CLpro), is essential for the cleavage of viral polyprotein. Nsp6, Nsp7 and Nsp8 are involved in replicative complex formation and alteration of cell membranes, while Nsp9, Nsp10 and Nsp11 exert their varied roles in the efficiency of viral replication. Nsp12 is the main RNA polymerase of the virus, Nsp13 acts as helicase, and Nsp14 and Nsp16 are involved in modifying and protecting viral RNA to avoid detection and degradation by the infected host [12,13].

1.2. Signs and symptoms in COVID-19

COVID-19 disease presents a variety of clinical manifestations, from mild to severe. Common symptoms include fever, cough, headaches and fatigue, and other manifestations may include breathing problems, loss of smell and taste. The incubation period of the virus generally ranges from one to fourteen days after exposure to it, and infected individuals can be contagious before showing obvious symptoms. A significant number of COVID-19 cases are asymptomatic, with around a third of infected people affected, complicating efforts to diagnose and control the spread of the virus [14,15].

Data indicates that most COVID-19 cases (about 81%) experience mild to moderate symptoms, while about 14% develop severe symptoms such as dyspnea, hypoxia or extensive lung damage. In rare but severe cases, approximately 5% of patients may experience critical symptoms such as respiratory failure, shock or multiorgan dysfunction, requiring intensive medical intervention and prolonged hospitalization. This variety of clinical manifestations highlights the complexity and severity of COVID-19 as a global public health problem, highlighting the importance of close monitoring, early diagnosis and proper management of infected patients. The severity of symptoms can be influenced by various factors, including the patient's age, older people being more susceptible to severe forms of the disease and associated complications [16].

Persistent or recurring symptoms, known as "lasting COVID" or "long-COVID," are a worrying aspect of COVID-19. These patients may suffer from chronic fatigue, shortness of breath and organ damage for a long time after the onset of the disease. SARS-CoV-2 can cause damage to the lungs, heart, kidneys, and brain, which can persist even after clinical recovery [17].

The virus affects various human cells, having a tropism specific to those expressing the ACE2 receptor, present in multiple tissues. The ACE2 receptor is abundantly expressed on the surface of type II alveolar cells in the lungs, explaining the severity of lung damage. In addition, the virus can infect and affect the upper and lower respiratory tract, including the sinuses, nose, throat, trachea and lungs. This diversity of tissue damage underlines the complexity and severity of the disease and highlights the need for effective therapeutic and preventive strategies for managing COVID-19 [18].

Therefore, COVID-19 is a multifactorial disease that affects a wide range of systems and organs in the human body, having the potential to cause serious complications, especially in people with pre-existing comorbidities. This complexity underlines the importance of an integrated approach in the diagnosis, treatment and management of patients infected with SARS-CoV-2 [19-23].

COVID-19 was originally thought to be a zoonosis, but later data show that the main transmission occurs between humans, with viral particles remaining airborne for several minutes or hours, favoring inhalation and infection. Asymptomatic or pre-symptomatic people can spread the virus without knowing it. In addition to transmission through aerosols and droplets, the virus can also be spread by touching contaminated surfaces and then the face or by contact with feces, suggesting a possible fecal-oral route. Vertical transmission from mother to fetus or through breast milk is rare and controversial [24-27].

Standard diagnostic methods for COVID-19 focus on identifying the genetic material of the virus. Molecular testing, especially RT-PCR, is the most common and sensitive method for directly detecting the presence of the virus. This involves collecting respiratory samples, usually by nasopharyngeal swabbing or using sputum samples. RT-PCR detects and amplifies specific segments of viral RNA, thus providing accurate confirmation of active infection. In addition, serological tests are used to identify the presence of specific antibodies against SARS-CoV-2 in patients' blood, providing information about the body's immune response and being useful in epidemiological and surveillance studies [28].

For imaging diagnosis of COVID-19, chest X-ray and computed tomography (CT) are commonly used modalities. Chest X-ray may reveal bilateral multilobar opacities with peripheral, asymmetric and posterior distribution. In advanced stages, lung consolidation, subpleural dominance and "crazy paving" pattern can be observed, indicating inflammation and extensive tissue damage [29].

1.3. Treatment and complications in COVID-19

At the beginning of the pandemic, in the absence of specific treatments, COVID-19 management focused on symptomatic treatment, offering therapies to relieve fever, cough and other associated symptoms. There was no clear protocol for combating the virus itself. Remdesivir, an antiviral drug originally developed to treat the Ebola virus, was one of the first agents repurposed for the treatment of COVID-19. Preliminary studies have suggested that remdesivir could shorten the length of hospitalization in patients with severe disease. However, its effectiveness in reducing mortality has been the subject of debate in the medical community. Other antiviral medicines used include umifenovir, lopinavir/ritonavir, favipiravir, with varying effects on efficacy. In addition, azithromycin and hydroxychloroquine were popular drugs, but with questionable effects [30].

Treatment with convalescent plasma, which involves administering antibodies recovered from people cured of COVID-19 to infected patients, was considered a promising option. It was thought that these antibodies could help fight infection in the body of treated patients. However, subsequent clinical trials have had mixed results regarding the effectiveness of this treatment [31].

Anti-inflammatory drugs such as dexamethasone and methylprednisolone have shown significant potential in reducing excessive inflammation and damage in patients with severe forms of COVID-19. Monoclonal antibody treatments, which directly target the virus, have also gained ground and have been authorized for emergency use in certain situations [32].

In the fight against the COVID-19 pandemic, the rapid development and distribution of vaccines was a turning point. The Pfizer-BioNTech, Moderna and others vaccines have been hailed as promising solutions, offering significant efficacy against the virus. However, unequal distribution, misinformation and skepticism were major challenges. However, the data showed that vaccines are crucial in reducing the risk of severe infection and have been vital in adapting to new variants of the virus. Education and transparent communication have been instrumental in promoting scientific trust and understanding, providing prospects for saving lives and adapting to the new reality [33].

COVID-19 can cause serious complications, including severe pneumonia and Acute Respiratory Distress Syndrome (ARDS), affecting the lungs and breathing capacity. It can also influence the heart, increasing the risk of myocarditis and other cardiovascular problems. The impact on the central nervous system can lead to strokes, brain inflammation, or symptoms such as dizziness and loss of taste or smell. In addition, the pandemic has had a significant impact on mental health, increasing the risk of anxiety, depression and other psychological disorders [34].

SARS-CoV-2 infection can impair kidney function, causing acute kidney failure and accumulation of toxins in the body. It can also trigger an excessive inflammatory reaction called a "cytokine storm," which can cause damage to organs and tissues. The infection can also disrupt the immune system in the long term, increasing the risk of other infections. Hematological complications may include cytopenia, venous thromboembolism and other thrombotic problems, as well as fluctuations in blood glucose levels and impaired thyroid and adrenal function [35-38].

Symptoms associated with long COVID include persistent fatigue, shortness of breath, joint and muscle pain, headaches, loss of taste and smell, sleep disturbances, and trouble concentrating or remembering. These symptoms may persist for weeks or months after initial recovery and affect patients' quality of life, limiting their ability to work or perform daily activities. Long COVID is a major concern for the medical community and researchers, who are conducting studies to better understand this condition and develop appropriate management and treatment strategies [39].

2. COVID-19 EPIDEMIOLOGY

The evolution and spread of SARS-CoV-2 has posed an unprecedented challenge to the global health community. The pandemic was officially declared by the WHO in March 2020, based on the emergence and rapid spread of the virus, with its origins in the city of Wuhan, China, in December 2019. In the first weeks of the outbreak in China, the rate of increase in cases was alarming, with the number of infections doubling in about seven and a half days. The spread of the virus has also been facilitated by social factors, such as Chinese New Year celebrations, when many people travel or meet in the traditional way. Italy was hit hard, reporting its first cases at the end of January 2020, and the increased number of cases and deaths drew the world's attention to the seriousness of the situation [40].

The United States has become the epicenter of the pandemic, recording an impressive number of cases and surpassing China and Italy in the total number of confirmed infections. Interestingly, genetic investigations of the virus showed that a significant proportion of cases in New York, one of the hardest-hit U.S. cities, came from European travelers, not directly from China or other Asian countries. Most governments around the world took drastic measures after the WHO declared COVID-19 a pandemic, which had long-term social, psychological, logistical, administrative and financial repercussions. The measures imposed included non-pharmaceutical restrictions such as social isolation, a ban on free movement during certain time intervals and the wearing of protective equipment. The spread and evolution of SARS-CoV-2 has been marked by significant

scientific discoveries and the emergence of new variants of the virus, such as the Alpha and Delta variants, which have raised concerns among medical communities and the general public [41,42].

The Omicron variant caused major concern in November 2021, with a faster replication rate and less severe manifestation than previous variants. COVID-19 vaccines have been widely distributed, helping to reduce the spread and severity of infection. However, by May 2022, the pandemic had caused the loss of approximately 15 million lives globally [43].

The period from the onset of infection to death varies significantly, but the median of this period is 14 days. The risk of death is higher in older people and those with pre-existing medical conditions, such as cardiovascular disease or diabetes. Protecting these vulnerable groups and implementing appropriate prevention measures are key priorities in tackling the COVID-19 pandemic [44].

The rapid spread of COVID-19 has had a significant impact on Romania, with millions of confirmed cases and thousands of lives lost. In an effort to control the virus, Romania has stepped up testing and administered more than half of its population at least one dose of vaccine. The pandemic has brought multiple challenges for Romania, affecting not only the medical system, but also other governmental and economic sectors. Rapid adaptation and collective efforts have enabled the country to meet these challenges. On 8 March 2022, all restrictive measures were lifted, signaling an important step towards a return to normality and national recovery [45].

The COVID-19 pandemic has disproportionately affected vulnerable populations, including older people, those with pre-existing medical conditions and marginalized communities. Factors such as geographical location and limited access to healthcare contributed to this disproportion. In dense urban areas, implementing precautionary measures has been difficult and lack of access to adequate medical services has delayed diagnosis and treatment, amplifying the impact of the pandemic on these groups [46].

The COVID-19 pandemic has amplified pressure on mental health, significantly affecting vulnerable communities and minority groups. Pre-existing socioeconomic factors, along with the direct impact of the pandemic, contributed to increased levels of anxiety, depression, and stress in these groups. Lockdown and social distancing measures have heightened the sense of isolation and uncertainty, amplifying the psychological impact of the pandemic. It is essential to address these issues by developing and implementing comprehensive strategies that provide mental health support, also taking into account socio-economic and structural factors contributing to the vulnerability of these communities [47].

The situation of vulnerable groups in front of the SARS-CoV-2 virus in Romania reflected global trends, with local peculiarities. A study identified counties with the highest and lowest vulnerabilities to the pandemic. The elderly were the hardest hit, with many deaths recorded, especially in aged care centers. Social isolation has amplified these problems, with significant medical, social and psychological consequences [48].

Health workers, as well as workers in public services and other essential professions, have been at increased risk of infection during the COVID-19 pandemic, given their frequent contact with patients or the public. In Romania, vaccine hesitancy was particularly evident among vulnerable populations. This phenomenon can be influenced by cultural, socio-economic and trust factors in the healthcare system. The country's recent history of health systems and the level of public trust in authorities may be an important factor in explaining this vaccine hesitancy [49].

Vulnerable populations in Romania, such as rural communities, Roma and people with limited education, are often more likely to be vaccine hesitant. Hesitancy can be fueled by misinformation, myths and negative perceptions about vaccines, including fears of serious side effects or the use of vaccines as control tools. The socio-economic context is another factor contributing to vaccine hesitancy, as people from disadvantaged backgrounds may have different health priorities or limited access to health services. To address this issue, it is crucial to increase education and awareness among vulnerable populations, providing accurate and accessible information about the benefits and safety of vaccination [50].

3. ASPECTS OF THE COVID-19 PANDEMIC IN VULNERABLE GROUPS

The COVID-19 pandemic has affected children by closing schools and transitioning to online learning, generating gaps in access to education and difficulties in adapting to the virtual environment. Children from disadvantaged communities or minorities were disproportionately affected. In addition, the pandemic has amplified children's stress and anxiety, raising concerns about their families' financial situation. It is essential to provide tailor-made solutions to address these problems and ensure adequate support for children affected by this unprecedented crisis [50].

The pediatric population has been extensively studied during the pandemic to understand how the virus affects different age groups and to develop appropriate management and prevention strategies. Although children initially appeared to be less affected, cases were subsequently reported, mostly mild or asymptomatic, but there were also severe cases, including Pediatric Multisystem Inflammatory Syndrome (PIMS or MIS-C), a rare but serious condition associated with SARS-CoV-2 infection in children and adolescents. Treatment with remdesivir has been shown to be helpful in shortening the length of hospitalization in children. In Romania, vaccination of children remained a disputed topic, although the World Health Organization recommended the administration of the vaccine from the age of 12 [51,52].

The elderly have been an intensely studied category in the context of the COVID-19 pandemic, being considered an increased risk group due to their weakened immune system and the presence of frequent comorbidities. Nursing homes have become hotbeds of rapid transmission of the virus due to proximity and close contact between residents. The isolation and restrictions imposed have brought a sense of loneliness and isolation, accentuating the psychological impact of the pandemic on this vulnerable group. It is essential to address not only medical risks but also the emotional and social needs of older people during the pandemic [53].

Older people are at increased risk of morbidity and mortality associated with COVID-19, especially if they have comorbidities such as hypertension, diabetes or cardiovascular disease. Their immune systems may be less reactive, and their ability to fight off infection is reduced. COVID-19 can cause serious complications, such as multiorgan failure, coagulopathies and neurological conditions, which can have long-term consequences and require ongoing medical care. Older age was a predictor of disease severity and increased risk of developing acute respiratory failure, which often requires treatment with oxygen therapy, mechanical ventilation or ECMO [54].

In terms of specific therapy for SARS-CoV-2, antiviral drugs such as remdesivir or ritonavir have been authorized for use under certain circumstances, but their effectiveness among the elderly still requires further research. Immunomodulators, such as corticosteroids and tocilizumab, are used in certain situations to reduce excessive inflammation associated with COVID-19. However, the use of high doses of corticosteroids may be associated with an increase in mortality. Tocilizumab is another commonly used immunomodulator with increased efficacy. Vaccination is essential for reducing the risk of infection and complications in the elderly. Authorized and recommended vaccines have shown efficacy in preventing severe disease and reducing the risk of death in this population. Most vaccines showed promising and safe results in this age group, and vaccinated geriatric patients showed less severe symptoms and faster recovery [55,56].

Minority populations in many countries are more exposed to COVID-19 due to socio-economic inequalities. These groups had higher rates of hospitalization, mechanical ventilation, and disease-related deaths. In the United States, African Americans, Hispanics and migrants were at higher risk, and in Western Europe, people of color had more severe outcomes. Vulnerable populations also showed vaccine hesitancy [48,57,58].

SPECIAL PART

4. The purpose and general objectives of the thesis

The COVID-19 pandemic has highlighted and accentuated existing social and economic inequalities, exposing the vulnerabilities of marginalized groups and minorities. These communities, which were already suffering from discrimination and lack of access to essential services, have been hit hard by the crisis. Disparities in health, education and social support have widened, affecting in particular the most vulnerable segments of society. The response to this crisis must go beyond the immediate management of the pandemic and address the underlying causes of inequalities. Governments, NGOs and community leaders should work together to develop and implement sustainable strategies. They should aim not only at mitigating the immediate effects of the pandemic, but also at rebuilding and revitalizing vulnerable communities, ensuring access to health services, social support and fair economic opportunities for all members of society.

The objectives of the paper are:

1. Identification of genomic variants of the SARS-CoV-2 virus in the Western region of Romania, using nanopore sequencing technique
2. Analysis of biomarkers and assessing their role as predictive factors by comparing them in children, adults and elderly, in the West of the country
3. Analysis of biomarkers and assessing their role as predictive factors by comparing them in Roma people and the general population in Western Romania
4. Discussions on the impact of COVID-19 on healthcare workers and the vulnerability generated by the field of activity in the Western part of Romania

4.1. Identification of genomic variants of the SARS-CoV-2 virus in the Western region of Romania, using the nanopore sequencing technique

In recent years, the COVID-19 pandemic, caused by the SARS-CoV-2 virus, has generated significant socio-economic repercussions globally. This virus, classified in the genus Betacoronavirus, shows a pronounced tendency to mutation, imposing the need for regular sequencing of its genome. These efforts are crucial for understanding the evolution of the virus, identifying variants and adapting diagnostic tests and vaccination strategies. Genomic sequencing has also helped elucidate the relationship between viral mutations and clinical manifestations of COVID-19, including cases of reinfection [59,60].

Sequencing of this virus was performed using various techniques, including metagenomics, specific capture, and PCR amplification. Metagenomics is used to identify and characterize novel pathogens without prior knowledge of their genomic sequence. On the other hand, sequencing based on capturing a specific region of interest requires detailed genome knowledge. These approaches are influenced by the viral load of samples, and PCR-based techniques are preferred for samples with low or damaged viral content because they are more sensitive and can detect small amounts of viral material. However, the PCR method can introduce artifacts and biases, requiring additional validation. PCR-tiling amplicon is a specific, effective and less expensive method for virus sequencing [60,61].

However, PCR-based methods are not suitable for the discovery of novel pathogens because they require prior knowledge of the genome sequence. A key aspect and major limitation of these methods relates to the effectiveness of primers, especially when new variants of the virus emerge. Interference with hybridization sequences can lead to reduced coverage or incomplete assembly of the genome, highlighting the need for continuous monitoring and adaptation of methodology according to the evolution of the virus and its variants. Short-read sequencing platforms such as Ion Torrent and Illumina are recognized for their accuracy and are

considered the current industry standard. However, long-read sequencing, such as those offered by Oxford Nanopore Technologies (ONT), is becoming increasingly valued for its combination of low cost and equipment mobility. The major advantage of nanopore sequencing is the elimination of the need for sophisticated laboratory infrastructure or personnel with extensive expertise, allowing real-time data analysis even in the field [59,62].

One outstanding aspect of Nanopore technology is its portability; for example, the MinION device does not require internet access, allowing researchers to use it in field studies or in remote regions. Results obtained in epidemiological events, such as the outbreak of Ebola, Zika or Lassa fever, have demonstrated the effectiveness of this technology in rapidly and accurately monitoring the spread of the pathogen, adapting to identify and characterize new viral mutations or to explore the molecular diversity of different types of RNA. The ability to adapt and respond effectively to various research challenges confirms the invaluable value of Nanopore technology in genome sequencing [60,62].

The ARTIC network played a key role in developing an advanced protocol for nanopore sequencing and subsequent data analysis. This method was successfully implemented in the management of Ebola and Zika epidemics, and was later adapted for rapid identification of the SARS-CoV-2 virus. The protocol was revised to introduce alternative primer schemes and adjust amplicon size, thus allowing for faster library preparation and a significant increase in multiplexing, with up to 96 samples analyzed simultaneously. Between 2020 and 2021, the ARTIC network developed the set of ARTIC V4 primers for sequencing the genome of the SARS-CoV-2 virus, in response to the emergence of new mutations. These adaptations were necessary to ensure complete genome coverage and maintain the protocol at the level of state-of-the-art sequencing techniques [63,64].

The fundamental goal of this study was to develop and validate a workflow for identifying mutated variants of SARS-CoV-2 through the Nanopore sequencing technique, with a special focus on vulnerable subpopulations. Using the MinION Mk1C device and the ARTIC protocol, we aimed to identify and analyze virus variants that circulated in Timis County, Romania, between August 2021 and May 2022.

As **materials and methods**, nasopharyngeal and oropharyngeal samples were collected from patients infected with SARS-CoV-2, hospitalized in the Victor Babes Infectious Diseases Hospital in Timisoara, Romania. To ensure the integrity of biological materials, each sample was placed in a specialized transport environment and quickly delivered within 3 hours to the Molecular and Biochemical Diagnostic Laboratory of UMFTVB. The samples were managed and stored anonymously at a temperature of -80°C to guarantee the integrity of the viral genome. The validation and support of the UMFVB Ethics Committee ensured respect for patients' rights and welfare in the research process. The viral RNA was extracted using Promega's Maxwell® RSC Viral TNA Kit, on Maxwell RSC's automated platform. After extraction, the viral RNA concentration was assessed with the HS RNA Analysis Kit, using Thermo Fisher's Qubit 2.0 instrument. To confirm the presence of SARS-CoV-2, ABM's GenomeCov19 Detection Kit was used, and RT-qPCR analysis was performed on the Bio-Rad CFX96 instrument. Positive and negative control was included, and the N and S genes were targeted in the viral genome. Samples with a threshold cycle (Ct) greater than 32 were excluded from the analysis.

For MinION testing, we adapted the SARS-CoV-2 sequencing protocol, called nCoV-2019 sequencing protocol v3 (LoCost) V.3, developed by the ARTIC team, using NEB reagents and ONT technology. Each step included 23 individual samples and a negative control, using ONT's SQK-LSK109 Kit. For the synthesis of cDNA, we combined LunaScript RT SuperMix with viral RNA and adjusted the concentration of samples according to the Ct value for optimal preparation. We generated overlapping fragments (~400 bp) by mixing Hot Start High Fidelity Master Mix with primer group V3 or V4, then performed separate reactions for each sample. After combining and diluting the samples, we used the NebNext Ultra II End Repair/dA-Tailing module for final preparation, followed by coding and purification. The samples were weighed using Qubit 2.0 and a dsDNA HS Analysis Kit, and for ligation of the adapters we used specific enzymes. The obtained library was loaded into an R9.4.1 stream onto a MinION Mk1C tool for further analysis. The process of base identification and demultiplexing was carried out with MinKNOW 20.10 software on the MinION Mk1C tool, ensuring the correct identification of each fragment amplified by barcodes. Further data analysis was carried out on the Epi2me platform developed by Metrichor Ltd., which integrates the Artic, Nextclade and Pangolin sections. Artic software evaluates the coverage of fragments and investigates individual amplicons that may not have been

amplified correctly. Nextclade identifies genetic variations, and Pangolin determines the lineage of the sample. The reference genome used was that of the Wuhan SARS-CoV-2 virus, codenamed MN908947.

For Ion Torrent testing, samples were selected for sequencing based on viral RNA quality and quantity, with Ct values < 32 and a minimum concentration of 7 ng/μL, determined with TaqMan 2019-nCoV Assay v1 Kit. Reverse transcription of viral RNA into complementary DNA was performed using the SuperScript™ VILO™ cDNA Synthesis kit according to the instructions. The fragments of interest for sequencing were obtained with the Ion AmpliSeq™ SARS-CoV-2 Panel, and the preparation of the library was carried out with the Ion AmpliSeq™ Library Kit Plus using the Ion Chef tool. The samples were sequenced on an Ion Gene Studio S5 machine using an Ion Torrent 540 chip. Data processing was performed with Iterative Refinement Meta Assembler (IRMA), and alignment, genetic line and building identification were performed with Nextclade and the SARS-CoV-2 reference genome (MN908947).

Nanopore sequencing results showed that most samples analyzed for SARS-CoV-2 viral RNA sequencing showed excellent quality, with Ct values between 11.9 and 29 and an average quality score above the standard threshold. Of the initial 103 samples, 96 provided clear and interpretable sequences, but 7 showed significant ambiguities. The sequencing process involved six distinct rounds, and some samples were subjected to double analysis using different panels. The overall average of readings obtained at all stages was approximately 1,646,673, of which an average of 390,414 were unclassified, highlighting the complexity and diversity of the dataset.

In the context of analyzing the average number of mutations identified, it was observed that clade 21L (Omicron) is distinguished by the presence of 59 mutations, marking the highest level of variation. Next, clade 21K (Omicron) was noted with 47 mutations, followed by clade 21J (Delta) with 34 mutations. The other clades, such as 20I (Alpha, V1) and 21J (Delta), had 32 and 26 mutations, respectively, while clade 20A had 24 mutations. Notably, clade 21B (Kappa) had minimal genetic variation with only one mutation identified. In terms of genome coverage, most samples showed values above 250×, with an average sequence length of about 519.17 bp. An interesting observation was related to a specific region of the genome, between 20 k and 22 k, which revealed less coverage. This phenomenon could be attributed to changes or variations in primer binding areas. For a better illustration of this phenomenon, Figure 4.2. detail the sequential coverage for barcode sample 5 of sequencing round 6, taking into account primer groups A and B used.

The results of Ion Torrent sequencing observed an average Spherical Ion Particle (IPS) load of 91%, and 99.5% of these contained the patient's actual library. Polyclonal IPS accounted for 28% of the total, and lower quality products accounted for 11.3%. No dimmer additions were identified, thus ensuring a final composition of the IPS library of approximately 60%. The mean length of the readings reached 190 bp, and almost all readings (98.6%) aligned accordingly with the reference genome. For most of the samples analyzed, the data were congruent in terms of identifying clades (90.90%) and determining genetic lines (72.72%). However, outside of clade 20I (Alpha), Ion Torrent technology revealed a higher number of mutations than Nanopore: on average, 27.9 and 39.8 mutations for Ion Torrent, compared to 24.1 and 30.9 for Nanopore, for clades 20A and 21I+J (Delta), respectively. In terms of correlation between the two techniques, the average correspondence percentage was 80%. An interesting aspect was the identification of 2 out of 3 samples that, having the same number of mutations identified by both methods, showed the same genetic changes.

The complete genome analysis provides detailed insight into the evolutionary path of the virus. Nanopore sequencing is distinguished primarily by its temporal efficiency: ONT technology requires less than 20 hours, while platforms such as Illumina and Ion Torrent require 36 hours, which is also associated with high costs [65].

The ARTIC protocol has become a popular choice among laboratories, including those that lack extensive expertise in next-generation sequencing. Its popularity is due to its intuitive nature, low cost and effectiveness in virus detection [66].

Genetic variation is often specific to certain populations, and analysis of diverse ethnic groups may reveal significant differences in alleles that may be clinically important. The study by Shen et al. identified genetic differences between ethnic groups in susceptibility to COVID-19 in Florida. MHC alleles in the European

group show protein E, while those in the African American group have protective alleles. These findings suggest that other regions of the virus outside the RBD domain are immunogenic. This could influence the development of vaccines and medicines for specifically affected groups [67].

Significantly increased expression of the F8A2 gene in African Americans, which codes for the HAP40 protein, may influence cellular response to SARS-CoV-2. Gene expression studies in African Americans have shown increased activity of certain cell types in the esophagus and lungs, including esophageal glandular cells and ACE2-positive basal keratinocytes. These findings provide important prognostic information that could help refine COVID-19 prevention and treatment strategies in this population. It is essential to include metadata about genomic and socio-environmental factors in human RNA sequencing studies to better understand the causes of divergence of molecular phenotypes [68].

4.2. Analysis of biomarkers and assessing their role as predictors by comparing them in children, adults and elderly in the West of Romania

COVID-19 infection has been associated with a number of significant biomarkers in the inflammatory response. Lactate dehydrogenase (LDH): An indicator of vascular permeability in immune-mediated lung lesions, closely linked to various conditions, including cardiopulmonary disease and cancer. C-reactive protein (CRP): Used to identify and evaluate inflammatory conditions and tissue damage. Creatinekinase (CK): Associated with mortality from COVID-19 infection. Vitamin D (25-OHD): May positively influence the immune system, slowing viral replication and reducing inflammation. Ferritin (FER): A valuable indicator, indicating the possible presence of secondary hemophagocytic lymphohistiocytosis and associated with an unfavorable prognosis in COVID-19. High-density lipoprotein (HDL) cholesterol: Its level has been observed to have an inversely proportional relationship with disease severity, being associated with reduced susceptibility to COVID-19. These findings underscore the importance of monitoring and correct interpretation of biomarkers in the management and prognosis of patients affected by infection with the new coronavirus [69,70].

Epidemiological data reveal the different impact of the COVID-19 virus on age groups. Children are generally less affected than adults, while the elderly, especially those in old age, are at increased risk of severe complications and death. This discrepancy can be attributed to several factors, including immune system functioning, pre-existence of other medical conditions, and socioeconomic factors. Adapting prevention and treatment strategies to the specifics of each age group is essential to reduce the incidence and severity of COVID-19. A comparative study between children, adults and the elderly could provide a deeper understanding of how SARS-CoV-2 infection affects these age groups and could help identify new personalized diagnosis and treatment strategies [69,71].

The materials and methods applied to this part of the study involved a retrospective cohort of children, adults and elderly who received a positive diagnosis of COVID-19. It is a multicenter study, as children were admitted to the Emergency Reception Unit (UPU) at the "Louis Țurcanu" Pediatric Hospital, while adults and the elderly were admitted to the UPU at the "Pius Brânzeu" County Emergency Hospital, both located in Timisoara, Romania. The study period was between March 1, 2021 and March 1, 2022 (1 year). Data was collected from hospital registers.

The severity of the disease was classified into five distinct categories: asymptomatic, mild, moderate, severe and critical. Epidemiological information such as age, gender and diagnosis were analyzed. The selected biomarkers

were: CRP, CK, LDH, FER, 25-OHD and HDL, analyzed on Cobas Integra 400 plus. Normal values were correlated with age.

Statistical analysis was performed using MedCalc software, version 20.218. The analysis was conducted using Kolmogorov-Smirnov, ANOVA, Kruskal-Wallis tests, Chi2 test and Spearman rank correlation coefficient. For the evaluation of the diagnostic value, characteristic curves of receiver operation (ROC), area under the curve (AUC) and Youden index for cutting point determination were used. The statistical significance was established at $p < 0.05$.

Test results in children showed a total of 381 patients, with a similar gender distribution ($M=55.64\%$). Most children had mild symptoms (53.28%). There were 14 (3.67%) cases of admission to the intensive care unit, of which 7 (50%) were classified as severe and 2 as critical. Three deaths were recorded (0.79%). There were only two critical cases, but none of them had a fatal end. Severity was associated with increased biomarkers, except for CK, and was excluded from further analysis. The strongest correlations were between CRP, FER (direct), HDL (indirect), and ICU admission. A moderate correlation involved ERF and death.

Results among adults showed a total of 614 patients, with a similar sex distribution ($M=52.12\%$). In terms of symptoms, most patients had mild symptoms (35.02%). There were 81 (13.19%) cases of admission to the intensive care unit, of which 33 (40.74%) were classified as severe and 30 (37.04%) as critical. In total, 53 (8.63%) deaths were recorded. It has been found that men tend to have lower levels of vitamin D (median = 24.28, $p = 0.0028$). Again, severity was associated with all but CK biomarkers. The correlations observed were: between admission to the ICU and CRP, LDH, IRON (direct), 25-OHD, HDL (indirect), respectively between death and CRP, LDH, IRON (direct) and 25-OHD, HDL (indirect).

The results of the elderly group showed a total of 243 patients, with equivalent distribution per sex ($M=42.39\%$). In terms of symptom severity, the majority of patients experienced severe symptoms ($n=73$, 30.04%). There were 112 (46.09%) cases of admission to the intensive care unit (ICU), of which 43 (38.39%) were classified as severe and 31 (33.04%) as critical. In total, 61 (25.1%) deaths among patients were recorded. A significant difference was noted for LDH, which was greater in males (median = 223, $p = 0.0462$). In this group, CK showed statistical value, along with the other biomarkers, but could not correlate with the Spearman analysis. CRP, LDH, FER (direct), 25-OHD, HDL (indirect) correlated with admission to ICU, and but CRP, LDH, FER (direct) with death.

As a risk of admission to ICU, **the results of curve analyses** showed significant differences between vitamin D 25-OH AUC in the elderly, which is lower compared to adults and children, and ferritin, where AUC in children is higher than in adults and the elderly. Lower levels of vitamin D 25-OH in the elderly could affect the test's ability to distinguish between positive and negative cases. In contrast, ferritin might be a more effective marker in the diagnosis or evaluation of children compared to adults and the elderly. In terms of risk of death, significant differences between AUCs for different biomarkers indicate variable ability to distinguish between age groups and different clinical importance depending on them. CRP appears to be a stronger indicator in adults than in the elderly, suggesting greater clinical relevance for detecting and monitoring certain conditions in adults. LDH has a better ability to discern in children than in adults and the elderly, indicating a different sensitivity of this marker in different age groups. Vitamin D 25-OH shows a lower capacity for discernment in the elderly, suggesting a possible association between its levels and age. HDL cholesterol has a weaker ability to discern in the elderly, indicating a possible change in the importance

of this marker depending on age. Ferritin has a very high capacity for discernment in all age groups, being useful in diagnosing and monitoring health status in all these categories, but may be particularly relevant in children.

4.3. Analysis of biomarkers and assessment of their role as predictors by comparing them in Roma people and the general population in the West of Romania

A similar analysis was conducted on a sample of Roma patients, compared to the general population in the Western part of Romania. Europe's Roma face poverty, discrimination and limited access to health services, exacerbating health problems such as chronic diseases and infant mortality. Vaccine hesitancy may be linked to these social problems and access to health services [72,73].

Demographic, epidemiological, clinical and biomarker data were analyzed to assess disease severity and outcome. It will be examined whether there are significant differences between the biomarkers measured in the general Romanian population and those in the Roma community. The aim is to provide information about variability in response to COVID-19 infection across different ethnic and socio-economic groups and to identify patients at increased risk of severe complications. The results of the study could contribute to improving strategies for diagnosis, treatment and management of COVID-19 patients, including in marginalized communities, such as the Roma population in western Romania.

As **materials and methods**, a retrospective research of patients hospitalized in Timisoara between March 2020 and August 2022 was applied, according to the database of the Timis County Public Health Directorate. Severity was estimated as in the previous study. Data were collected on age, gender, geographic location, body mass index (BMI), as well as tobacco and alcohol habits. Regarding COVID-19 aspects, vaccination status and source of transmission were also noted. In laboratory analysis, parameters such as CRP, FER, interleukin 6 (IL-6), D-dimers, LDH, HDL and 25-OHD were investigated. The statistical analysis followed the same pattern as the age-related study.

As **results**: the study included a total of 578 patients, of which 144 (24.91%) were Roma and 434 (75.09%) were Romanian. The gender distribution was 243 (42%) females and 335 (58%) males, and the median age was 57.52 years. Significant differences were observed between groups in BMI, location and source of transmission, with p values < 0.05 . The Spearman test revealed significant correlations between Roma ethnicity and BMI (directly poor) and rural place of origin (directly poor). For the entire sample, varying degrees of COVID-19 symptoms were recorded, with a significantly higher proportion of severe and critical cases among Roma patients. However, there were no significant differences in intensive care admission or mortality between Roma and non-Roma groups. A correlation test revealed a very weak association between Roma and intensive care admission, but no significant correlation was found between Roma and symptom severity.

Significant differences were noted between CRP ($p = 0.0245$), IL-6 ($p < 0.0001$), HDL ($p = 0.0008$) and 25-hydroxyvitamin D ($p = 0.0299$), the first two being higher in the Roma group, while the other two were lower. The results of the Spearman test showed several statistically significant correlations with Roma. CRP showed a very weak relationship, IL-6 a weak relationship, HDL an indirect weak relationship, and 25-OHD a very weak indirect relationship. All links were found to be statistically significant in terms of severity. Certain correlations were

considered direct and moderate: D-dimers for Roma and CRP, FER, D-dimers for the general population. Others were considered direct and strong: CRP, IL-6, LDH for Roma and IL-6, LDH for the general population. All reverse relationships were strong. There is a significant difference in rho values for CRP and HDL between the two groups, while the others were similar.

Multivariate logistic regression analysis showed that male gender, advanced age and smoking status were significantly associated with likelihood of intensive care admission and death. However, ethnicity did not demonstrate significant influence in this analysis. Broken down by ethnicity, in relation to ICU admission, increased CRP was involved in both groups, whereas the lack of increase in HDL and 25-OHD was significant only among the general population. Relative to death, increased CRP and lack of 25-OHD increase were implicated in both groups, whereas no increase in HDL was only among the general population.

The results of the ROC-AUC analysis indicate that CRP levels had the highest predictive capacity for intensive care admission among the Roma population, with an AUC of 0.985, sensitivity of 93.33% and specificity of 96.43%. The threshold value for this group was around 28.98, suggesting greater accuracy in detecting the need for admission to intensive care. Although no other significant differences were observed between groups, all individual ROC tests were statistically significant, with a different AUC of 0.5, demonstrating some predictive capacity in all patients.

4.4. Discussions on the impact of COVID-19 on healthcare workers and the vulnerability generated by the field of activity in the Western part of Romania

By the beginning of 2021, most European countries classified SARS-CoV-2 infection as an occupational disease or work-related accident, with Romania following this direction until February 2022. However, only a fraction of the cases of infection of medical personnel could be directly linked to the medical environment, most of them being the result of community transmission. Studies have revealed variable incidence rates among healthcare workers, with varying levels of hospitalization, intensive care admissions and deaths reported [74].

Each wave of the pandemic has amplified risks for healthcare workers, with increased exposure seen in each successive wave. Globally, surveillance data points to thousands of deaths among health workers, with estimates suggesting that the actual number of deaths could be significantly underestimated. In Romania, tackling the pandemic has been accompanied by unique challenges, including low vaccination coverage due to factors such as legislative issues, emigration of medical staff and limited health infrastructure, especially in rural areas. Despite vaccination efforts targeting high-risk healthcare workers as early as December 2020, vaccine hesitancy remained an issue, contributing to continued difficulties in managing the pandemic among healthcare workers in Romania [49,74].

The study published in 2023 investigated infection and vaccination against COVID-19 among healthcare workers in western Romania [49]. The analysis showed a significant association between the incidence of COVID-19 recorded in all categories of medical personnel and the evolution of the pandemic in the community, highlighting the link between the medical environment and the transmission of the virus in society. Auxiliary and mid-level medical staff showed the strongest correlations with the pandemic trend in the community, indicating increased vulnerability

to infection. Also, socioeconomic status and medical education level were significant predictors for acceptance of SARS-CoV-2 vaccination among healthcare workers, suggesting that those with higher socioeconomic status and advanced medical training were more likely to get vaccinated.

Non-vaccination status or incomplete vaccination was highlighted as an independent risk factor for COVID-19 reinfection among healthcare professionals. In particular, people who received a smaller number of vaccine doses had an increased risk of reinfection, while those who received a higher number of doses had a reduced risk. Worryingly, suboptimal adherence to subsequent vaccine doses was prevalent among middle and auxiliary staff, increasing their susceptibility to infections with emerging variants such as Omicron. These findings underline the continued importance of vaccination efforts and support for full vaccination coverage to reduce the risk of reinfection and build resilience against new threats in the evolving COVID-19 pandemic.

CONCLUSIONS

The present paper had the research theme: "The impact of the SARS-CoV-2 pandemic on patients from vulnerable groups in western Romania". This theme was chosen both because of the complexity it possesses, but also because of the burning desire to contribute to improving the understanding of the research field through the expansion of knowledge about the COVID-19 pandemic.

The COVID-19 pandemic has had a devastating impact on the health and well-being of the global population. In western Romania, patients from vulnerable groups have been severely affected, being more susceptible to severe forms of disease and admission to intensive care units. This is due to the high prevalence of comorbidities, limited access to health services and living conditions conducive to the spread of the virus. On the economic front, the pandemic has hit economic sectors hard, having a particular impact on those with low incomes and vulnerable groups in western Romania, contributing to increased poverty and food insecurity.

The Nanopore sequencing method has emerged as an affordable and intuitive technique, suitable for implementation in laboratories even with limited expertise in next-generation sequencing. This provides a robust solution for accurate and rapid surveillance of the spread of COVID-19 infection. Although its sensitivity is comparable to that of Ion Torrent technology, Nanopore has notable advantages, including simplicity in sample preparation, real-time data analysis capability, low cost and high accuracy of the information provided. These features make Nanopore a preferred option for effectively monitoring and managing emergencies associated with COVID-19.

Several biomarkers have been identified as useful for severity analysis, intensive care admission, and COVID-19-associated mortality. Among them, CRP had a significant correlation with outcomes across all age groups, with threshold values that were higher for adults than for the elderly. LDH also showed a good correlation with results in all age groups, especially in adults and the elderly. In contrast, CK had limited utility as a biomarker in assessing the health of pediatric COVID-19 patients because its values were largely within the normal range and did not provide significant correlation results. 25-OHD was a significant marker for assessing severity, intensive care admission, and mortality, particularly in adults. Ferritin had the strongest correlation with outcomes across all age groups, with more pronounced utility in pediatric patients and slightly less utility in the elderly. In addition, HDL cholesterol showed consistent inverse correlations with severity, intensive care admission, and mortality in all groups studied, reinforcing its value as a predictive biomarker.

Integrating laboratory data into clinical decision-making enables healthcare professionals to provide personalized and effective care for Roma patients, thereby contributing to the effective management of the COVID-19 pandemic. Regarding the impact on minorities, Roma patients were found to face multiple challenges, including being overweight, predominantly from rural areas and difficulties in identifying the source of transmission of the virus. These patients were admitted to intensive care units more frequently and had more severe symptoms compared to others. Analysis of laboratory parameters at the time of admission indicated that levels of inflammatory markers such as CRP and IL-6 were significantly higher among Roma patients, while levels of HDL cholesterol and 25-hydroxyvitamin D were lower. These data suggest that laboratory parameters may be useful as predictive indicators for intensive care admission and mortality, and CRP showed greater sensitivity in Roma patients. However, further

studies and data collection are needed to better understand the specific needs of these communities and develop more effective interventions in managing the pandemic.

The correlation between the incidence of COVID-19 among healthcare workers and the evolution of the pandemic underlines the complexity of transmission of the virus. Auxiliary and intermediate-level health workers require improved training in preventive practices, and adherence to vaccination, especially among them, requires increased attention to strengthen protection against new variants of the virus. In conclusion, the SARS-CoV-2 pandemic has had a significant impact on patients from vulnerable groups in western Romania, causing devastating consequences on health, economy and social well-being

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