PORTOFOLIU DE LUCRĂRI ȘTIINȚIFICE

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1. **Cocuz, M.E.**; Cocuz, I.-G.; Rodina, L.; Filip, R.; Filip, F. Clinical Outcomes and Characteristics of COVID-19 in Neonates: A Single-Center Study in Romania. *LIFE-BASEL* **2024**, *14*, doi: https://doi.org/10.3390/life14121650. – **PRIM AUTOR**
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Brief Report

Clinical Outcomes and Characteristics of COVID-19 in Neonates: A Single-Center Study in Romania

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Abstract: Background: SARS-CoV-2 infection is generally associated with less severe forms of disease in children, where most cases only require symptomatic treatment. However, there is a paucity of information regarding the impact and clinical course of COVID-19 in neonate patients. This study aimed to analyze the epidemiological and clinical aspects of COVID-19 in this particular age group who were patients treated in our department. Materials and methods: This is a retrospective observational study that includes neonates (aged less than 1 month) who were diagnosed with COVID-19. The patients were admitted between 1 January 2022 and 31 December 2023, to the Infectious Diseases Pediatric Department of the Hospital Clinic of Pneumophthisiology and Infectious Diseases in Brașov, Romania. All the patients were tested for SARS-CoV-2 infection at admission, using either a real-time PCR (RT-PCR) or rapid antigen testing, according to the national COVID-19 protocol in use at the time. We collected the following data: demographic data, clinical picture and laboratory values at presentation, clinical course, complications, and other significant data. All the data were extracted from existing hospital administrative databases or electronic medical records. Results: Nine neonates were hospitalized with COVID-19, of which five were boys, and four were girls; the mean age was 18.89 days (ranging between 6 and 28 days). The clinical picture at admission mainly consisted of fever (eight cases) and nasal obstruction and cough (five cases each). Only one patient required oxygen support. Co-infections with *Streptococcus pneumoniae* and *Haemophilus influenzae* (one case), respiratory syncytial virus (RSV, one case), and rotavirus (one case) were identified. Complications were represented by acute bronchiolitis in three patients. Biologically, lymphopenia was found in three cases, monocytosis in five cases, and increased ferritin values in five cases. The clinical outcome was favorable in all the cases. The patients were discharged in improved condition after an average stay of 5.11 days (ranging between 3 and 10 days). Conclusions: Our data support the observation that infection with SARS-CoV-2 in neonates is a relatively benign condition with a good prognosis. Our study has several limitations and establishes a foundation for future studies on a larger sample of term and premature neonates with different comorbidities.

Keywords: SARS-CoV-2 infection; COVID-19; neonates

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1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic seriously challenged healthcare systems worldwide [1,2]. Children and adolescents experienced less severe forms

of the disease than adults, and most cases only required symptomatic treatment or no treatment [1–4]. The mortality rates in children were related to certain risk factors and were low [5–7]. However, there are few reports on SARS-CoV-2 infection in young children, especially in neonates. Most articles on this subject include a small number of cases or present isolated experiences from single institutions. This article presents our experience with SARS-CoV-2 infection in nine children aged less than 1 month, and analyzes the epidemiological and clinical aspects of COVID-19 in this age group. We consider an important aspect, namely, the lack of an approved etiological therapy for the treatment [8,9] of COVID-19 in newborns at the time of the hospitalization of the patients included in this study. Thus, our study presents an analysis of the natural evolution of SARS-CoV-2 infection in the absence of a specific etiological treatment, possibly only influenced by the pathogenetic therapies targeting the co-infections, providing information to better monitor and treat COVID-19 in newborns.

2. Materials and Methods

We conducted a retrospective study on newborns who were hospitalized with COVID-19 in the Pediatric Infectious Diseases ward of the Clinical Hospital for Pneumophtysiology and Infectious Diseases in Brașov, Romania, between 1 January 2022 and 31 December 2023. The diagnosis of COVID-19 was established on admission to the hospital in all the patients by means of a rapid antigen test and/or RT-PCR, according to the national protocols for COVID-19 in place at the time. Informed consent for admission, treatment, and the use of data for scientific research was obtained at admission from the legal guardians of the patients. The approval of the Ethics Commission of the hospital was obtained for this study (No. 1871, 7 February 2024). The patients were de-identified. The data used in this study were extracted from the patients' clinical observation sheets and electronic medical records. The collected data were the age (at the time of the clinical onset of the disease, at the time of the diagnosis of COVID-19, and at the time of admission to the hospital), sex, personal pathological history, type of birth (natural or by cesarean section, at term or premature), co-infection with SARS-CoV-2 in the mother, clinical manifestations, clinical form of the disease, co-infections, hospitalization in the pediatric ward or intensive care unit (PICU), the need for additional oxygen (O_2), and the length of stay (LoS). We analyzed the values of selected parameters determined by laboratory analyses at admission (serum leukocyte count, C-reactive protein, alanine aminotransferase/glutamate–pyruvate transaminase (ALT/GPT), lactate dehydrogenase (LDH), ferritin, serum creatinine, and thrombocyte count), as well as any changes in the imaging investigation results, namely, chest X-ray images. All the laboratory values presented in our study were interpreted from a qualitative point of view, defining them as normal, increased, or decreased in comparison to average values, due to the fact that many of the laboratory tests were performed by different laboratories (Clinical Hospital for Pneumophthysiology and Infectious Diseases in Brașov and the Laboratory of the Emergency Clinical Hospital for Children in Brasov) with different ranges of normal values, according to each kit that was used.

The severity of the clinical form of COVID-19 was evaluated according to the existing national protocols for the diagnosis and treatment of COVID-19 at the time of the hospitalization of the patients [8,9]:

- Mild form: general and/or upper respiratory tract symptoms, without manifestations that are evocative of pneumonia and without lung damage;
- Medium form: patients with pneumonia confirmed by imaging but without hypoxemia (if there was no respiratory impairment prior to the current illness);
- Severe form: respiratory distress with arterial oxygen saturation (SaO_2) below 94% in atmospheric air and imaging abnormalities indicative of lung damage;
- Critical form: patients with severe respiratory failure requiring ventilatory support, septic shock, and/or multiple organ dysfunction.

3. Results

Between 1 January 2022 and 31 December 2023, 278 children with COVID-19 were admitted to the Pediatric Infectious Diseases Department, out of which 9 (3.23%) were newborns (2 were admitted in 2022, and 7 in 2023). The diagnosis of SARS-CoV-2 infection was established at hospital admission using a rapid antigen test for six children, an RT-PCR for two children, and both types of tests for one child.

The time interval between the clinical onset of the disease and the diagnosis of SARS-CoV-2 infection was 0–2 days for eight of the patients, who were hospitalized on the same day as the diagnosis. A single newborn who was initially asymptomatic tested positive for COVID-19 on her first day of life, likely due to the mother having the infection prior to delivery. At six days old, the infant developed clinical symptoms that were suggestive of COVID-19 and was subsequently admitted to the Pediatric Infectious Diseases ward. Birth occurred at term in all cases, naturally in six cases and by cesarean section in three cases. Two children had a history of acute illnesses, one with measles, and another with an episode of acute enterocolitis. All the patients were admitted to the pediatric ward (Table 1).

Table 1. Epidemiological and clinical characteristics of nine newborns (1–9) admitted with COVID-19.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Age athospital admission (days)	6	9	10	19	22	25	25	26	28
Gender	F	M	F	M	M	M	M	F	F
Age at the clinical onset ofthe disease (days)	5	9	10	19	21	23	24	26	27
Age at the moment of diagnosis—quick antigen test (QAT), RT-PCR* (days)	1	9	10	19	22	25	25	26	28
Mother positive for COVID-19?	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No
Type of COVID-19 diagnostic test	QAT *, RT-PCR *	QAT *	QAT *	QAT *	QAT *	RT-PCR	QAT *	QAT *	RT-PCR *
Delivery on time (T) or premature (P)	T	T	T	T	T	T	T	T	T
Natural delivery (n) or C-section (CS)	CS	N	N	N	CS	N	CS	N	N
Weight at delivery (grams)	3160	3300	3600	3670	2498	3280	2200	3460	2600
Admission to clinical ward (CW) or ICU	CW	CW	CW	CW	CW	CW	CW	CW	CW
Personal pathological history	-	-	-	-	Measles	-	-	Acute enterocolitis	-

* RT-PCR = real-time PCR; ICU = intensive care unit; QAT = quick antigen test.

The clinical manifestations at admission were fever (8/9 cases) and, less often, cough (5/9 cases), nasal obstruction and rhinorrhea (5/9 cases), and diarrheal stools (2/9 cases). Dyspnea was rare (2/9 cases) and associated with acute bronchiolitis; in one case, it required the administration of oxygen through a facial mask.

Co-infections were identified in three of the patients: one case with *S. pneumoniae* and *H. influenzae* (identified by means of an RT-PCR from respiratory secretions), one case

with respiratory syncytial virus (identified by means of a rapid antigen test from nasal secretions), and one case with rotavirus (identified by means of a rapid stool test) in a patient who also had diarrheal stools (Table 2).

Table 2. Clinical and radiological characteristics of newborns admitted with COVID-19.

Patient No.	Fever	Nasal Obstruction/Rhinorrhea	Coughing	Dyspnea	Diarrhea	Other Clinical Manifestations	Oxygen at Admission	Co-Infections	Associated Diseases	Chest X-Ray
1	X *	X	X	- *	X	-	-	-	-	Accentuation of the interstitial markings in the right pulmonary basal area
2	X	X	-	-	-	-	-	-	-	Normal
3	X	-	-	-	-	-	-	-	-	Not performed
4	X	-	-	-	-	-	-	-	-	Not performed
5	X	-	X	-	-	-	-	-	Acute conjunctivitis Anemia	Normal
6	X	X	X	X	-	-	X	*S. pneumoniae*, *H. Influenzae* (RT-PCR—pulmonary secretion)	Acute bronchiolitis	Normal
7	X	X	-	-	-	-	-	RSV (quick test)	Acute bronchiolitis	Diffuse and bilateral accentuation of the interstitial markings
8	X	-	X	-	X	-	-	Rotavirus (quick test from stool)	Acute enterocolitis with rotavirus Anemia	Normal
9	-	X	X	X	-	-	-	-	Acute bronchiolitis	Not performed

* "X"—present, "-"—not present.

Imaging evaluations were performed in six patients, with a normal appearance found in four cases, and pulmonary interstitial accentuation in two.

The laboratory data and the therapeutic and evolutive characteristics of the newborns admitted with COVID-19 are presented in Tables 3 and 4.

Table 3. Laboratory data for newborns (1–9) admitted with COVID-19.

Parameter/Patient No.	1	2	3	4	5	6	7	8	9
Seric leucocytes at admission	N	N	N	N	N	N	N	N	N
Seric lymphocytes at admission	↓	N	↓	↓	N	N	N	N	N
Monocytes at admission	↑	↑	↑	N	↑	↑	N	N	N
C-reactive protein at admission	N	N	N	N	N	N	N	↑	N
ALT (GPT) at admission	N	N	N	N	N	N	N	↑	N
LDH at admission	N	-	-	N	N	N	N	N	N
Ferritin at admission	↑	-	↑	N	↑	N	N	↑	↑
Seric creatinine at admission	N	N	N	N	N	N	N	N	N
Thrombocytes at admission	N	N	↑	N	↑	↑	↑	-	N

Legend: N—normal; "-"—not performed, "↓"—decreased, "↑"—increased, ALT—alanine aminotransferase; GPT—glutamate pyruvate transaminase; LDH—lactate dehydrogenase.

Table 4. Therapeutic and evolutive characteristics of newborns (1–9) admitted with COVID-19.

Parameter/Patient No.	1	2	3	4	5	6	7	8	9
Corticotherapy	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Antibiotics	No	No	No	No	No	Yes	Yes	Yes	Yes
Fever duration after admission (days)	2	1	1	2	3	1	1	2	-
Hospitalization period (days)	6	3	3	3	6	10	4	6	5
Evolution	F Discharge at personal request	F Discharge at personal request	F	F	F	F	F	F	F

Legend: F—favorable.

4. Discussion

COVID-19 in children is associated with less severe forms of the disease and fewer complications compared to adult patients [1,2,4]. These findings can be explained by several factors, such as children's decreased expression of the ACE2 receptor, the primary target of the SARS-CoV-2 virus; differences in the levels of transmembrane protease serine 2 (TMPRSS2) between children and adults; and the larger number of other viruses residing in the lung and airway mucosa of children, which could limit the replication of SARS-CoV-2 by means of direct virus-to-virus competition [10]. In addition, children may have stronger innate immune responses and fewer comorbidities, such as smoking-related dysfunctions and obesity [11–13]. There are studies that have shown that in the epithelial cells of their upper respiratory tract, children exhibit a higher basal expression of some cellular sensor proteins, such as MDA5, which, upon binding to viral RNA, activate a stronger early innate antiviral response to infection with SARS-CoV-2 than in adults [14,15]. Finally, fetal hemoglobin is speculated to have a role in protecting against coronavirus infection in neonates [16]. Infants with COVID-19 develop mild gastrointestinal or respiratory infections. Pneumonia is not a rare condition, however, and some patients require oxygen therapy. Infant appetite disorders may have a significant impact and be responsible for hospitalization [17,18]. Infants with eating disorders should thus be considered for COVID-19 testing; this could also help control disease spread, as infants may be involved in the transmission of SARS-CoV-2 infection in households [19,20]. The clinical picture of COVID-19 in infants changed significantly between the first two waves of the pandemic [17]. There has been little experience with COVID-19 in young children and neonates. Significant data on COVID-19 in neonates and young children became available during the fifth wave of the pandemic, which was largely caused by the Omicron variant and was more transmissible [21]. Shaiba et al. [22] performed a retrospective study of children younger than 90 days who tested positive for SARS-CoV-2 between March 1 and August 2020 in three hospitals in Saudi Arabia. A total of 36 children were identified; most of them were mildly symptomatic, with fever being the most common presenting feature, similar to what was observed in older children [1,2,5]. Only four (11%) patients had a severe infection, corresponding to a slightly increased incidence compared to that of 6% in previous reports [1,6]. Of these four cases, two were considered to be affected by multisystem inflammatory syndrome in children (MIS-C). Only one death was recorded in this group. The authors argued that the severe course and need for critical care or respiratory support in some of these patients might be explained by the presence of MIS-C, possibly diagnosed in two patients in the post-neonatal age group, and of other conditions, such as cyanotic heart disease and bacterial co-infections. Khoury et al. [23] reported on their experience with 52 COVID-19 patients who were younger than 6 months and admitted during the fifth wave of the COVID-19 pandemic (Omicron variant infection). Of these, 10 patients were younger than 1 month, 33 patients were aged 2–3 months, and 9 patients were aged 3-6 months. The mean age was 8.6 weeks (range of 2–20 weeks). The disease was mild in the neonates and young children, and admission was only required for observation.

No patients required ventilation or O_2 support, their laboratory results were within normal ranges, and there were no complications, including no documented bacterial infections. There were no 30-day readmission cases. They concluded that further studies are needed to reach more definitive conclusions on the severity of COVID-19 in young children, especially regarding the need for full sepsis workups and antibiotic treatments in neonates who are less than 28 days old. Similar results were published by Chen et al. [21]. Panetta et al. [7] reported on 27 infants with COVID-19 who were younger than 1 year, of whom 14 were below 3 months of age, and 13 were 3–12 months of age. The report showed that most of the infants below the age of 3 months presented with mild symptoms (86%), and only eight of them were hospitalized. Most of these infants presented with gastrointestinal symptoms; none of the infants required ICU admission, and only 10 were hospitalized.

Our study evaluated different clinical, biological, therapeutic, and evolutionary aspects of the neonates who were hospitalized for COVID-19. In the epidemiological context of COVID-19, most patients with a SARS-CoV-2 infection are adults, and a low proportion of pediatric patients have been treated; thus, less experience in the management of this disease in neonates has been gained. All nine newborns, four girls and five boys, included this study were born at term, naturally or by cesarean section, with normal birth weights in eight cases and a low birth weight in one, according to the WHO and CDC specifications [24]. Testing for SARS-CoV-2 infection was performed in eight of the patients due to the presence of symptoms that were suggestive of an acute infection; the remaining newborn was tested immediately after birth via cesarean section in the absence of any symptoms, because her mother had COVID-19 before giving birth. Testing in the epidemiological context of the persistence of a SARS-CoV-2 infection and infectious contact enables the isolation of asymptomatic cases of the disease, which are possible sources of infection for susceptible people in the vicinity of a patient, and can trigger cases of healthcare-associated infections, an aspect that is also mentioned in the specialized literature [25]. Later, the newborn presented with suggestive symptoms, a situation that determined her admission to the Infectious Diseases ward.

The clinical onset of the disease occurred at a variable interval after birth, between 5 and 27 days. In most of the patients, their infection with the SARS-CoV-2 virus most likely occurred through direct contact with a source of infection in their immediate neighborhood. An exception was the newborn who tested positive immediately after birth while being asymptomatic, because her mother had a confirmed infection. It is important to mention that if a mother is SARS-CoV-2-positive, her infant should be immediately tested after birth and possibly admitted to the hospital on the same day. The mothers of the children were also hospitalized with their children, and seven mothers tested positive for SARS-CoV-2 infection. The patients were admitted to the Pediatric Infectious Diseases Department, as they did not require intensive care interventions. Regarding the pathological history of the newborns, one of them, aged 22 days, had recently had measles, and another one, aged 26 days, had experienced an episode of acute enterocolitis.

The clinical manifestations at admission were dominated by fever, cough, nasal obstruction, and symptomatology that were suggestive of an acute respiratory tract infection of an unspecified etiology, including SARS-CoV-2 infection. This situation once again emphasizes the need for testing for COVID-19 to achieve an early correct diagnosis for the appropriate interventions. Two children presented with dyspnea at admission, with one of them also requiring oxygen administration at admission. Another two children presented with diarrheal stools as part of the clinical picture of febrile respiratory infection. The diagnosis of co-infections by means of various laboratory tests (RT-PCR of respiratory secretions, respiratory syncytial virus rapid antigen test of respiratory secretions, and rapid stool antigen test for rotavirus) allowed for the appropriate therapeutic interventions, rigorous monitoring, and, very importantly, patient isolation in separate rooms to prevent the occurrence of infections associated with medical assistance, which is a well-known issue that has been mentioned in the specialized literature [26–28]. Three patients presented with associated acute bronchiolitis. A co-infection with respiratory syncytial virus, which is

known to be frequently involved in this condition, was identified in one of the children [29]. Another patient with bronchiolitis was diagnosed with an *S. pneumoniae* and *H. Influenzae* co-infection, which is very rarely involved in the production of bronchiolitis in young children, and the third had no identified co-infection. This last observation may suggest the direct involvement of SARS-CoV-2 in the production of bronchiolitis, although some studies have shown that infection with SARS-CoV-2 rarely produces acute bronchiolitis, whose evolution is not severe. This is also supported by the finding that during the COVID-19 pandemic, there was a marked decrease in bronchiolitis cases [30].

Regarding the radiological changes that may be detected in patients with an acute SARS-CoV-2 infection, Kurian et al. [31] mentioned that the most common radiological changes encountered in pediatric patients with COVID-19 are ground-glass opacities, consolidations, and peribronchial thickening. Of the six patients in our study who underwent lung radiography, changes were identified in only two of them, represented by an accentuation of the pulmonary interstitium, a finding that is suggestive of a viral infection [32]. In accordance with the existing national protocols for the diagnosis and treatment of COVID-19 at the time of the hospitalization of the patients, the clinical forms of COVID-19 were interpreted as mild and medium [8,9].

The hematological changes found in children with COVID-19 show some differences compared to those that appear in adults. The most common aspects in newborns and infants with COVID-19 with hematological changes are leukopenia and lymphocytosis; lymphopenia is rare and mainly present in older hospitalized children. Thrombocytopenia is rare [33]. In our study, all the patients had normal leukocyte counts; lymphopenia was only present in three children, monocytosis in five, and thrombocytosis in four patients. Different scientific studies have identified certain risk factors for the severe evolution of COVID-19 in children, among which are young age; comorbidities; and elevated C-reactive protein, ferritin, and ALT values at hospitalization [34,35]. The patients in our study did not exhibit changes in the biomarkers that would suggest a severe evolution at admission. Thus, eight of the children had normal C-reactive protein and ALT values. The serum ferritin was elevated in five of the eight patients who underwent this investigation, but the course of the disease was favorable. Regarding possible kidney damage during COVID-19, Saygili et al. [36] highlighted the fact that even pediatric patients with mild or moderate forms of COVID-19 may be at risk of acute kidney injury (AKI), indicating that they require clinical monitoring and monitoring of their biomarkers to detect subclinical renal damage. In our study, all the patients presented with normal values of serum creatinine and, clinically, their diuresis was normal.

No patient received an etiological treatment with remdesivir. During the period in which the patients in our study were hospitalized, remdesivir was not approved for the treatment of SARS-CoV-2 infection in newborns (it was approved for use in newborns by the FDA in February 2024 [37]). The patients in our study were treated with corticotherapy (eight of the children), antibiotics (four of the patients), and symptomatic and supportive therapies (all of the patients). It should be noted that even in the absence of an etiological therapy, the evolution of the disease in the newborns hospitalized with COVID-19 was favorable in all cases, including those cases with viral or bacterial co-infections, which could have produced severe complications [38]. No patient required intensive care interventions. The average length of the hospital stay was 5.11 days, with a longer duration for the patients who had associated acute bronchiolitis or enterocolitis with rotavirus.

Limitations: Our study was a single-center, retrospective study, limited to a time interval of 2 years. The size of our study group was small (nine patients), and there was no control group. There was no long-term follow-up, as no child returned after discharge; so we cannot draw any conclusions regarding any possible long-COVID status in these patients.

5. Conclusions

Our study contributes to the accumulation of medical experience regarding different aspects of SARS-CoV-2 infection in newborns in the absence of an etiological treatment. Our data support the observation that infection with SARS-CoV-2 in neonates is a relatively benign condition with a good prognosis. We advocate for the need to carry out investigations into the diagnosis of viral or bacterial co-infections, which could worsen the prognosis, require specific therapeutic interventions, and require isolation in order to prevent the occurrence of healthcare-associated infections. Our study has several limitations and establishes a foundation for future studies on a larger sample of term and premature neonates with different comorbidities.

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Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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Treatment with Remdesivir of Children with SARS-CoV-2 Infection: Experience from a Clinical Hospital in Romania

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Abstract: Background: The COVID-19 pandemic was characterized by mild-to-moderate disease in children and adolescents, with low incidences of severe cases and mortality. Most of the information on drug therapy in COVID-19-positive children was derived from research in adult patients. Remdesivir, an inhibitor of viral RNA polymerase, was shown to be effective in COVID-19 patients with moderate-to-severe disease. In this study, we present our experience of the use of remdesivir in pediatric patients hospitalized with COVID-19. Materials and methods: This retrospective study was based on the early use of remdesivir in 14 children with mild, moderate, and severe clinical forms of COVID-19, who were hospitalized between 1 January 2022, and 30 September 2023. Results: The patients included eight infants and six children older than 1 day (the age range was 2 months to 17 years). Most of them (92.85%) had documented pneumonia. Four patients had associated acute laryngitis, and another had bronchiolitis. Coinfections with Streptococcus pneumoniae were diagnosed in two patients. The clinical course was favorable in 12/14 (85.71%) children. Two patients were transferred to the pediatric intensive care unit because of aggravation of associated acute diseases (acute laryngitis and bronchiolitis, respectively). Mild increases in alanine aminotransferase levels occurred in two patients, with no increase in serum creatinine, during treatment with remdesivir. Conclusion: The appropriate use of remdesivir proved safe and efficient in our group of patients. However, further studies are required to support the efficiency, tolerability, and safety of remdesivir in children.

Keywords: remdesivir; COVID-19; children; SARS-CoV-2

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1. Introduction

The coronavirus 2019 (COVID-19) pandemic represented a serious challenge to healthcare systems worldwide. Children and adolescents experienced less severe disease compared to adults, with most cases requiring only symptomatic treatment or no treatment [1,2]. Mortality at young ages was related to certain risk factors and had a low incidence [3–5]. The choice of the appropriate therapy for children was a significant issue during the pandemic. Few clinical studies have examined drug therapy for children and adolescents with

SARS-CoV-2 infection; the limited number of studies available have generally included adult patients [6–10].

Remdesivir, a nucleotide prodrug analog that inhibits viral RNA polymerase, displays in vitro and in vivo activity against SARS-CoV-2 [1,11,12]. It has shown clear benefits in moderate-to-severe pediatric cases of COVID-19, especially for children of all ages with an increase in supplemental oxygen [13]. The use of remdesivir has been approved for pediatric patients by the US Food and Drug Administration and for adolescent patients ($\geq$12 years) by the European Medicines Agency (EMA) [14,15]. The criteria for applying remdesivir treatment are a weight of at least 40 kg and age of at least 12 years, the onset of disease a maximum of 7 days ago, the lack of need for additional oxygen, and the presence of risk factors for severe disease evolution [16]. In most cases, treatment with remdesivir has been evaluated in randomized clinical trials with adults. Regarding the safety of using remdesivir, a study carried out with adults highlighted biological changes, represented by increased serum creatinine and serum alanine aminotransferase values, as well as clinical side effects, namely, bradycardia, in addition to the mortality benefit [17,18]. Experience with the safety and efficacy of remdesivir use in pediatric patients is limited [19]. This article presents our experience with the use of remdesivir in 14 pediatric patients admitted with SARS-CoV-2 infection between 1 January 2022, and 30 September 2023. Remdesivir was prescribed according to the national medical protocol in use at the time of admission.

2. Materials and Methods

We performed a retrospective study of COVID-19-positive children admitted to the Pediatric Infectious Diseases Department of the Clinical Hospital of Pneumology and Infectious Diseases in Brasov, Romania, between 1 January 2022, and 30 September 2023. Patients aged between 1 day and 17 years received remdesivir during hospitalization. Informed consent for treatment, data sharing, and publication was obtained at admission in all cases from the legal guardians. The patients were deidentified, and the study data were extracted from existing hospital administrative databases or electronic medical records by the primary author. All patients were tested for SARS-CoV-2 infection at admission, using either RT-PCR or rapid antigen testing. The data collected were age, sex, time from onset of symptoms to admission, comorbidities or previous conditions, severity of SARS-CoV-2 infection, clinical manifestations, admission to the pediatric ward or pediatric intensive care unit (PICU), need for oxygen administration, length of stay, and clinical course and outcome. We also included significant laboratory data: WBC, CRP, and liver and kidney function tests (alanine aminotransferase ALT and serum creatinine, respectively), measured at the beginning and end of remdesivir treatment. As the normal values of the laboratory analyses show some differences depending on the age of the patients and the laboratory where it was performed (in our case, the laboratory of the Pediatric Clinical Hospital in Brasov and the laboratory of the Clinical Hospital of Pneumology and Infectious Diseases in Brasov), to obtain a homogeneous evaluation of the changes in the analyses, a qualitative assessment was made of, respectively, a normal, low, or increased value. The severity or clinical form of COVID-19 was established according to the standard protocols used at the time of the patient's admission [20,21]:

- mild form: general or upper respiratory tract symptoms, without manifestations evocative of pneumonia, without lung damage;
- medium form: patients with imaging-confirmed pneumonia, but without hypoxemia (with no respiratory impairment before the current illness);
- severe form: respiratory distress with oxygen saturation below 94% in atmospheric air and imaging abnormalities suggesting lung damage;
- critical form: patients with severe respiratory failure requiring ventilatory support, septic shock, or multiple organ dysfunction.

According to the same protocols, children with a history of chronic diseases (neurological pathology, genetic syndromes including trisomy 21, obesity, chronic cardiopulmonary

diseases), immunocompromised children, and older adolescents (aged over 16 years) could be considered at high risk of severe disease.

Remdesivir doses were administered according to EMA recommendations: 5 mg/kg on the first day and 2.5 mg/kg on the following days [22].

3. Results

We identified 246 COVID-19-positive children who were admitted to our department between 1 January 2022 and 30 September 2023. Of these, 148 (60.16%) were younger than 1 year, and 98 (39.84%) were older than 1 year. The diagnosis of SARS-CoV-2 infection was obtained at admission using either the RT-PCR test or the rapid antigen test. Only 14 (5.69%) patients, representing our study group, received remdesivir (Table 1). In the remdesivir group, 7/14 (50%) cases presented various comorbidities (recurrent wheezing, congenital ichthyosis, congenital heart malformation) or had a history of acute, predominantly infectious conditions (acute tonsillitis, bronchiolitis, pneumonia, infectious mononucleosis). The interval from the onset of disease to hospitalization was less than 3 days, with five patients presenting the same day and six patients the day after the onset of symptoms (Table 2). No patient required initial admission to the PICU. The clinical picture was dominated by fever (13/14, 92.85%) and cough (8/14, 57.14%). Dyspnea was rare (4/14, 28.57%) and always associated with pneumonia, bronchiolitis, or laryngitis. Only 4/14 (28.57%) children had digestive symptoms (vomiting or diarrhea). The initial clinical picture was represented by convulsions in one patient and syncope in another (Table 3). During the clinical course, 13 children had documented pneumonia, one patient developed bronchiolitis, and another four had acute laryngitis, all associated with pneumonia. In two patients, bacterial coinfections were diagnosed by RT-PCR from the respiratory secretions: two cases of infection with *Streptococcus pneumoniae*, associated with *Haemophilus influenzae* in one case.

Regarding the laboratory data (Table 4), the WBC level was normal in 12/14 (85.71%) cases, with associated lymphopenia in 8/14 (57.14%). CRP levels were measured at admission in 11/14 (78.57%) patients and were elevated in only 2/11 (18.18%). Alanine aminotransferase (ALT) values at admission were measured in 12/14 (85.71%) patients and were normal in 11/12 (91.66%); only one child had elevated values, but he had also experienced a recent episode of infectious mononucleosis with associated hepatitis. Serum creatinine was normal on admission in all patients for whom it was measured (11/14, 78.57%).

Remdesivir treatment was initiated at the onset of hospitalization in 3/14 (21.43%) patients and after 1–6 days in the remaining 11/14 (78.57%). The duration of remdesivir treatment varied, from 1 day in a patient who required transfer to PICU the day after admission to 7 days in a patient who presented with a severe clinical form of COVID-19, with associated pneumonia, bronchiolitis, and coinfection with *S. pneumoniae*. Remdesivir was administered to most of the patients (8/14, 57.14%) for five days. The patients received other simultaneous therapies. Parenteral corticosteroid therapy was administered to 12/14 (85.71%) patients, and antibiotics were also given to 12/14. Remdesivir, cortisone, and antibiotic-associated therapy were applied to 10/14 (71.42%) patients.

The clinical course of patients treated with remdesivir was favorable in 12/14 (85.71%) patients, with discharge in an improved condition after variable periods of hospitalization (Table 5). Oxygen administration was required in just 2/14 (14.28%) cases: one with associated pneumonia and bronchiolitis and the second with associated pneumonia and laryngitis. The severity of COVID-19, as well as the need for associated antibiotic or cortisone treatment, influenced the duration of hospitalization. Two patients required transfer to the PICU: one for worsening bronchiolitis and the requirement of oxygen after 12 days of hospitalization and the other after displaying a rapid worsening of associated acute laryngitis after the first day of hospitalization.

Table 1. Distribution by gender and age group of pediatric patients diagnosed and admitted with COVID-19.

	Gender		**Treatment with Remdesivir** *n* **(%)**
	Male *n* **(%)**	**Female** *n* **(%)**	
Infants and newborns	66 (44.59%)	82 (55.41%)	8 (5.41%)
Children aged 1–17.9 years old	70 (71.43%)	28 (28.57%)	6 (6.12%)
Total	136 (55.28%)	110 (44.72%)	14 (5.69%)

Table 2. Epidemiological and clinical characteristics of pediatric patients diagnosed and admitted with COVID-19.

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Age (Year, Month)	5 M	4 M	2 M	1 Y 1 M	1 Y 2 M	3 M	2 Y	15 Y	1 Y	4 Y	5 M	1 Y 10 M	2 M	5 M
Gender	M	M	M	F	F	F	M	M	M	M	M	F	M	F
Onset: Admission Interval (days)	1	Same day	Same day	1	Same day	1	2	Same day	2	Same day	1	3	1	1
PPH	Recurrent wheezing	-	*Enterococcus* spp. infection		Congenital ichthyosis	-	Acute angina Urinary infection	-	Bronchiolitis Pneumonia	-	-	Mononucleosis with hepatitis	Congenital heart malformation	-
Fever	X	X	-	X	X	X	X	X	X	X	X	X	X	X
Cough	X	X	X	X	X	-	X	-	X	-	-	X	-	-
Dyspnea	X	-	-	X	-	-	-	-	-	-	-	X	-	X
Nasal obstruction	-	-	X	-	-	-	-	-	-	-	-	-	X	-
Vomiting	-	-	-	-	-	X	X	-	X	-	-	-	-	-
Diarrhea	-	-	-	-	X	-	-	-	X	-	-	-	-	-
O_2 administration	X	-	-	-	-	-	-	-	-	-	-	-	-	X
Other	-	-	-	-	-	-	-	Syncope		Convulsion Headache				

Legend: M—month, Y—year, PPH—Personal Pathological History, M—male, F—female, X—present, "-"—absent.

Table 3. Clinical characteristics of pediatric patients diagnosed and admitted with COVID-19.

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Clinical form of COVID-19														
Mild								X						
Medium		X	X	X	X	X	X		X	X	X	X	X	
Severe	X													X
Associated diseases														
Pneumonia	X	X	X	X	X	X	X		X	X	X	X	X	
Bronchiolitis	X													
Laryngitis		X		X								X		X
Others			Conjunctivitis Alergo-dermitis			Anemia	Anemia							
Admitted to ICU														
	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
SARS-CoV-2 Infection Confirmation														
RT-PCR	X		X											X
Rapid Antigen Test		X		X	X	X	X	X	X	X	X	X	X	

Legend: ICU—Intensive Care Unit. X—present, "NO"—Not admitted to ICU.

Table 4. Laboratory data and therapeutic data of pediatric patients diagnosed and admitted with COVID-19.

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Leucocytes	N	N	N	N	N	D	N	N	N	N	N	N	D	N
Lymphocytes	N	N	N	N	D	D	D	D	N	D	D	N	D	D
CRP	N	D	N	N/A	N/A	N	N	N	I	N	N	N/A	N	N
ALT (TGP) at admission	N	I	N	N/A	N/A	N	N	N	N	N	N	I	N	N
Serum creatinine	N	N	N	N/A	N/A	N	N	N	N/A	N	N	N	N	N
Onset: Administration of Remdesivir interval (Days)	6	1	Same day	1	1	2	3	Same day	3	Same day	2	5	1	2
No of days of Remdesivir administration	7	5	2	1	5	5	5	3	4	5	5	4	5	5
Corticotherapy	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Antibiotics	Yes	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes

Legend: CRP—C Reactive Protein, N—Normal Value, I—increased value, D—decreased value, N/A—not assessed.

Table 5. Evolutive data of pediatric patients diagnosed and admitted with COVID-19.

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Clinical evolution after Remdesivir administration														
Improvement and discharge from hospital		X	X		X	X	X	X	X	X	X	X	X	X
COVID-19 worsening and transfer	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Worsening of the associated disease and transfer to PICU	X Bronchiolitis		X Laryngitis											
Hospitalization days														
n	12	5	2	1	8	7	8	3	14	5	5	6	9	6
ALT (TGP)—at discharge														
n	N	N	N	N/A	I	I	N	N	N	N/A	N	D	N	N
Serum creatine														
n	N	N	N	N/A	N	N	N	N	N	N/A	N	N	N	N

Legend: N—Normal Value, I—increased value, D—decreased value, N/A—not assessed, X—present.

4. Discussion

The mechanism of action of remdesivir functions by inhibiting viral replication through the premature termination of RNA transcription following binding to a viral RNA-dependent polymerase [12,23]. The antiviral effect of remdesivir on some RNAs was demonstrated by studies conducted before the SARS-CoV-2 pandemic. Furthermore, clinical studies have highlighted the usefulness and safety of using remdesivir in various contexts, including in Ebola virus infection [24,25].

For the treatment of SARS-CoV-2 infection in children, remdesivir is recommended for patients meeting certain conditions: they must be at least 12 years old and weigh at least 40 kg, the onset of symptoms must be at least 7 days ago, they must not need additional oxygen administration, and they must present risk factors for severe disease evolution [26]. The dosage differs according to the weight of the patient. For those weighing over 40 kg, 200 mg is administered on the first day and then 100 mg/day in a single dose. For patients weighing less than 40 kg (3–40 kg), the dose is calculated according to weight: 5 mg/kg on the first day and then 2.5 mg/day in a single dose (10,25). In adults, the initiation of remdesivir treatment in the first 7 days of the disease has been associated with a decrease in mortality at 28 days and a reduction in the need for mechanical ventilation [27].

Studies on remdesivir treatment in children and adolescents were carried out at the beginning of the COVID-19 pandemic in the context of its compassionate use [28,29]. Goldman et al. conducted a study on a group of 77 pediatric patients from six countries who underwent treatment with remdesivir for severe forms of COVID-19 between 21 March 2020, and 22 April 2020. The patients were of various ages, with a median of 14 years, and 79% had at least one comorbidity. Of these children, 90% required additional oxygen from the beginning, and 51% needed invasive ventilation. By day 28, 83% had recovered, and 73% were discharged. Of the children who required invasive ventilation, 80% recovered, and 67% were discharged. Of the four recorded deaths, three were interpreted as being caused by SARS-CoV-2 infection. The study highlighted the good level of tolerance to remdesivir therapy, the high recovery rate of the treated children, and a low incidence of serious adverse events (16%) [28].

Mendez-Echevarria et al. published a study conducted on eight infants and older children with severe COVID-19 who received remdesivir [29]. Six required admission to the PICU, five required mechanical ventilation (14 to 23 days), and one case required noninvasive ventilation. The evolution was favorable in seven patients. Patient 8 presented with multifactorial renal failure and died of COVID-19 and severe complications 10 days after remdesivir administration, initiated late after the onset of SARS-CoV-2 infection symptoms. No increase in liver enzymes was found in any patient.

A narrative analysis type of study, published in 2021 by La Tessa et al., discussed that at that time there were few studies on the use of remdesivir in the treatment SARS-CoV-2 infection in children, especially in severe or critical forms of the disease. They also noted the effectiveness of remdesivir therapy, administered as early as possible in the course of the disease, as well as the low rate of serious adverse events [30].

A study of a large number of children hospitalized with COVID-19 in the intensive care unit in 2020 found that most received therapies targeting the SARS-CoV-2 virus, including remdesivir, despite little data at the time on the use of new antiviral agents in pediatric patients [31]. Another multi-center study, conducted on 52 patients with COVID-19, highlighted that remdesivir treatment was generally well-tolerated; 82% of patients were discharged, and many showed clinical improvements based on a 7-point ordinal scale [32]. Adverse events were recorded in 21 patients, comprising acute renal failure, increased serum alanine aminotransferase values, hyperglycemia, and increased blood pressure.

Data from the specialized literature suggests that remdesivir treatment is safe, as demonstrated by the lack of adverse effects directly attributable to the drug, in the treatment of pediatric SARS-CoV-2 infections. Patients who received multiple doses of remdesivir had fewer symptoms and lower median World Health Organization Ordinal Scale scores for clinical improvement [12]. The use of remdesivir was also correlated with a reduced

need for invasive mechanisms of additional oxygen administration. Less than a quarter of patients required an increase in oxygen support while receiving remdesivir [12,33,34].

In our geographical area, one paper analyzed the use of remdesivir in children. The study was performed in Bucharest and showed that the use of remdesivir in the treatment of COVID-19 in children was not associated with serious adverse reactions [34].

Our study evaluated the efficiency and safety of remdesivir treatment in 14 pediatric patients aged between 2 months and 15 years, who were diagnosed with mild (1 patient), moderate (11 patients), and severe (2 patients) forms of COVID-19. Remdesivir was used in only 5.69% of all children hospitalized with COVID-19, in similar proportions in infants and children over 1 year old (5.41% of hospitalized infants and 6.12% of hospitalized patients aged over 1 year). A high proportion of young infants received remdesivir (7/14, 50%), the youngest aged 2 months.

Hospitalization occurred early after the onset of symptoms in most patients. Only one child was hospitalized three days after onset. The speed with which the diagnosis of SARS-CoV-2 infection was established is notable, occurring in all cases on the day of admission, regardless of the test used for the diagnosis. This allowed the rapid establishment of treatment, including remdesivir, in most cases. In just two patients, the remdesivir treatment was initiated five and six days after the onset of symptoms due to the aggravation of symptoms attributed to SARS-CoV-2 infection. The presence of some comorbidities, along with young age and clinical form, determined the decision to treat with remdesivir. All patients were initially hospitalized in the pediatric ward, not the PICU.

Notably, the dominant clinical manifestations were uncharacteristic of COVID-19, namely, fever and cough, which are symptoms also found in other respiratory diseases of various etiologies. Testing for SARS-CoV-2 was imposed in a pandemic epidemiological context, a situation that must be considered in the future, to properly manage cases. The patients were also tested by RT-PCR of respiratory secretions for other respiratory, viral, and bacterial infections. No viral coinfections were identified, but bacterial coinfections were identified in two children: one with mono-infection with *S. pneumoniae* and one with double infection with *S. pneumoniae* and *H. influenzae*.

Only two patients, with severe clinical forms, required administration of oxygen with a simple face mask at the time of hospitalization. One, a 5-month-old infant with associated pneumonia and bronchiolitis, coinfection with *S. pneumoniae*, and a background of recurrent wheezing, was transferred to the PICU on the day of admission. The second patient who required oxygen from admission, with a medium clinical form of COVID-19 associated with bronchiolitis, evolved favorably, with discharge. This suggests the need to correctly evaluate the involvement of various associated pathologies in a certain evolutionary modality in patients with COVID-19.

The analysis of changes in serum leukocytes revealed interesting information. Most patients had a normal number of leukocytes, and lymphopenia, a marker of the severity of the SARS-CoV-2 infection, was identified in only some of the patients: one with a mild form, one with a severe form, and six with moderate forms of the disease. In only two cases was lymphopenia associated with leukopenia, in two patients with moderate forms of the disease. These findings strengthen the evidence from the specialized literature, according to which dynamic monitoring of the number of lymphocytes is required to assess the risk of severe evolution [35].

To identify possible adverse effects of remdesivir, the values of ALT and serum creatinine were analyzed at the initiation and completion of treatment. These two analyses were not performed at admission or at the end of treatment for all patients, for administrative reasons. The patients had normal ALT values at the beginning of antiviral treatment in most of the investigated cases; only one had elevated values. After completing the treatment with remdesivir, normal values were found in 9/12 patients, with increased values below two times the normal value in two patients. In the patient who had an increased value initially, it remained increased but decreased compared to the initial level. These data must be interpreted with caution because the patients simultaneously received other potentially

liver-toxic medications, namely, paracetamol (acetaminophen), which is frequently used in the treatment of fever. Serum creatinine had normal values at admission in the patients for whom this analysis was performed and remained within normal limits even after the completion of the antiviral treatment, reflecting the lack of renal toxicity among the patients in our study group.

Patients in the study received etiological treatment for SARS-CoV-2 infection with remdesivir. The initiation of therapy with remdesivir fell within the interval recommended by specialist forums: in the first seven days from the onset of the disease. Twelve patients received remdesivir within three days of the onset of symptoms. This was due to their rapid presentation to the hospital, early diagnosis, and rapid assessment of the need for antiviral treatment. The duration of remdesivir treatment varied: eight patients received five days of treatment; five patients received between one and four days; only one patient, with severe COVID-19 and associated pneumonia and bronchiolitis, received remdesivir for seven days. No patient experienced a hypersensitivity reaction to remdesivir. All these findings suggest that both the timing of the therapeutic intervention and its duration represent important factors for the evolution of the disease.

The patients in the study group mostly benefited from other simultaneous treatments, comprising corticotherapy (12/14) and antibiotics (12/14). The associated therapies may have contributed to the favorable evolution observed in most patients, namely, the discharge of 12 of them, after variable periods of hospitalization due to COVID-19 and associated diseases.

Limitations: Our study had several limitations. In terms of the study type, our study was a retrospective, single-center study in which a small number of children patients received treatment with Remdesivir. In addition, the analysis was performed on the existing medical record, which was in some cases incomplete in terms of laboratory investigations. Due to the complex treatment administered to children with COVID-19, which also included corticotherapy and antibiotic therapy, the conclusions regarding the efficacy and amplitude of side effects of remdesivir as a single therapy should be used with precautions. Another limitation that should be mentioned in terms of treatment with Remdesivir in children is that each patient should be treated in concordance with the particularity of the case. The approach of combined therapy should be analyzed taking into consideration any aspects that could further influence the condition of the patient.

5. Conclusions

Our study contributes to the development of medical experience regarding the usefulness of remdesivir treatment in pediatric patients with COVID-19. Remdesivir contributed to the favorable evolution of SARS-CoV-2 infection in the majority of patients and was safe in terms of adverse effects. Further studies are required to support the efficiency, tolerability, and safety of remdesivir in children.

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Pharmacological Management of Cholera: A Century of Expert Opinions in *Cecil Textbook of Medicine*

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Background: Cholera is a potentially lethal diarrheal disease produced by *Vibrio cholerae* serotypes O1 El Tor and O139. Known since antiquity, the condition causes epidemics in many areas, particularly in Asia, Africa, and South America. Left untreated, the mortality may reach 50%. The crucial therapeutic intervention is intravenous or oral rehydration and correction of acidosis, dyselectrolytemia, and renal impairment. Antibiotic use represents the main pharmacological intervention.

Study Question: What are the milestones of the antibiotics use recommended by experts for the pharmacological management of cholera in the past century?

Study Design: To determine the changes in the experts' approach to the management of cholera and particularly the use of antibiotics as presented in a widely used textbook in the United States.

Data Sources: The chapters describing the management of cholera in the 26 editions of *Cecil Textbook of Medicine* published from 1927 through 2020.

Results: Sulfonamides were recommended in 1947, followed by the introduction of tetracyclines, chloramphenicol, and furazolidone in 1955. The options were restricted in 2000 to doxycycline. In the past decade, patients infected with strains known to have a degree a resistance to tetracyclines were treated with azithromycin or ciprofloxacin. Antibiotic use decreases the volume of stool and the duration of diarrhea but has not been considered lifesaving. Drugs with antimotility, antiemetic, or antisecretory properties are not useful.

Conclusions: The utility of antibiotic use in cholera has been endorsed by experts, but only as an adjunct to rapid and complete fluid and electrolyte replacement.

Keywords: cholera, pharmacological management, antibiotics

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S. Hassoun and F. Leasu have contributed equally to this work.

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BACKGROUND

Cholera, a fecal-oral infectious disease, remains a major public health problem, especially in countries from Asia and Africa, and can affect both locals and people who move to those areas for various reasons, from lucrative activities to tourism.[1]

Known since antiquity, cholera is characterized by a large geographical distribution, a risk of reintroduction into the territories from which it was eradicated, the possibility of persisting for a long time without causing infection, but also by major public health

problems induced by the short incubation period and the risk of rapid occurrence of a large number of cases. From the point of view of public health, the disease has entered a new era, in which attempts are made to ensure the control of the disease and even its eradication.[2,3]

Cholera is an indicator of socioeconomic inequities, of the lack of respect for human rights to access decent living conditions, being found, both for morbidity and mortality, in countries where, along with poor infrastructure, we have at least 1–2 other economic and social risk factors, such as conflicts, poor infrastructure, malnutrition, and a health system unable to update.[4,5] At global level, the Global Task Force on Cholera Control was established. This body has already developed the strategy until 2030, the main preventive direction still remaining WASH: water, sanitation, and hygiene.[6]

According to WHO, although the target internationally proposed is to reduce local transmission in at least 20 countries, in the past 3 years, there has been an increase in cholera cases. Compared with 2021, in 2022, the number of countries that reported cholera cases increased from 23 to 29, being reported even a higher number of cases than in the previous period and outbreaks in countries that had not previously reported such situations in past years.[7]

Cholera is still a condition that affects a large number of people. Although the actual number is unknown, it is estimated that more than 2.5 million people are affected annually and more than 90,000 die.[2]

Cholera poses major care challenges especially because it occurs in countries with poor health systems, where both drinking water provision and capacity to provide sanitation or patient care are limited. The true impact of cholera, mortality, and actual morbidity are not really known. At the beginning of an outbreak, the number of deaths is higher than later when adequate rehydration measures begin to be taken, but also during that period, the reported deaths include only those occurring in health facilities, without taking into account those occurring in places other than health facilities.[8,9]

Produced by cholera vibrion, the disease evolved in the form of 7 pandemics (1817–1824, 1827–1849, 1852–1859, 1863–1879, 1881–1896, 1899–1923, respectively, and 1961 to date). The most common serotypes that cause disease is *Vibrio cholerae* O1 (the 7 pandemics) or O139, biotype O1 El Tor (the seventh pandemic), being discovered different variants of alleles, ctxB1 represents the classic biotype O1, ctxB3 the El Tor biotype, and ctxB7 the recent El Tor variants.[7,10]

Cholera is a disease whose spread is known since antiquity, with writing evidence from Greece and India. It also represents a disease whose spread has been reduced based on a correct epidemiological investigation by John Snow (1854).[10,11] Cholera vibrion was discovered in 1854 by Filippo Pacini, but it was not recognized its importance until 1883 when Robert Koch managed to establish a connection between the agent and the disease; cholera toxin was discovered in 1959 by Sambu Nath De, thus explaining how vibrion works in the intestine.[10,12] However, the major change came when Rogers introduced intravenous hydration.[13]

Cholera, a potentially fatal condition in the absence of rapid intervention, is manifested by diarrhea, most commonly unaccompanied by fever and severe dehydration, as a result of hydroelectrolyte losses through diarrhea that can reach 13.5 L/day for adults and 368 mL/kg body weight in children.[7] The patient in serious condition also has changes in hematocrit, hyponatremia, and other changes related to dehydration (increased concentration of nitrogen, creatinine, calcium, and magnesium in the blood), but the most severe complication remains hypovolemic shock leading to death. Another serious complication, especially in children, is hypoglycemia.[14]

The risk of serious illness varies depending on a number of factors such as infective dose or route of exposure. So, it becomes important to diagnose as soon as possible (with rapid tests), based on which to institute treatment as soon as possible.[3]

Rehydration and hydroelectrolyte rebalancing are the basis of treatment, often requiring large amounts of intravenous solution and even more than 1 intravenous line. The introduction of intravenous solutions, accurate assessment of losses by methods such as Watten elbow to restore water-electrolyte balance, was a step forward in reducing mortality.

In fact, research related to cholera hydration, water, and electrolyte transport mechanisms has led to solutions that have not only saved the lives of cholera patients, but also those suffering from other forms of diarrhea, and especially children.[2,3,15]

Antibiotics are indicated only in serious situations or for people at risk (malnutrition, pregnancy, coinfections: HIV, malaria, diabetes, kidney failure, etc.), and unlike other diseases, it only has the role of reducing the duration of the disease and the period of contagiousness. Some research has shown that there is a beneficial effect of administering, especially to children, of 20 mg/d of zinc for 10 days.[16,17]

The use of antibiotics is also limited by the fact that *V. cholerae* has acquired resistance to aminoglycosides (streptomycin), amphenicols (chloramphenicol),

nitrofurans (furazolidone), or sulfonamides (cotrimoxazole), but these have been replaced by sensitive drugs, such as tetracyclines, doxycycline (tetracyclines), erythromycin, azithromycin (macrolides), or ciprofloxacin and norfloxacin (fluoroquinolones), multiresistance occurring by transfer of plasmids, integrous, or the presence of a megaplasmid.[18]

Protection by natural immunity or vaccination is limited to 5 years in case of vaccination and 3 years after infection with a homologous serogroup, cross-protection being asymmetric and incomplete; protection is provided by antilipopolysaccharide antibodies, the response to toxin being insufficiently documented.[7]

Worldwide, there are 3 types of oral vaccine available, which combined with 3 sanitation measures are supposed to reduce the frequency of diseases. High costs, inaccessibility, the need for boosters, and also the lack of clear evidence of their long-term effectiveness prevent those vaccines to be widely used.[3]

METHODS

Data sources

The primary data sources for this work were the chapters on the management of pulmonary tuberculosis in the 26 consecutive editions of *Cecil Textbook of Medicine*. The first edition of the textbook was published in 1927, whereas the latest became available in 2020. Secondary sources were publications retrieved from Medline that clarified technical issues related to the development, regulatory approval, and utilization of the drugs mentioned in the *Cecil Textbook of Medicine.*

Data collection and analysis

We started by identifying the pharmacological interventions proposed in the first edition of the textbook, after which we recorded the changes in subsequent editions. We also noted the year in which previously endorsed interventions were no longer recommended. Finally, we assessed the duration of latency periods during which the management was not changed.

RESULTS

Main findings

The main pharmacological advance in the management of cholera was the use of antibiotics. Sulfonamides were recommended in 1947, followed by the introduction of tetracyclines, chloramphenicol, and furazolidone in 1955, followed by the *tetracyclines period* which started in 1955 and has continued to the present day. *Rescue antibiotics* (ie, chloramphenicol, furazolidone, erythromycin, azithromycin, and ciprofloxacin) have been recommended for tetracycline-resistant *Vibrio* organisms (Table 1). Antibiotic use decreases the volume of stool and the duration of diarrhea but has not been considered lifesaving. Drugs with antimotility, antiemetic, or antisecretory properties are not useful, and no effective antitoxin serum exists.

The preantibiotic period (1927–1943)

The clinical presentation of cholera was well-established by the time Andrew Watson Sellards[19] from the Department of Tropical Medicine at Harvard Medical School described it in the first edition of *Cecil's*. Regarding the treatment, Sellards clearly outlined its priorities, as "(1) the relief of toxemia, (2) the restoration of fluids, and (3) the prevention of acidosis and uremia." He indicated that none of the many antitoxin sera preparations had been effective. The empirical use of kaolin suspension, which was believed to absorb the toxin present in the small bowel, was similarly considered to lack therapeutic value. For restoration of fluid, he recommended the intravenous administration of

Table 1. Chronology of pharmacological interventions for cholera recommended in *Cecil Textbook of Medicine* (1928–2020), correlate with the timeline of cholera pandemics

Antibiotic Class /Drug (s)		1927	1931	1934	1937	1942	1943	1947	1951	1955	1959	1963	1967	1971	1975	1979	1982	1985	1988	1992	1996	2000	2004	2008	2012	2016	2020
Sulfonamides	Sulfaguanidine							■																			
	TMP-SMX*																		■								
Tetracyclines	Oxytetracycline									■																	
	Tetracycline											■															
	Doxycycline																			■							
Nitrobenzenes	Chloramphenicol									■																	
Nitrofurantoins	Furazolidone															■											
Macrolides	Erythromycin																							■			
	Azithromycin																							■			
Fluoroquinolones	Ciprofloxacin																							■			

*Trimethoprim-sulfamethoxazole.

saline solution, starting with 2 L given over 15–20 minutes and continuing with 2 L every 2–8 hours for 1 or 2 days. Acidosis was treated with intravenous injections of sodium bicarbonate up to 90 g in the first day. Small doses of cocaine were used to control vomiting, and digitalis was believed to be very effective in "regulating the action of the heart." Sellards continued as the author of this chapter for 5 additional editions of the textbook without making any changes in the therapeutic protocol.

The antibiotic period (1947-present)

The use of sulfonamides was first mentioned by Francis Dieuaide[20] from Columbia University in 1947 and was considered of uncertain value. The only specific recommendation was for *sulfaguanidine*, administered at a dose of 5 g 4 times daily for 4–5 days, but only in patients whose urinary output had been restored after hydration. *Sulfaguanidine* was no longer recommended by 1963. The last mention of a drug from this class was *trimethoprim-sulfamethoxazole* in 1988.[21]

The most important change in the management of cholera occurred in the early 1950's following the discovery of tetracyclines. At the same time, other antibiotics were added as needed in cases in which the *Vibrio* organisms developed resistance to tetracyclines. The initial antibiotics of this period were *oxytetracycline* and *chloramphenicol*, mentioned in 1955.[22] The expert was skeptical, stating that there was no clinical benefit despite the rapid disappearance of the bacteria from stool.[22] Writing in the next edition, in 1959,[23] he acknowledged as possible benefits the reduction of the infection risks to other individuals and a shorter course of illness. By 1967, *tetracycline* was considered a standard component of the therapeutic intervention, because it shortened the duration of diarrhea and decreased the fluid requirements.[24] The drug was administered intravenously at a dose of 100 mg mixed in 1 L of IV fluid for a total of 500 mg in the first day of the diarrheal illness. The recommended dose was later modified to 40–50 mg/Kg/d divided in 4 doses and given for 2 days.[25] In 1992, *doxycycline* replaced tetracycline as the drug of choice, but mention was made of rapidly acquired resistance to it.[26] Macrolides and fluoroquinolones were added as rescue antibiotics by 2000. The current recommendations are for single dose treatments with doxycycline 300 mg, *azithromycin* 20 mg/Kg, or *ciprofloxacin* 1 g.[27] (Figure 1).

DISCUSSIONS

Cholera, intestinal infection caused by *V. cholerae*, known since ancient times, causing pandemics, clinically manifested by diarrheal stools produced by secretory mechanism, associated with different

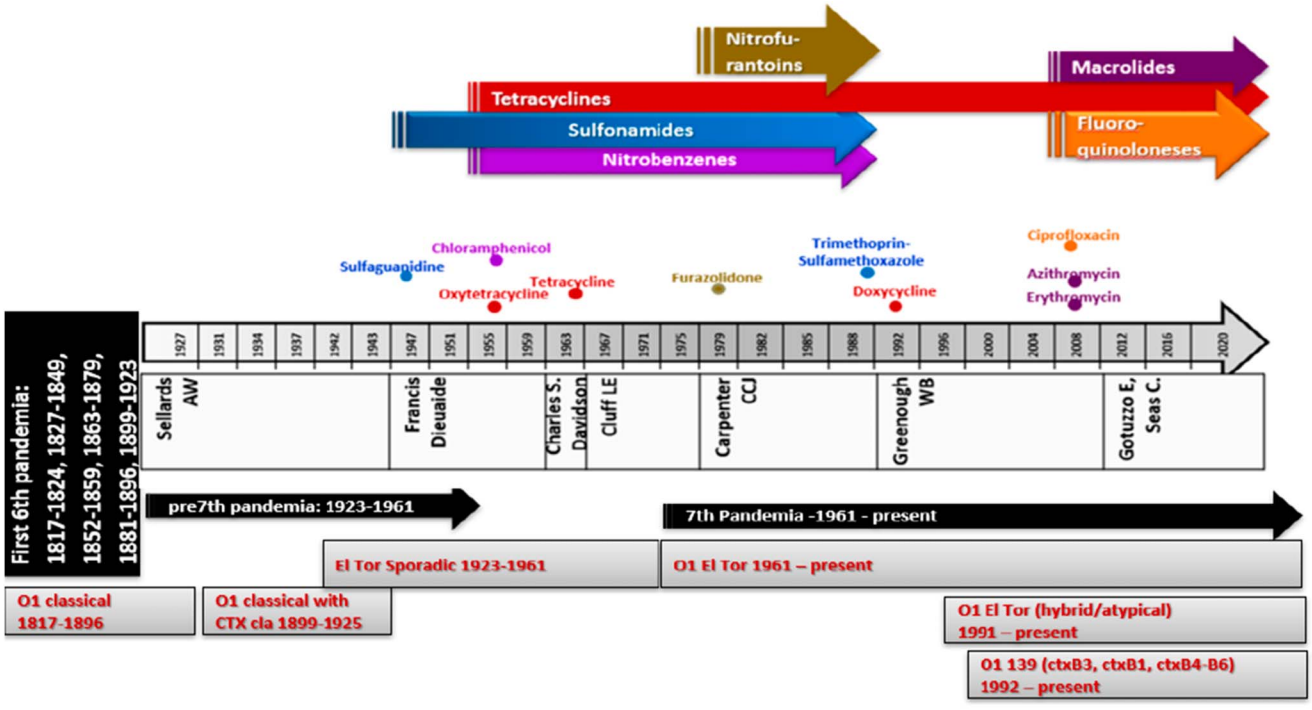

FIGURE 1. Chronology of drug administration for cholera recommended in Cecil textbook of Medicine (1928–2020).

degrees of acute dehydration, with various clinical forms, from asymptomatic and mild to severe, with risk of death within a few days, remains a major public health problem worldwide due to the large number of cases of disease and associated deaths, in many developing countries. The priority in the therapeutic management of the disease is the rapid replacement of hydroelectrolyte losses, together with etiological treatment with antibiotics when indicated.

Antibiotic therapy has a number of beneficial effects in the sense that it decreases the duration of disease evolution and improves the severity of the clinical image by reducing the volume of watery stools, which implicitly leads to a decrease in the amount of fluids needed to compensate for losses, with a favorable impact on financial costs. Etiological treatment also influences bacteriological healing, along with clinical healing, thus reducing the risks of environmental contamination.[28,29]

Over time, several classes of antibiotics, namely, tetracyclines, quinolones, and more recently, azithromycin, have been used in the etiological treatment of cholera.[29]

The use of these antimicrobials has been hampered over time, especially in recent years, by the emergence of antimicrobial resistance. Therefore, the WHO does not recommend their use in all cases of cholera, but only in those with severe dehydration, because of the risk of increasing resistance and limiting treatment possibilities.[30,31]

Resistance to multiple antibiotics and high resistance rates has led to the assessment of cholera vibrion as a multidrug-resistant pathogen or even extensive drug resistant (XDR), situation considered as a suggestive example of bacterial evolution.[29]

The emergence of multidrug-resistant pathogens and the decrease in the effectiveness of antibacterial therapy constitute a global threat to public health. In this context, antimicrobial resistance of cholera vibrion has become a global concern.[32]

Multidrug-resistant strains have been isolated from both patients and the environment, leading to the idea that antibiotic therapy should be recommended selectively and that complementary methods of cholera targeting should be found.[28,33]

The mechanisms by which *V. cholerae* acquires resistance to antibiotics are multiple. Active discharge of the drug from the bacterial cell by efflux pumps and preventing access to the target by decreasing membrane permeability are mechanisms involved in resistance to erythromycin, azithromycin, tetracycline, fluoroquinolones, chloramphenicol, and ampicillin. Resistance through structural modification of the antibiotic target occurs through accumulations of mutations in the gene coding for the target, with changes in the environment where antibiotic–target interactions take place and thus decreases the effectiveness of the antibiotic. This mechanism is found in quinolone resistance. Antibiotic inactivation is another antibiotic resistance mechanism, produced by its hydrolyzing, mechanism identified for macrolides, quinolones, and chloramphenicol.[29]

In addition, the biofilm that choleric vibrion forms for the purpose of protection from the environment, including antibiotics, favors and increases antibiotic resistance. This mechanism would explain the failure in some cases treated with antibiotic type ciprofloxacin and azithromycin, with particular reference to bacteriological clearance. In the same context, the possibility of antimicrobial escape associated with biofilm cannot be excluded.[34]

Tetracyclines were and still are among the most widely used antibiotics in the treatment of cholera.[35] Tetracycline-resistant strains have been reported worldwide, causing epidemics in some areas of the world.[36]

Resistance to tetracyclines is currently at high levels, which limits their effective use. Average resistance rates reach 50% for serogroup O1 for isolates worldwide. At the same time, it was highlighted that the tetracycline resistance rate is variable in different geographical areas, for example, in Asian countries between 25.7% and 66.7% and in African countries between 65.3% and 35%, which underlines the need for regional and local antibiotic sensitivity testing before initiating etiological treatment, to avoid therapeutic failures.[37]

The antibiotic therapy currently recommended in the treatment of cholera is addressed mainly to patients with severe forms of disease, pregnant women (regardless of the degree of dehydration), and people with comorbidities, who are at risk of severe evolution. Both in children younger than 12 years and in children older than 12 years, adults, and pregnant women, therapeutic regimens contain doxycycline as the first choice, with a single oral dose of 2–4 mg/kg, respectively, 300 mg. The alternative treatment option (alternate drug choice) is represented by azithromycin, also a single oral dose, at a dose of 20 mg/kg, respectively 1 g, or ciprofloxacin, a single oral dose, 20 mg/kg, respectively, 1 g.[3]

Concomitant administration of zinc may reduce the absorption of antibiotics, especially ciprofloxacin, so it is recommended to take it away from each other.[3]

Resistance to antibiotics used in the treatment of cholera (tetracycline, doxycycline, cotrimoxazole, ciprofloxacin, ampicillin, and chloramphenicol) has also been identified in isolates of cholera vibrion from

environmental samples (drinking water, storage tanks, wells, and seafood) in varying proportions.[33]

In the context of the emergence and expansion of cholera vibrion resistance to antibiotics, effective alternative therapies are sought in the adjuvant treatment of cholera, such as phage therapy[38–40] and probiotics. The study of the effects of probiotics in the therapeutic management of cholerae has yielded promising results. *Bifidobacterium animalis* BF052 has shown inhibitory activity against pathogens including Salmonella typhimurium and *V. cholerae*.[41] There are data that the use of probiotics could limit antibiotic use and thus bacterial resistance to antimicrobials.[42]

CONCLUSIONS

Antibiotic treatment has been and continues to be a valuable and effective therapeutic resource in the treatment of cholera, but it was unable to replace or reduce the importance of hydration. The current antibiotic resistance of cholera vibrions, varied in antibiotic classes and varying in intensity and regional spread, requires the assessment of sensitivity to antibacterial drugs before initiating etiological therapy, together with a correct decision regarding the criteria for prescribing this therapy. Studies of alternative pharmacological therapies are needed to limit antibiotic use in the threatening global context of growing bacterial resistance to antimicrobials.

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Article

Using IoT Assistive Technologies for Older People Non-Invasive Monitoring and Living Support in Their Homes

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Abstract: Many western societies are confronted with issues in planning and adapting their health policies due to an ageing population living alone. The "NOt Alone at Home—NOAH" project aimed to involve older people in the Agile co-creation of services for a collaborative monitoring and awareness notification for remote caregivers. Our research aim was to create a scalable and modern information system that permitted a non-invasive monitorization of the users for keeping their caregivers up to date. This was done via a cloud IoT (Internet of Things), which collects and processes data from its domotic sensors. The notifications generated by the system, via the three applications we developed (NOAH/NOAH Care/Admin Centre), offer caregivers an easy way of detecting changes in the day-to-day behaviour and activities of their patients, giving them time to intervene in case of abnormal activity. Such an approach would lead to a longer and more independent life for the older people. We evaluated our system by conducting a year-long pilot-study, offering caregivers constant information from the end-users while still living independently. For creating our pilot groups, we used the ABAS (Adaptive Behaviour Assessment System) II, which we then matched with the pre-profiled Behavioral Analysis Models of older people familiar with modern communication devices. Our results showed a low association between daily skills and the sensors we used, in contrast with the results from previous studies done in this field. Another result was efficiently capturing the behaviour changes that took place due to the COVID-19 Lockdown measures.

Keywords: AAL programme; REST; sensors; microservices; behavioural analysis models; ABAS; cloud; Agile

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1. Introduction

An ageing population is a vital aspect in planning and adapting public health policies within a society. The projects developed under the European Active Assisted Living (AAL) program aim to keep people connected, healthy and active until old age, under the slogan: "Ageing Well in the Digital World", providing older people with a safe, yet independent living environment. The most important aspect within this framework is the activities of daily living (ADL) component, a component with a significant impact on the adaptation of the environment to the older people's needs, taking into account also the psycho-social factors through human–technology interaction.

The research presented in the following section was motivated by the need to extend the time that older people spent actively living in their home, thereby reducing institutionalization. The Cloud/Edge modern technologies bring artificial intelligence and centralized resource-intensive computation in cooperation with distributed IoT (Internet of Things) WSAN (Wireless Sensors and Actuators Networks), which have become pervasive in smart buildings. The older-people-centered approach was supported not only by the Agile co-creation of assistive services, but also by the psychological assessment of volunteers based on the ABAS (Adaptive Behaviour Assessment System) methods of selection for the pilot groups and on Behavioral Analysis Models pre-profiling (BAM "tuning").

The Active and Assisted Living (AAL) program supports projects through which applied research on innovative ICT-based services is carried out and implemented for the benefits of aging well, targeted to older people, who are increasing in number and in the fully deserved esteem of modern society.

The AAL program financed the "NOt Alone at Home—NOAH" project, which contributes with solutions for older people's independent living, providing them the opportunity to manage their daily activities self-sufficiently. This can be done by ensuring a permanent connection of the end-users with caregivers who can help them (relatives, friends, professional caregivers) and by providing helpful tools to keep them safe and by providing them with increased confidence.

Being able to live independently also depends on subjective perceptions, such as feeling safe and comfortable while alone at home. For instance, in the AAL Strategic Research Agenda from March 2010 [1], a holistic list of requisites for independent life, was given, which includes aspects such as:

- a secure environment;
- constant contact with friends and family (to provide reassurance);
- physical, social and mental stimulation;
- knowledge that, if needed, carers will come to support them;
- relying on appropriate responses when in trouble.

We tried to address all these needs in the NOAH project, by developing a socio-technological system and embedding a practical, usable and accessible monitoring technology, which is not intrusive and is suitable for inspiring users' trust.

This was achieved by the implementation of the users' suggestions and feedback received during co-design sessions.

Our research aim was to create a scalable and modern information system that permitted a non-invasive monitorization of the users for keeping their caregivers up to date. This was done via a cloud IoT, which collected and processed data from its domotic sensors. The notifications generated by the system needed to offer caregivers an easy way of detecting changes in the day-to-day behaviour and activities of their patients. This would offer them ample time to intervene in case of abnormal activity, which would lead to a longer and more independent life for the older people.

The rest of the paper is organized as follows. In Section 2, we present the background and related works of our project within the AAL context and, based on these, the hypotheses to be proved by our work. Section 3 is dedicated to the NOAH solution, with the innovative platform, the unified Cloudant database and Kibana, as an advanced analytical tool. Section 4 shows the system architecture, prototyping and the microservices-based variant. In Section 5, the database is described, with the tables storing the data being detailed. Section 6 presents the sensors comprising the NOAH kit and its associated library and communication. Two mobile applications for the end-users and caregivers (NOAH and NOAHCare) and one web application for administrators (Admin Centre) are presented in Section 7. Section 8 contains a detailed explanation about the methods, instruments, procedure and data analysis used in the project. Section 9 evaluates the descriptive and correlation results and analyzes the differences between the periods. Section 10 is dedicated to the discussion and some limitations, and finally, in Section 11, the work finishes with some conclusions.

2. Background and Related Works

The AAL classification comprises four basic categories: smart homes, smart nurses, portable devices and robotics [2], integrated in different kinds of solutions enabled by technologies in areas such as health and care, living and building and safety and security.

All these categories have warnings about data security laws and common frameworks for interoperability, privacy, security management [3] and about confidentiality with information security and unauthorized threats to accessibility [4]. Data collection is based on the critical performance of Internet-aware technologies for monitoring older people, such as the city/home data capture layer, the centralized Cloud-based data, the management repository and the risk analysis and prediction module [5].

The Internet of Things (IoT) aims to integrate various sensors and applications that allow users to share information, data and resources and which can provide information about a person's level of functioning, by measuring and analyzing physiological and environmental parameters. These IoT systems are intended for use in areas such as health care, hospital health systems and safety and sound monitoring [6].

Such systems would trigger alarms in the case of some medical conditions that are critical for the user. For example, if the patient does not respond to a prescribed medication, the Cloud interprets data collected by different sensors and triggers an alarm, so the medical staff can intervene [7].

This type of architecture and use of that Cloud environment is a technology that has become more and more popular among researchers due to its great flexibility and power [8,9].

The context of IoT-based systems oriented to the monitoring of older people has been exploited in several works following a similar design and combining both hardware and software components of many forms, being implemented with different technologies. Many of these papers follow a similar pattern, consisting of a cloud-based architecture that involves sensors of any kind that collect data into the cloud to be processed and provided to the users involved.

In recent years, several research projects started using IoT-based systems that aim to monitor older people. These works follow a similar design and combine both hardware and software components but use a wide variety of technologies. Many of these papers follow a similar framework, consisting of a cloud-based architecture that involves sensors of any kind that collect data into the cloud. This data is then processed and provided to the users.

CoSHIE [10], for example, is a system presented that gathers data from non-invasive sensors through a home gateway into the cloud, where they are processed and stored in a non-relational database. However, it has some limitations regarding the direct monitoring of older people by their caregivers. Similarly, SW-SHMS [11] is a system separated into three main components: a user environment collecting data from wearable sensors through a gateway and a cloud datacenter to compute the data and provide information as alerts to the third component, which is a monitoring platform for the caregivers. The City4Age Project [12] is intended to support older people's daily life but on a different scale, being oriented also to outdoor monitoring. It is built on a similar architecture, which was enhanced by adding a behavioral analysis and risk management component. Additionally, Wang, Y. and Jang, S. [13] presented another way of implementing such a system and described it as being composed of a home gateway (RaspberryPi 3B+) directly connected with sensors (via Arduino UNO). The gateway communicates with a cloud component to save the data into a non-relational database and provide information to its users through mobile or web applications.

These projects mainly have the same goal, and even though they follow similar architectures, they combine different technologies and present some particularities for different topics. Nevertheless, in terms of development, these systems have been divided into more independent components, which can be implemented separately and incrementally, presenting a standard communication interface for interaction among them.

An important goal is the independent operation of these systems, reducing the strain on doctors (or specialized medical staff) by using biometric information obtained from users. This information could be used as a supplement to existing data acquired by relatives/caregivers in order to obtain a better image that enables comprehensive care for older people [14,15]. A possible integration of these systems could be reached using an interdisciplinary approach, by standardizing the data used on human-oriented studies. Two key directions in the implementation of IoT systems would be a) digitization of public health systems and enhanced security; social isolation prevention; improving quality of life and b) technology-based economic development for older people; development of new markets for smart devices; encouragement of private investment in the smart IoT [15].

All these references provide valuable information about the guidelines of the AAL program from the perspective of the use of sensors and IT equipment but less information about the involvement of older people in activities that require physical activities to maintain executive functions. Small positive effects were observed, regardless of health and physical activity, for the older people who were initially sedentary; they seemed to benefit from training interventions of exercises in relation to the executive function performed [16]. The effects of a sedentary lifestyle were observed, especially during the COVID-19 crisis, and one of the recommendations, especially important for the older population, was to maintain regular physical activity during self-isolation to prevent frailty and deterioration of health [17]. Together with these aspects of systems' interconnection and the interdisciplinary approach, the discussion remains open about the lifetime of using these methods and their benefits in long-term studies exploring effects and benefits [4].

The development and implementation of life improvement systems through AAL programs is an ongoing challenge. New data was continuously collected during and after the completion of the project [18]. In this regard, Calvaresi, D. et al. [19] indicated two clear needs in streamlining these systems by understanding and validating new solutions and re-evaluating the relationship between the system user and the proposed solutions.

Sensors are fixed in the same position on household objects (bed, refrigerator, living room, bathroom and bed) being able to detect human activity in the home. The sensors can analyze the activity levels of the older people in order to identify behavioral changes that can detect abnormal situations [20,21].

Co-creation allows the involvement of users [22] in a project team by using personal experience to design solutions using the ideas of future users in possible real solutions [23] which will contribute to user well-being [24]. In carrying out a co-design, the involved researchers must know the objectives of the project in order to match the development with them [25]. In the NOAH project, the participatory validation of ideas [26] was accomplished based on information generated in the context of "health and sustainability" for people lives. These users were encouraged to actively participate in the development of the future products [27]. The co-creation was carried out in successful AAL projects, such as "CaMeli" [28], "Rose" [29] and "Cognivitra" [30], by validating the results with the help of all users involved (end-users, caregivers and stakeholders).

The social evaluation and technical approach will be considered together for providing more coherence and displaying the interconnected nature of our methodology.

After reviewing the studies conducted on the topic mentioned above, with results presented in [19,20], we decided on the following research questions:

(1) Do domotic sensors have long-term relevance and utility?
(2) Did using the system lead to changes in the lifestyle of the older people participating in our pilot?
(3) Was the users' behaviour, in their homes during their daily live, affected by the Covid crisis?

3. Solution Overview

Our research was focused on improving elder people's independence and providing relief for caregivers based on technological designing and solution implementing.

The innovative platform developed within the project was implemented as an integrated solution for the use of mobile devices in a Cloud computing environment. This environment allows the import and distribution of developed applications, allowing users to access them directly.

The platform:

- Uses open-source software products, both in terms of programming languages, programming environments, tools, databases and architectures offered and as standardization, to ensure interoperability and interchange;
- Ensures the safe storage of data, the scalability of resources necessary for the correct and uninterrupted operation of applications and the possibility of using advanced data analysis services to obtain the necessary information, both from the analysis of stored data and from the devices from which they are collected;
- Offers the possibility to choose the desired software products and to optimize the maximum costs by the fact that only exactly what is used is paid (the Pay Per Use, PPU, principle).

The solution optimizes five important criteria: benefits, costs, flexibility, scalability and risks. It integrates services; proves usability; uses state-of-the-art devices and technologies (Section 4) and collects, stores and processes data (Sections 5–7), ensuring the security of the system regarding the use of devices.

According to its architecture, the NOAH system implements the client–server model, combining multiple technologies to achieve the best outcome. The core purpose of monitoring implies that there are sensors gathering data and feeding them to a server, where they are processed in several ways and, in the end, delivered to a final user (via mobile applications) to be interpreted [31,32]. For these features, the NOAH system takes advantages of Cloud technology, meaning that data collection, processing and delivery are achieved in the Cloud [11].

A unified database was designed, developed, and implemented using the IBM Cloud with Cloudant database. Basic algorithms were implemented for the behavioral analysis module (BAM) and the Cloud-based data mining application, using the IBM Watson IoT Platform and the Data Science Experience (DSX) tool. To analyze the data, Kibana was used to perform advanced analytical tasks. Kibana is an open-source analysis and visualization platform, used to monitor, search, analyze and visualize data through a variety of graphs, for example, charts and tables.

Artificial Intelligence tools were used to validate the sensory data in real time based on the model implemented with Watson Machine Learning.

The devices associated with the applications were tested throughout their development, using the tools provided by the IBM Watson IoT Platform, which provides REST APIs. The models offered by Watson were used for training, evaluation and implementation. We used IBM Watson Studio, offering a high degree of security, both in terms of operation and of persistence over time, and the possibility of recovery. This approach allowed us to offer critical notifications in case of abnormal readings from our sensors regarding the users' health and safety.

Feedback from the end-users gathered through co-creation sessions is a valuable tool, which facilitated the improvement of the project design. These sessions provided an important insight into the users' interaction with the system, and researchers received suggestions on how to better satisfy the needs of the older people and their caregivers. Furthermore, the feedback was successfully collected throughout the pilot run through a set of standardized questionnaires for assessing the users' satisfaction in all pilot sites.

With great support from the Brasov City Council's Department for Social Services, two groups of users were involved, one for the older people end-users and one for their caregivers, with each of them for their specific application. The aim of this co-creation phase was to identify their specific needs and see how useful it would be for them to use mobile devices and these applications. The same model was applied to both user groups, resulting in 14 older people and seven caregivers providing feedback on their own version

of the application. This led to a set of specifications and a set of redesign requirements adapted to users' needs that were later implemented. All these requirements improved the system, with some referring to the sensors' communication and data collection, while others referred to some of the application's features. After discussion sessions with the users, improvements were made for receiving the alerts from the sensors and other notifications and for the application itself, with the user now being able to select, from an improved configuration page, what features/modules they want to use or see.

The experience and results of the NOAH project provide a good basis for further applied research on the definition and implementation of new services for the benefit of older people, adding to issues related to personal safety and issues related to the security of their neighborhood. As a result, another AAL-funded project, "SAfety of elderly people and Vicinity Ensuring—SAVE", is currently underway.

4. System Architecture

The NOAH system was designed with Cloud technologies in mind, either being used as a software ecosystem (SaaS—*Software as a service*) or as infrastructure (IaaS—*Infrastructure as a service*). The main technologies within the NOAH system are Java, Android, MySQL, IoT, MQTT, JavaScript and REST API, all of which are Cloud-related.

There were two iterations of the design. The first (Figure 1—Var 1) had the purpose of developing the features required by the system as fast as possible and obtaining a prototype that could be evaluated with real users, without the need to develop a protocol and environment-related adapters. This variant worked in the SaaS context and made use of readily available tools, boilerplates and software systems in the IBM Cloud (*IoT Service* and *Compose for MySQL*), formerly known as Bluemix. The programming was done in a low-code environment (Node-RED, also offered by the IBM Cloud). The second variant (Figure 1, Var 2), working in the IaaS context, focused on the optimization of costs, scalability and performance. This variant required the development of the final form APIs (*Application Programming Interfaces*) and related software modules in general-purpose programming languages (Java, Python, etc.). The architecture of the latter was based on microservices 12 [33] and allows the deployment of the NOAH Cloud application in a containerization environment (such as Docker).

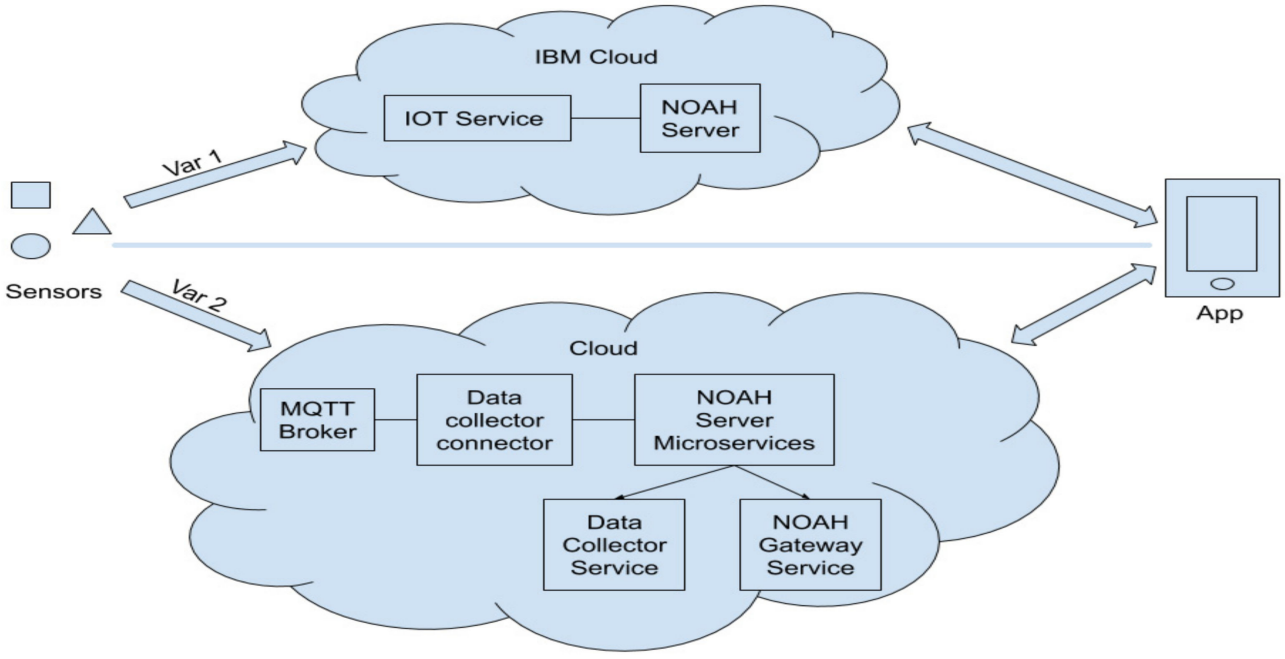

Figure 1. Architecture overview.

For both variants, the flow is the same: the sensors send their data through the Internet using the MQTT protocol (*Message Queuing Telemetry Transport*), secured by PKI infrastructure (certificate-based security and hybrid cryptosystems). An MQTT broker (in the prototyping phase, this was included in IBM Cloud's IoT Service) receives the data and forwards it to the collector software component (a web application that exposes an API), which communicates with the MySQL relational database management system, which retains the data. The user interface connects through dedicated REST APIs (*Representational state transfer APIs*) (the gateway component of the system) to the Cloud application. In the prototyping phase, the collector and the gateway were merged into a Node-RED web application. In the final implementation, they were two separate microservices.

The structure used to exchange data between the gateway and the user interfaces, formalized using the JSON (*Javascript Object Notation*) format, is identical for both variants of the architecture, such that the mobile applications can connect to both variants.

The security of these communication channels is ensured by the Public Key Infrastructure (PKI), the use of SSL/TLS certificates and by a hybrid cryptographic system. Regarding the user interfacing, the secured HTTPS protocol is involved, authentication is based on username–password pairs and authorization uses JSON Web Tokens (JWT).

The gateway component provides four types of information:

- User-related information;
- Alerts: warnings related to technical or non-technical aspects of the devices. Alerts provide details about the status of the sensors, the connection to the system or the battery level and situations in which the sensors are in a certain position, which is unnatural for a person's routine. Examples include situations in which the front door or the refrigerator door have been open for too long;
- Notifications: warnings related to changes in the behaviour of the monitored person. They are generated by the behavioral analysis module integrated in the system and represent an aspect of the person's way of life, which must be analyzed and on which it must be intervened with in an appropriate way;
- Statistical data: a log of change in the data taken from the set of sensors, over a certain period, which may be relevant for an analysis performed by specialized personnel.

The microservices-oriented architecture and the chosen protocols and technologies (Spring Boot) allow the deployment of the system on many different types of infrastructures (from a simple server to cloud services, such as IBM Cloud, Amazon's AWS, Microsoft's Azure, etc). Even if the prototyping was done on IBM Cloud, the final version of the services could run easily on other infrastructures. The IoT devices make use of the MQTT protocol, so they are not dependent on a particular proprietary technology.

4.1. Prototyping Architecture

The server side was developed and is hosted by the IBM Cloud, as shown in Figure 2. This requires the continuous running of two main services: *Internet of Things Platform* (IoT) and *Compose for MySQL*.

The IoT platform communicates with the registered equipment and has the role of collecting data from the sensors and transmitting them to the server application. The *Compose for MySQL* service provides support for storing and querying data needed to run the system.

The BAM (*Behavioral Analysis Module*), developed in Python, processes data from sensors to identify behavioral patterns and provides an estimate of the well-being of monitored individuals; the server application reads the BAM outputs and converts them into useful notifications or alerts for users.

The client part of the system is represented by two mobile applications: one for caregiver users (relatives, social workers, friends) and one for end-users (the older people). These applications are developed natively in Android, which can be used on a wide range of smart mobile devices (smartphone or tablet PC).

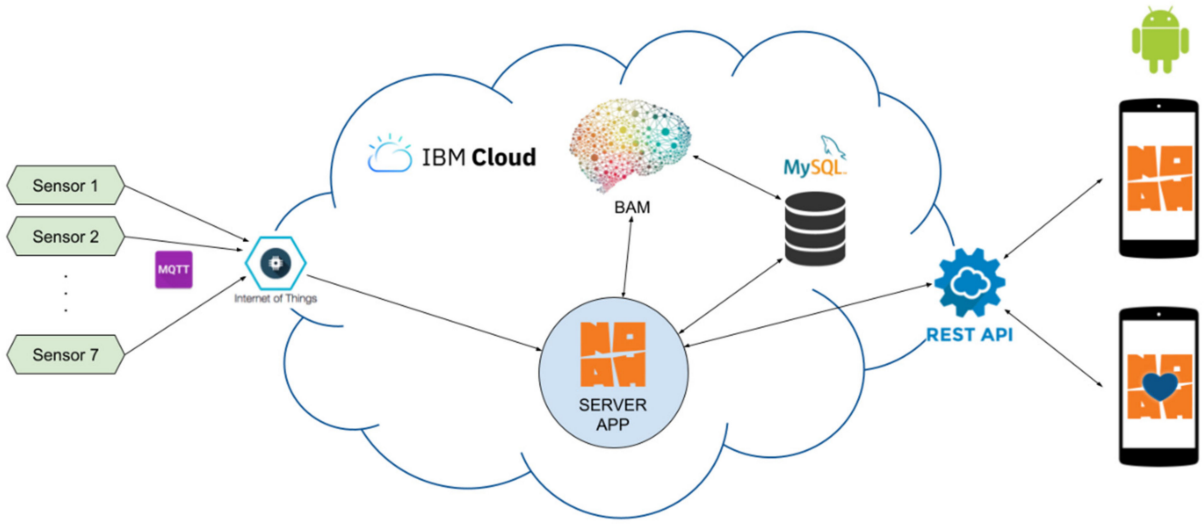

Figure 2. The architecture of the prototype system.

The communication between the application components, respectively, between the client and the server, is made through the REST type APIs on the HTTPS protocol. Android applications build HTTPS requests corresponding to user intentions and commands and interpret the response, providing information through the user interface.

Figure 3 depicts the flow of the collector implemented in Node-RED. Most of the blocks used to create the flow were readily available and needed minimal configurations. Some JavaScript was required to adapt and enrich the data structures. The collector includes a buffer for the current status of all the sensors, which is very useful for top performance in the mobile applications.

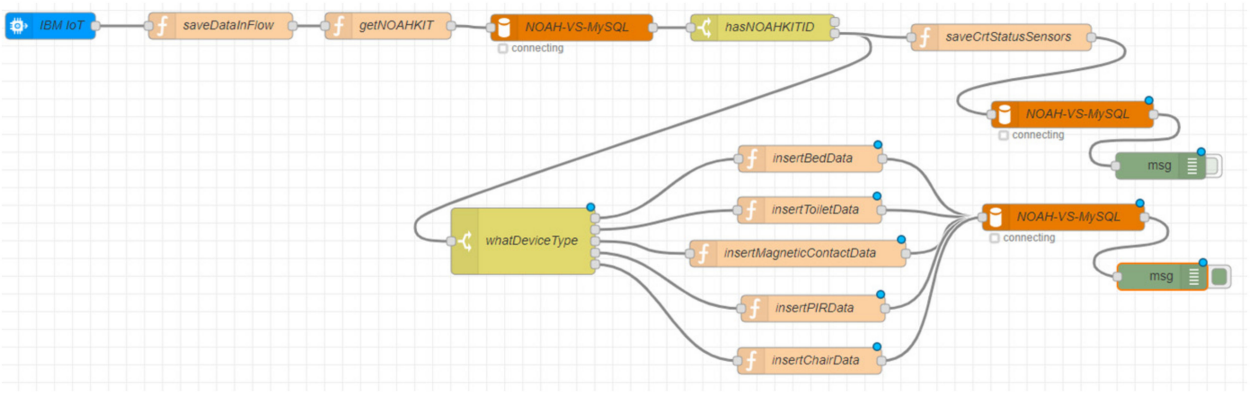

Figure 3. Collector flow in Node-RED.

4.2. Microservices-Based Architecture

The architecture implemented (see Figure 1) involves a server application built by interconnecting three microservices that fulfil different functionalities. Thus, there is a service that facilitates communication with the connected sensors by incorporating an MQTT broker and which, through a connector, sends the information to the second microservice, which collects this information and stores it. The third microservice is the interface between the server and the client, which exposes the paths for HTTPS requests that serve the information to Android mobile applications.

The server component of the NOAH system is a modular application, with the microservices being developed in different technologies. For instance, the communication part with the connected devices was developed in the Python programming language, and

the collection, data processing and customer service were developed in the Java language, using the Spring Boot framework.

This application aims to transfer and process data from the database and serve them in a standardized form in JSON format to Android applications for end-users and caregivers. When a request arises, a path is called to a resource that will process the request and make the necessary transformations to build the response.

5. Database

To store the data received from the sensors and other details required for running the NOAH Cloud application, the MySQL relational database management system was chosen.

The sensors are grouped into kits (tables *noah_kits* and *noah_kits_sensors*) and the sensor data are partitioned in different tables by kit; the structure of such a table (*NOAH_RO_00_log_values*) is given in Figure 4. The partitioning helps the access time performance, but it also helps with the users' data isolation.

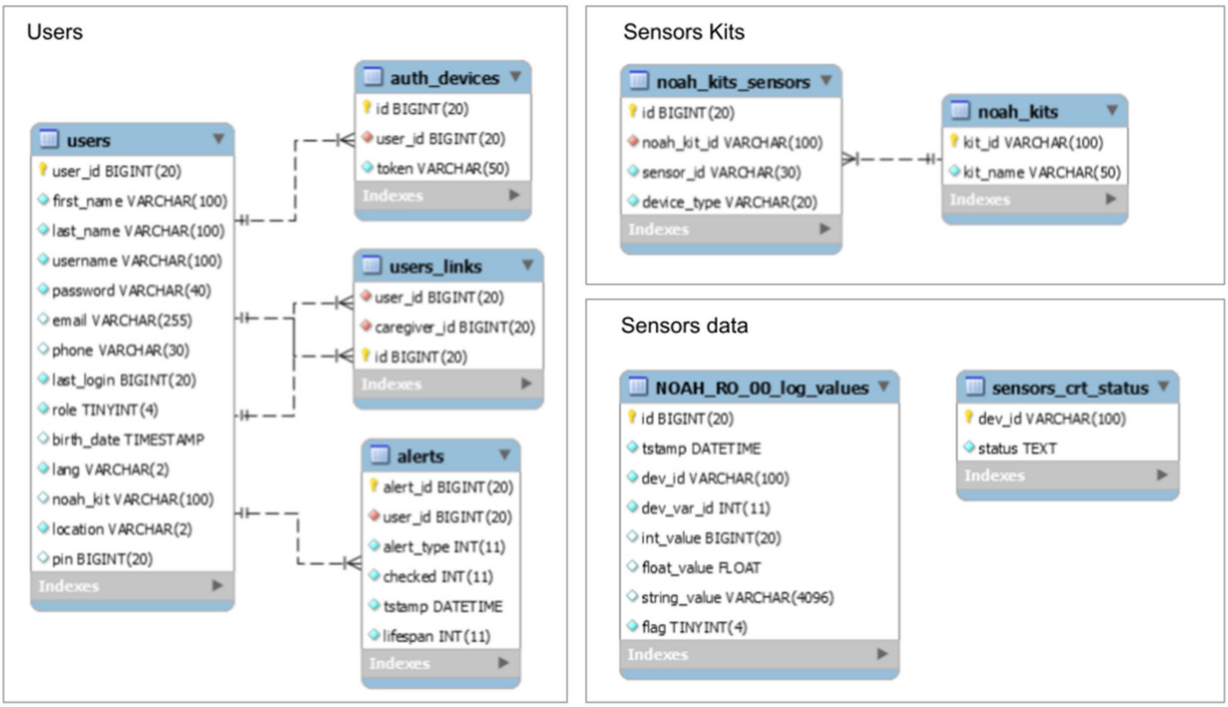

Figure 4. NOAH database.

The tables store data as follows:

- alerts: stores the alerts generated by the system for users (technical is related to the infrastructure, and notifications are generated, for example, by the BAM module);
- auth_devices: this is used by the auto-login system; when a user logs into the application (either for the caregiver or the end-user), a record with a unique device token is associated with the user; the record is deleted from the table at logout;
- contacts: each end-user can choose two contacts to call directly from the application, in case of emergency; this table stores data about these people;
- noah_kits: stores system-recognized kits (ID and name);
- noah_kits_sensors: contains data about all installed sensors (identifiers and types) and membership in a NOAH kit;
- {noah_kit} _ {number} _log_values (for example, NOAH_RO_00_log_values): stores the data obtained from the sensors associated with the corresponding kit;
- sensors_crt_status: keeps the latest values of the parameters of the sensors connected to the system;

- users: stores details about registered users and their preferences (for example, user interface language); if the user is an end-user, then he has an associated code, which allows caregiver users to associate it in the NOAHCare application, so they can view and receive data about it;
- users_links: contains the associations between caregiver users and end-users.

This solution optimizes the persistence of the collected data. With this partitioning logic, there is a visible link between sensors and their data, and there are no concerns about performance when querying the database to save or retrieve data to be processed. It also provides a faster way to aggregate data in order to build charts for the users.

From the performance perspective, there is also a table that stores the last received values from sensors to provides this information faster to the user, rather than a query for a large amount of data.

It must be highlighted that the collected data have no direct connection to the user's identity that they belong to in order to ensure the user's privacy and add a supplementary layer of security.

6. Sensors

Each NOAH kit consists of several IoT sensors:

- BED: pressure sensor for detecting when the end-user sleeps; it is placed under the mattress of the end-user's bed.
- CHAIR: pressure sensor placed under the cushion of the end-user's favorite chair/couch (that the older person usually sits on during the day, e.g., the one in front of the TV).
- TOILET: a presence sensor placed near the toilet, used to detect toilet usage.
- CONTACT: contact sensor (magnetic) that can be installed on doors or drawers (e.g., the fridge door, if culinary habits need to be monitored, or the entrance door of the home, if safety is concerned).
- PIR: presence sensor (infrared) for detecting the older people's presence in the living room.

The IoT components were developed especially for this project by WiMonitor SRL, Parma, Italy, for the University of Parma, a partner in the project. The sensing elements were readily available on the market (PIR, pressure sensor, magnetic contact) and were coupled with a WiFi-capable microcontroller development board. The internal details are proprietary to WiMonitor SRL. The estimation cost for installation is 900 EUR for each location in the case of large-scale implementation and a 50 EURO monthly subscription.

The sensors make use of a MQTT library to communicate with the MQTT broker included in the cloud application. The communication is encrypted using a hybrid cryptosystem and PKI's certificates. The data are sent in batches, at a fixed interval (1 h), to save energy and prolong battery life. However, it is possible to power the sensors using power adapters.

The values provided by the system are of type on–off (1–0) and are tagged with the timestamp of the event; only transitions from on to off and off to on are recorded. The sensors also send the value (charge) of the battery, to be able to generate alerts for replacing them. The different measurements provided by the sensors are identified through the *dev_var_id* field in the data tables (0 for the value of the sensor and 100 for the battery voltage).

The sensors' primary goal is to monitor the behaviour of the user, not to influence their life. However, by offering access to the data to the caregivers and users (via the visualization feature on the SAVE application), the system could lead to behaviour changes, the creation of healthy habits and a more active and fulfilling life.

7. Client Applications

The interaction among the users happens through three applications: Two mobile applications for the end-users and caregivers (NOAH and NOAHCare, respectively) and one web application for administrators (Admin Centre). These applications aim to provide

information about sensors' data and processing outputs as alerts or notifications, as well as managing the sensors within the system.

The main goal of the NOAH system is to gather data from the older people's environment and process it to information for their caregivers (formal or informal). The system provides statistical information about sensors' data, notifications in case there were some changes in the end-user's behaviour, and alerts about the environment (e.g., the door has been left open).

Depending on their role (caregiver, end-user or administrator), users can see the relevant information to their own interest. The end-users choose which caregiver to trust, by furnishing them with a secret PIN that they must input into the NOAHCare app; thus, they decide who is able to see their data.

Access to the system's features (through UIs) is granted on roles (rights), and the authentication is done using the username–password pair.

7.1. NOAH Application

The NOAH application is developed for older people in order to receive a series of useful alerts (e.g., when a fridge door is left open). They have the possibility to send feedback to the system about how they are feeling in a certain moment, to set two contact persons and call them when needed and see general statistics about their sensors.

The NOAH application consists of four main features:

- End-user authentication: To authenticate to NOAH app, an end-user must submit the credentials received from a system administrator.
- Contacts: An end-user has the possibility to set two contacts from his/her phone contact list (Figure 5) to be set as quick dial options within the app homepage.
- Alerts: An end-user will receive alerts describing some events happening in his/her environment, such as the entrance door being left open (Figure 6).
- Feedback—An end-user can provide feedback about how he/she is feeling in that moment (Figure 7).

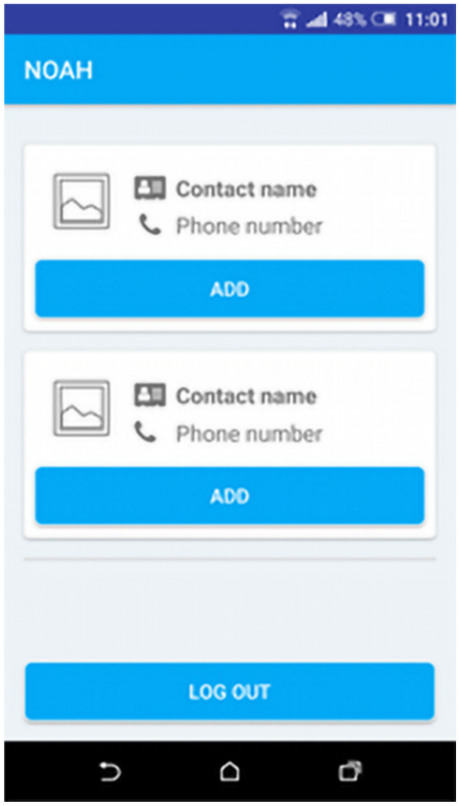

Figure 5. Contacts settings.

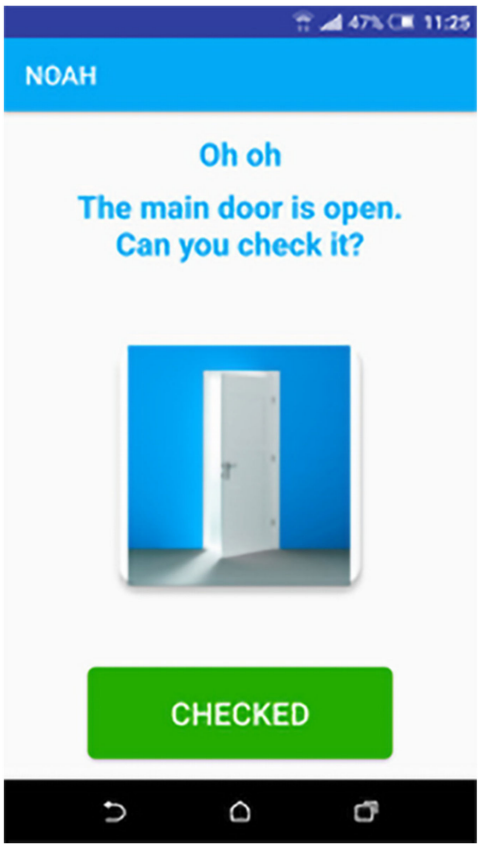

Figure 6. End-user alert.

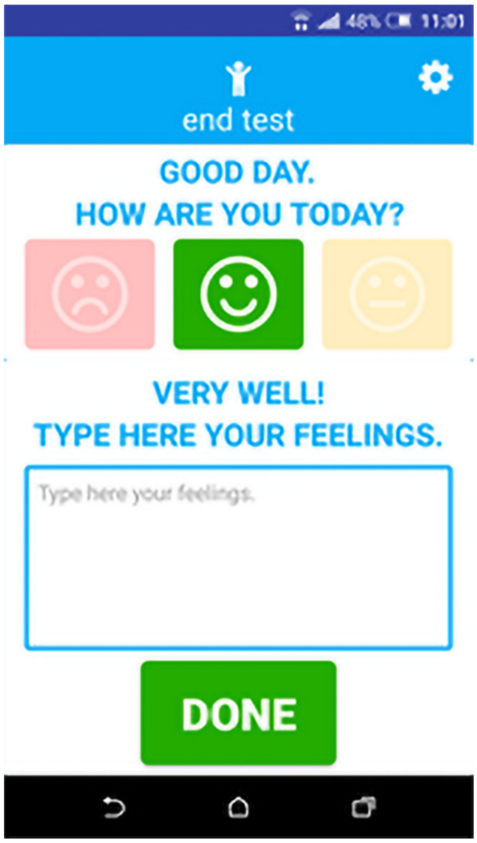

Figure 7. Feedback.

7.2. NOAHCare Application

The NOAHCare application is used by the caregivers to keep the older people they take care of under observation. The application offers them access to information, such as the state of the sensors connected in the end-user's home if there are some changes in the end-user behaviour. They also receive alerts and notifications about sensors, and they can view statistics from different time periods regarding stored data.

The caregiver mobile application usage flow starts with registering an account, logging in and selecting an end-user (from the ones he previously added using the PIN provided by the end-users). At this step, a caregiver can access an application feature to monitor the end-user, such as Sensors' information (current or statistics over a period), Notifications and Alerts. The notifications are generated by the BAM component and target the end-users' behaviors; they take the form of advice (e.g., "Today you walked less. You could visit a friend."). The alerts refer to the state of the sensors and can be related to an action of the end-user (e.g., "You forgot the door open") or to the functioning of the sensor (e.g., "low battery", "sensor is disconnected").

NOAHCare application consists of four main features:

- Caregiver account register and login: A caregiver has the possibility to register a new account by filling their personal information, and after, that they can log into the NOAHCare application.
- End-users management: The flow of a user in this application continues by associating one or more end-users to their account. This step is mandatory for a caregiver user in order to use the application's facilities. An end-user is linked using a unique PIN number (Figure 8).
- End-user monitoring: The caregiver user can see an overview of data for a specific end-user, the sensor's status, the changes in their behaviour (notifications) (Figure 9) and alerts.
- Application configuration: A caregiver can configure the application through activating or deactivating features (Figure 10), shown or hidden, respectively, in the app home page.

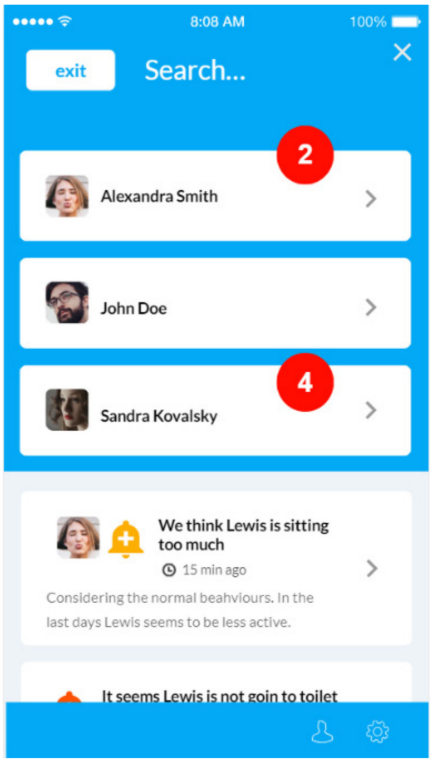

Figure 8. Set end-user.

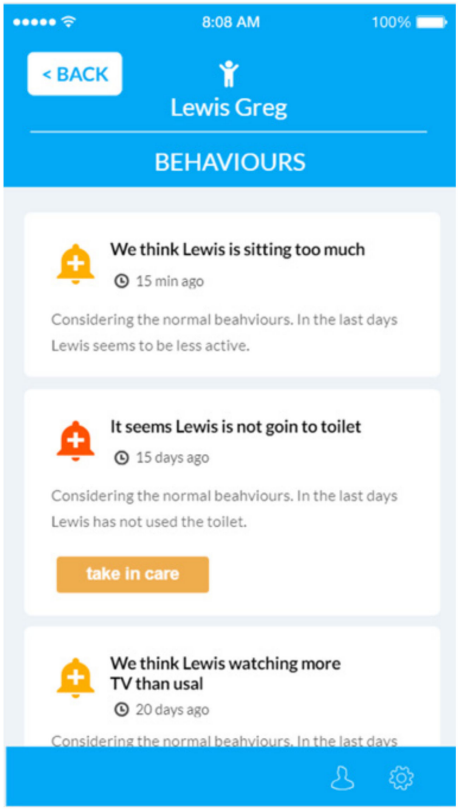

Figure 9. Behaviour.

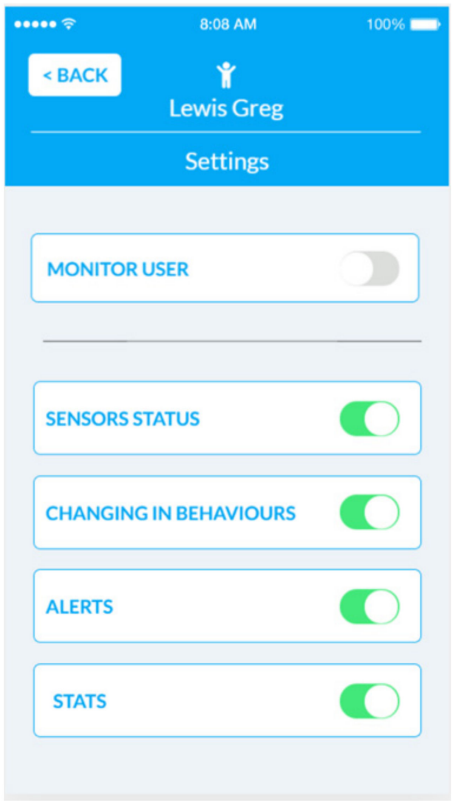

Figure 10. NOAHCare App configuration.

7.3. Admin Centre

The Admin Centre application of the system is a multi-purpose component. It has the role of extracting the information that sensors send, processing it and display it on a friendly interface. The component also offers the possibility of an easy administration of the sensors and kits.

Two types of users, a simple user and an administrator user, can operate it. A simple user can only visualize information, while the administrator user can also insert, edit or delete kits and sensors or the related details.

Once they are logged in, any user will be redirected to a dashboard page containing information about the sensors grouped by kits. The kits are grouped by country for easier filtering (refer to Figure 11).

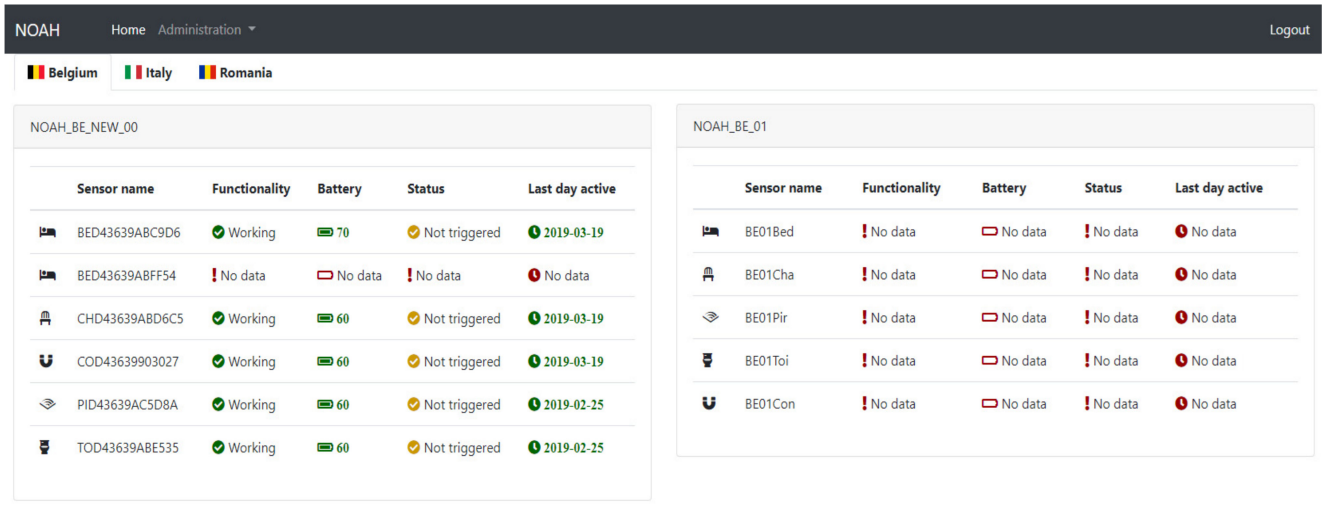

Figure 11. Admin Centre Dashboard.

An administrator user can manage the sensors and the kits from the interface. It is possible to insert a new kit (Figure 12) or a new sensor (Figure 13), to edit an existing one and to establish a link between these two entities.

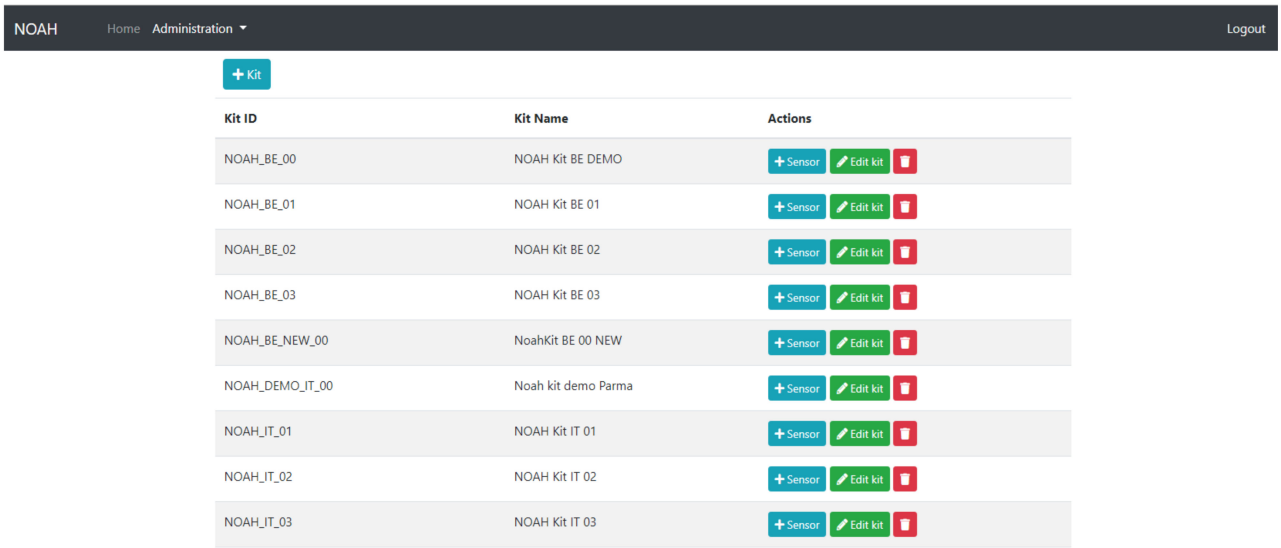

Figure 12. Manage Kits.

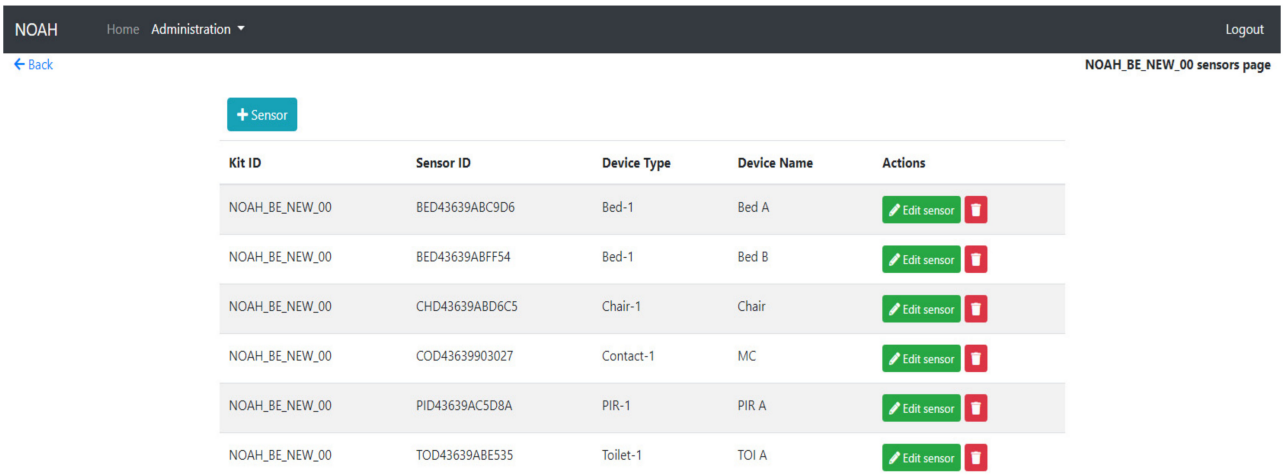

Figure 13. Manage sensors.

8. Methods

8.1. Participants

The subjects were selected by the Department of Social Services (DSS) within the Brasov City Council, following the NOAH project criteria. The DSS is specialized in working with members of this community. They also supervised the activity of the subjects within the project.

The project activities involving end-users and data collection were made according to the collaboration protocol between DSS and Transilvania University of Brasov. The study and research design were in line with the Declaration of Helsinki.

All subjects were informed of this and signed the written form of consent after having all of their questions answered. All data were rendered completely anonymous. During the NOAH project, a professional caregiver was designated from the DSS for each activity with the end-users.

Originally, 14 Romanian elderly individuals, who participated voluntarily, were included in the present study (2 males and 12 women; Mean age = 72.89 years, Standard Deviation (SD) = 5.08). This group of older people participated in our research study investigating the impact of the IOT system in their own environment, including analyzing behavior in association with sensors' detection. Only people older than 65 years of age, living alone in their home and not suffering from major chronic diseases or severe disabilities, were included in the study.

8.2. Instruments

The participants completed the Adaptive Behaviour Assessment System—Second Edition (ABAS II) [34]. The ABAS II is a questionnaire standardized and validated by an age-stratified Romanian sample [35]. The aim of ABAS II is to assess an individual's adaptive skills and their ability to live independently. This assessment plays a central role in the evaluation of sensor usage by older people in their homes. It measures functional states of daily skills in areas such as general adaptive composite skills (GAC), conceptual skills (communication, functions, self-direction), social skills (free time, social, practical community usage) and practical skills (including community use, home living, health and safety, self-care) used in the present study. The form used in this study had 239 items and was completed in two sessions. The psychologists from the Department of Social Services carried out the hetero-evaluation for each participant, separately. The inter-rater reliability coefficients on the GAC scores and for the daily skills areas are $\alpha = 0.80$ s [35]. The test was chosen based on the protocol that is described in the procedure.

8.3. Procedure

At the beginning of the procedure, a research team approached the Social Services Department of Brasov City Council and our participants. Participation in the study was exclusively voluntary. Written informed consent from older people and their caregivers was obtained prior to participation in the study. To be included in this study, an individual had to fulfil the following criteria: (a) being more than 65 years old; (b) living alone at home; (c) not suffering from major chronic diseases or severe disabilities; (d) receiving occasional care from relatives or professional caregivers; (e) having a smart phone and internet connection in their home; (f) agreeing to have their home equipped with the home kit and being paired to (at least) one caregiver person in their personal support network. The measurements were collected over a period of 1 year. The caregivers were comprehensively informed about the research project and the conditions of participation. Based on the procedure carried out by Moraru, S.A. et al. [36] and Kristály, D.M. et al. [37], data were collected between October 2019 and September 2020 from sensors installed in the participants' homes, with an emphasis on the period between mid-February and mid-May 2020, which was the Covid Lockdown period in Romania.

The procedure was as follows: the informed consent process; installation of a new router in the house; installation of sensors (bed, seat, contact, presence and toilet sensor); installation of the telephone application for the older people; presentation of the application; checking the system infrastructure (internet connection, batteries, ergonomic placement in the house, checking the cloud).

8.4. Data Analysis

The normal distribution of each variable was tested using the Kolmornov–Smirnov Test. Non-parametric testing, such as the Spearman Coefficient, was used to test the correlation between the bed, chair, contact, presence and toilet sensors associated with the behaviour of the participants. To determine two conditions in which the same people participated in each condition, the Wilcoxon signed-rank test was used. Due to the low number of participants, the observed cases were unusual. The significance level was set at 0.05. The statistical analysis was carried out using SPSS 26.

9. Results

9.1. Descriptive Results

Information regarding age, sex, educational background (primary school, lyceum, university), family structure (widowed, divorced) and their current health status was collected before starting the measurements. These aspects are recorded in Table 1; the descriptive statistics for all the variables are in Table 2.

Table 1. Descriptive statistics of the participants (older people).

Title 1	Age (Mean; SD)	Sex	Marital Status	Education	Health Issues *
Participants	72.89; 5.08	12 women 2 men	78.6% widowed 21.4% divorced	Primary School 7% Lyceum 71.5% University 21.5%	Yes Yes Yes

* Health issues = all the participants have different age-related chronic illnesses (such as hypertension, heart diseases or diabetes).

Table 2. Descriptive statistics * for all the variables included in the study.

Variable	N	Mean	SD	Skewness	Kurtosis
Communication	14	74.07	1.26	−0.944	−0.890
Community use	14	68.57	4.39	−0.962	−0.242
Functional	14	78.79	2.63	−0.817	−0.601

Table 2. *Cont.*

Variable	N	Mean	SD	Skewness	Kurtosis
Life/Family	14	68.29	1.06	−1.1	−0.295
Safety/Healthy	14	59.07	1.26	−0.944	−0.890
Leisure time	14	63	6.18	−0.541	−1.10
Self-care	14	74.07	1.14	−0.884	−0.18
Self-Direction	14	72.29	2.86	−0.495	−1.62
Social skills	14	67.5	1.65	−0.597	−1.33
GAC	14	111.07	5.04	0.404	−1.273
Conceptual	14	36.71	2.09	−0.794	0.443
Social	14	24.14	3.67	−0.436	−0.960
Practical	14	46.5	5.17	−1.23	0.684
Bed Sensor	14	27,112.14	16,222.15	0.333	−1.187
Chair Sensor	14	18,935.15	9863.57	0.731	0.255
Contact Sensor	14	13,877.08	5316.97	0.025	−0.916
Presence Sensor	14	39,760.31	24,659.54	−0.082	−1.182
Toilet Sensor	14	10,763.45	6711.73	−0.598	−1.826
Sensors Presence 1–6 months	14	19,780.64	8742.90	−0.771	−0.468
Sensors Presence 7–12 Months	14	16,798.57	9765.60	0.247	−1.476
Sensors Presence Covid Lockdown	14	8920.92	5814.62	0.438	0.597

* Besides the statistical values, the last rows express the number of occurrences.

9.2. Correlation Results

Community use was significantly related to using sensors from the bed ($r_s = -0.76$, 95%BCa CI [−0.976–1.00], $p = 0.27$) and social skills were related to using a contact sensor ($r_s = -0.79$, 95%BCa CI [−0.044–0.179], $p = 0.19$). There was no other significant association between the variables.

9.3. Differences between Periods

To test the differences between the activity recorded by the sensors during the one-year evaluation, we split the data into two six-month periods: October–March 2020 (first analysis) and April–September 2020 (second analysis). Furthermore, we tested the difference between first 6 months and the Covid lockdown period. The results based on the differences between scores using Wilcoxon signed-rank test [38] are presented in Table 3.

Table 3. Wilcoxon signed-rank test results.

Differences	N	Median	*z*	*p*	*r*—Effect Size
Sensors Presence 1–6 months vs. 7–12 months	14	22,357	−1.35	0.177	-
Sensors Presence 1–6 Months vs. Covid LD	14	10,653	−3.29	0.001	−0.87

N (number of participants); *z* (*z*-scores); *p* (*p*-value, significance level is 0.05); *r* (effect size).

10. Discussion

As mentioned in the background section, although the use of the IoT system has been shown to have a significant positive impact on maintaining the safety of older people at home, the effect on adaptive behaviour remains unclear. Therefore, the present research investigated the ability of sensors to analyze the behaviour of our participants in their homes during their daily life and in the context of the Covid crisis.

The results showed a low association between daily skills and sensors. Contact sensors were positively associated with social skills, and bed sensors were negatively associated with community use. In previous studies [39,40], the researchers' reports showed a correlation between using different sensors and behaviour adaptability. Similarly, our

results report differences between the activity of sensors during the daily life of the older people in real time [14]. The results also showed differences between first 6 months' activity and during the 3 months of Covid lockdown, and for the team's research, it was a positive sign that the data collected were real and that we would receive correct data from our users [4].

In the Background and related work Section, we discussed the relationship between sedentary behaviour and daily activities, but the best protocol for analyzing this process is yet to be determined [41]. In our study, we have reported basic values of continuous activity for the older people group carrying out daily home activities in real time, with each sensor capturing data every hour [5,14]. The results also showed important activity in March and May, a period associated with the lockdown in Romania [17].

Another important discussion is about the efficiency of the sensors while they were functioning. We started collecting data in October 2019, and for the system, it is an important step to understand the needs and gaps between the concerns of our participants and our ability to improve security and the IoT system [15]. To the best of our knowledge, our older people from Social Assistance Services, Brașov, were the first unit in our region to employ the use of bed, chair, contact, presence and toilet sensors, all integrated in one cohesive system.

As a corollary, these answer our research questions:

(1) Do domotic sensors have a long-term relevance and utility? The sensors did detect changes in user behaviour, even one year after installation (October 2019–September 2020).
(2) Did using the system lead to changes in the lifestyle of the older people participating in our pilot? Based on the data collected from the presence sensor, no changes (please see Table 3) were observed during the first and last six months of the study (prior to the Covid restrictions).
(3) Was the users' behaviour, in their homes during their daily lives, affected by the Covid crisis? The presence sensor was significantly higher during the Covid lockdown period than in the first six months of measurements (Table 3), with a large effect size. We can conclude that during the Covid lockdown, older people were less active and used their personal space (the familiar environment in which the end-user can exercise his autonomy and self-management, i.e., their home) less.

Limitations

The main limitation of our study is the small number of data points. The aforementioned national lockdown period interfered with the regular functionality check of the sensors, as leaving one's home was seriously restricted. Our data were collected in real time, but unpredictable system errors could have interfered with the accuracy of the data. Even though we have been in constant contact with the participants, we cannot be sure that they did not keep the sensors in the house incorrectly, which could have potentially led to the interruption of data collection from certain sensors. We cannot discuss prejudices in this study, because contact with the older people was maintained by telephone, and their involvement was based on the feeling that they were not alone, especially during the period of forced isolation.

11. Conclusions

In this paper, we combined social evaluation with the technical approach so that the implemented system could acknowledge the users' feedback. Presenting both aspects together provides more coherence and displays the interconnected nature of our methodology.

Our system focused on a closer interaction between healthcare (caregivers/stakeholders) and technology researchers in order to ensure that this new system addresses the unsolved needs of elders, especially in difficult periods.

In the general context of a system that can exploit an "Internet of Things" approach, NOAH, the powerful system described in this paper, has dedicated home sensors suitable for capturing a vast array of facets from day-to-day living activities in a non-intrusive

manner. Sensors connect directly to the Cloud through the home Wi-Fi network, requiring no aggregator node and avoiding the need for dedicated sensor networking.

Commercial Cloud infrastructure was exploited to gather data. The power of the Cloud platform has proven to be the ability to integrate all the sensors ("things") that can be connected to it. The integrative approach of our system provides all tracked data via a central interface. Data acquired via sensors are transmitted, stored, selected and processed in the Cloud, enabling the user to see it all at a glance. The security of the data in the Cloud is preserved by using on the server-only high-speed encrypted transmissions.

The Agile approach of NOAH resulted in pilot solutions' co-creation, with role-based user interfaces ranging from simple authentication and prompt/ping to caregiver alerting and administration dashboards. Given the efficiency of the sensors employed and data collection and interpretation since October 2019, this study has proven that this Agile approach was efficient in addressing the needs and gaps between the concerns of our participants and our ability to improve security and the IoT system. To the best of our knowledge, the older people from the Social Assistance Services, Brasov, were the first unit in our region to employ the use of bed, chair, contact, presence and toilet sensors, all combined in one cohesive and fully functional system.

In our study, a significant number of users participated, and we used an industry-standard instrument to conduct, control and validate the experiment on an age-stratified Romanian sample by a procedure that was agreed with our partners in the project. We proved that all the technology used was appropriate, relevant and valued for such a project. Validation of our research was done by confirming that the use of domotic sensors has long-term relevance and utility. We also proved that the sensors detect changes in user behaviour, even one year after installation, by comparing the first 6 months and afterwards, within the Covid lockdown period.

As future work, an evaluation is planned to assess the end-users' behaviour associated with the use of sensors, by conferring the corresponding significance and by information extraction and discrimination from the context. In addition, more users will be involved to extend the degree of confidence in the results. We also intend to build an overview of our past and present AAL projects, emphasizing the efficiency of the system architecture by adapting the development and operation to modern technologies and by calibrating the offered services to the end-users' and caregivers' specific needs, ultimately proving that AAL projects have utility and consistency.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. Ethical review and approval were waived for this study because, since it was an observational, non-clinical study, it was not mandatory.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

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Review

Analysis of Research Directions on the Rehabilitation of Patients with Stroke and Diabetes Using Scientometric Methods

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Abstract: The multidisciplinary approach to the rehabilitation of patients with stroke and diabetes has been followed in this article by a review of the literature published in the Web of Science in the last ten years. A review of the literature was performed using scientometric methods. VOS Viewer software was used to determine the research directions in this area. Scientometric analysis has extracted relevant published scientific output that treats diabetes and stroke. Studies based on qualitative research and the conclusions of these studies were analyzed. The clusters with the keywords used in the title and abstract by the authors who published in the Web of Science were reviewed and research directions in the field were formulated. The proper care of diabetes and its numerous consequences, including stroke and its neurologic complications, necessitates the fast identification of research findings in various types of medicines and their efficacy when applied to various patient groups, such as diabetic patients, whose recovery after a stroke is similar to that of a nondiabetic patient following hemodynamic stabilization, although it takes longer and has poorer outcomes. The limitations of the study refer to the fact that the data reviewed are from the Web of Science only.

Keywords: diabetes; stroke; scientometrics; Web of Science

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1. Introduction

1.1. Multi-Perspective Approach

The multi-perspective approach involves exploring the challenges and opportunities involved in developing rigorous and coherent research methodologies to capture complex phenomena by using multiple perspectives to explore the same phenomenon [1]. This multi-perspective approach was designed to facilitate patient-centered design and evaluation. We used diabetes and stroke scientific production to analyze field observations, structured interviews, and document analysis to collect and analyze user workflow patterns, decision support goals, and diabetes patient interaction preferences.

The purpose of this meta-analysis using scientometric methods is to identify possible approaches to the recovery of post-stroke diabetic patients.

Although there are still ongoing studies to identify the particular approach of the diabetic patient in post-stroke recovery to date, no particular method has been identified for them compared to patients without diabetes. The only difference is the extension of the recovery period in diabetic versus non-diabetic patients.

Diabetes mellitus may influence the post-stroke clinical evolution, especially in the initial phase, increasing the extension of the cerebral injured area [2]. Few studies have been aimed at studying the influence of diabetes on functional outcomes after stroke, and their results are not conclusive [3].

Diabetes is a chronic disease with a growing prevalence that affected a population of approximately 415 million in 2015, and will reach approximately 642 million in 2040 [3]. Diabetes increases the risk of stroke four-fold.

Analyzing the literature shows that there are various implications that diabetes has on different patients. We present some situations that the authors in the field presented in their published articles:

- For stroke, diabetes is an independent risk factor and is associated with increased mortality and morbidity [4].
- In diabetic patients, there is a high proportion of ischemic strokes versus hemorrhagic strokes, and heart attack is the most common type of stroke in these patients. This fact is due to the multiple microvascular damages and the simultaneous presence of arterial hypertension in diabetic patients [5,6].
- Diabetic patients have a greater functional disability, long-term hospitalization, and also a higher risk of dementia [7,8].
- Multicenter studies have shown that approximately 20–33% of stroke patients have diabetes [9–11].
- However, some studies suggest that diabetes has no influence on motor and functional outcomes within the acute and post-acute phase after stroke. Further research should be conducted to investigate motor recovery in a longer-term period and with larger samples.

In conclusion, the multidisciplinary approach that involves the diabetologist and neurologist in the case of a diabetic patient with a stroke is the key to success for a complete, functional, and integrated rehabilitation.

1.2. Scientometric Methods

Scientometric methods involve the quantitative analysis of the generation, dissemination, and use of scientific information, and allow the determination of large and emerging trends in scientific research in a particular field of research, based on statistical analysis of databases and the use of qualitative filters (topics, keywords, magazines). At the same time, it allows the review of the development of research over time or the geographical and organizational distribution of scientific production. The primary scientific data used for scientometric research are the authors, their papers, bibliographic references, and quotations received [12].

Scientometric examinations allow the identification of the most current research topics in a certain field, and the identification of the most quoted papers and authors who have addressed a specific topic. It is possible to determine which countries, institutions, and journals have the greatest influence on the development of science in a particular field and to analyze how the interest in a particular scientific discovery varies over time [13,14].

"A fast-growing trend is the increase of systematic reviews conducted with the assistance of science mapping tools" [13]. A science mapping tool typically takes a set of bibliographic records of a research field and generates an overview of the underlying knowledge domain, very quickly. "A scientometric overview of a field of research provides a valuable source of input to conducting systematic reviews, especially in situations when relevant and up-to-date systematic reviews may not be readily available or accessible" [14].

2. Materials and Methods

The Web of Science database was chosen as a representative sample of the scientific population in the field of diabetes and stroke. Using bibliographic data from this database, this paper seeks to identify current and future directions of research in the field of "diabetes" AND "stroke recovery". In this respect, the search in the database was limited for the time period 2011–2021, the last 10 years. The works were searched by topic, respectively (("diabetes" OR "diabetes mellitus" OR "type 2 diabetes" OR "type 1 diabetes") AND "stroke recovery"). The search was performed on 28 May 2021, and the resulting works were refined according to the type of documents, and only the scientific articles, in English and in Open Access, were selected, resulting in 46 papers.

Primary data were downloaded as plain text files from the Web of Science (WoS) database. The results were examined using VOS Viewer software version 1.6.16 [15],

which allows scientific mapping to analyze the content of titles and abstracts of scientific publications. Thus, the VOS Viewer term identification function was used to systematically identify key terms in the database (co-word analysis) and organize large amounts of text in a semantic map, ignoring the elements related to the structure of abstracts and copyright statements that might be included.

To prepare the terms for mapping purposes, VOS Viewer measures the link between the terms using the power measurement and suggests how many terms should be included in the map.

In this case, of the 1663 terms identified, 90 were used more than 2 times. The groups were analyzed, and the research directions were identified. In addition to this analysis, collaboration and quotation networks have been identified, details of which will be provided in the next section.

The scientometric study includes seven steps, according to Table 1, starting with the formulation of the problem, the establishment of protocols and research criteria, and the extraction of data, which were subsequently analyzed, synthesized, and discussed.

Table 1. Scientometric study stages.

No.	Steps	Description
1	Formulation of the problem	Mapping, bibliometric analysis of publications using descriptors and identification of research directions.
2	Research criteria	Subject: ((“diabetes” OR “diabetes mellitus” OR “type 2 diabetes” OR “type 1 diabetes”) AND “stroke recovery”)
3	Database used for research	Claryvate analytics, Web of Science (WoS) Accessed on 28 May 2021
4	Eligibility criteria	Filter 1: years of publication (2011–2020) Result: 1663 documents Filter 2: articles Filter 3: English Filter 4: Open Access Result: 43 documents
5	Data extraction	Bilingual format
6	Analysis and synthesis of results	Qualitative (descriptive) and quantitative (bibliometric) using VOS Viewer
7	Discussions	Analysis of the data gained

Analysis and interpretation of scientometric research data.

A total of 214 authors contributed to the writing of the 46 papers in the 10 years analyzed. The main authors are presented in Figure 1 according to the number of publications. There were 90 terms that appeared at least twice, and they are distributed in 6 clusters, according to Figures 2–4.

Using the keyword list and Excel, the keyword figure was obtained (Figure 5).

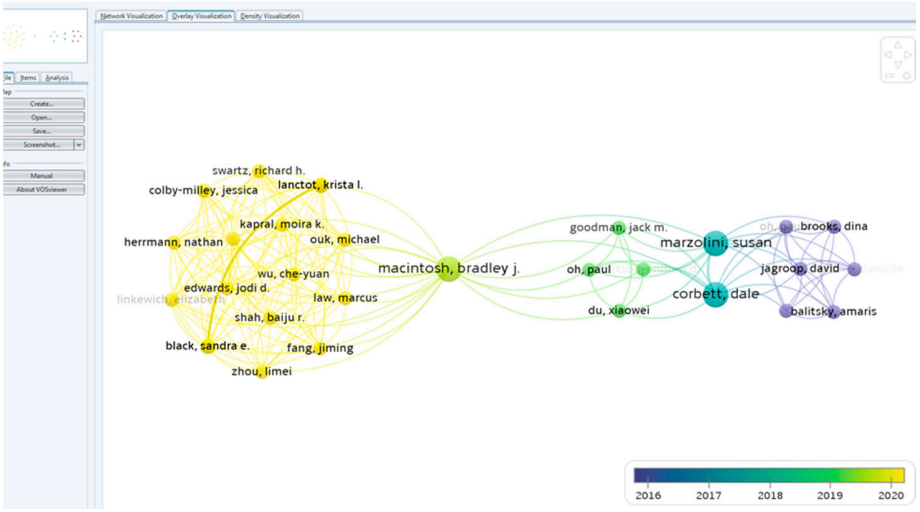

Figure 1. Authors, visual map according to number of publications.

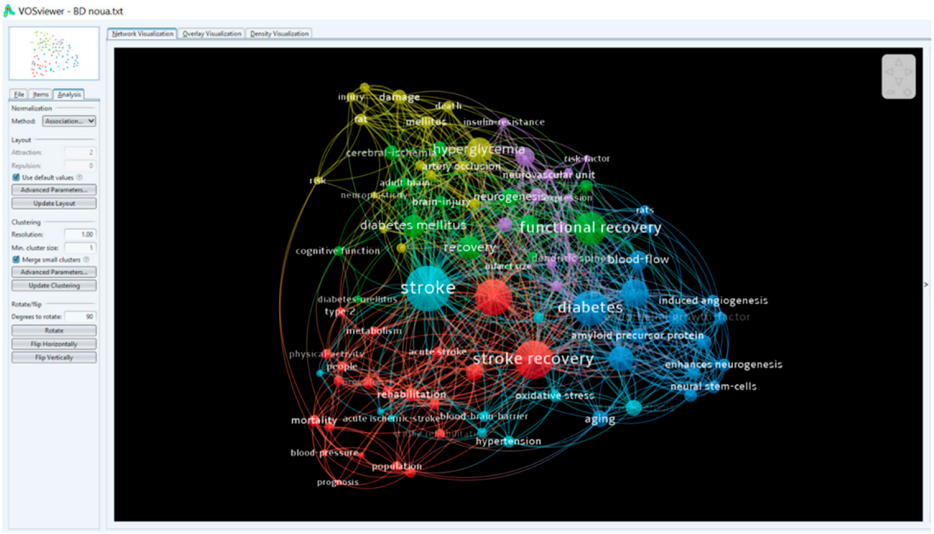

Figure 2. Keyword density map.

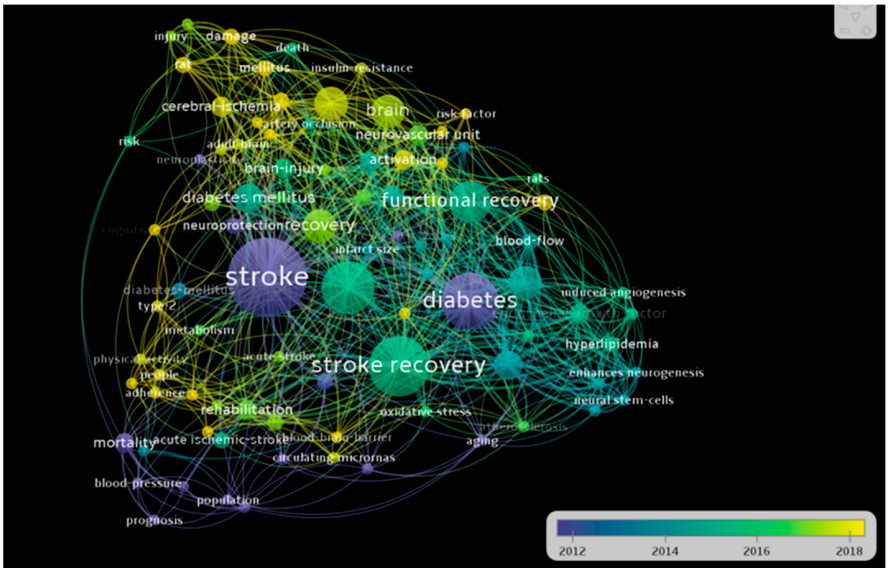

Figure 3. Keyword density map.

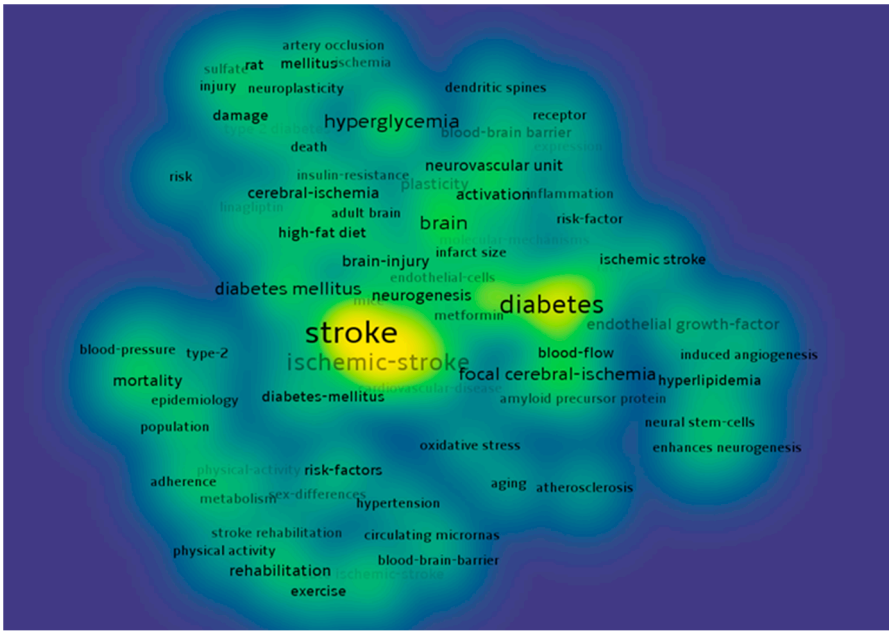

Figure 4. Keyword density map.

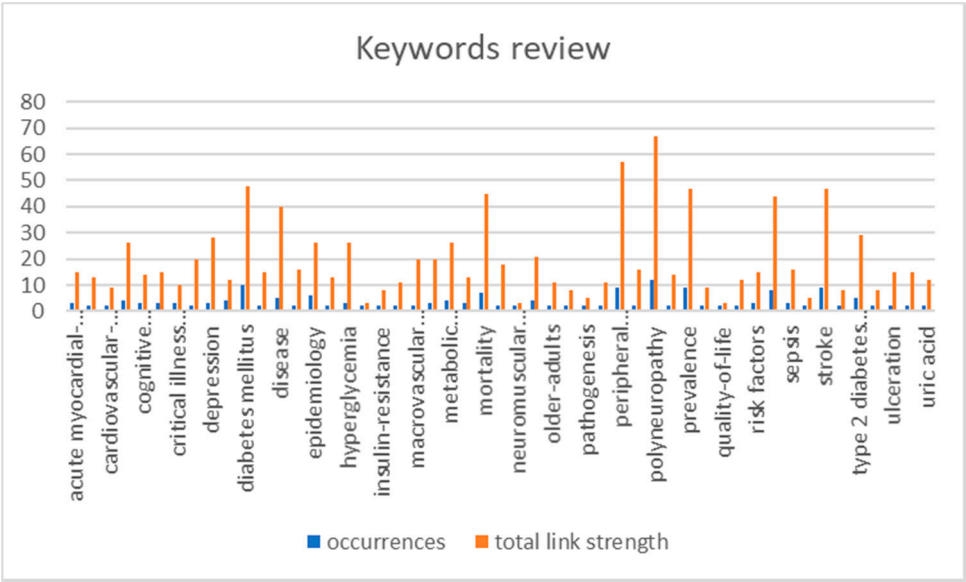

Figure 5. Keywords' occurrence. The words that have the highest rate of occurrence are: neuropathy, disease, and type 2 diabetes mellitus. Epidemiology, diabetes mellitus, mortality, risk factors, peripheral neuropathy, prevalence, stroke, polyneuropathy.

3. Results

Following the review of the 46 papers, the authors identified 21 studies and articles reviewing the specific literature (Table 2). The articles were reviews, randomized studies, and meta-analyses. Some articles indicate the number of participants, while others do not, indicated as n for not specified in Table 2.

The authors have also reviewed the articles delimiting the research directions in the six clusters presented in Figures 2–4. Table 3 summarizes the results.

Table 2. Studies and reviews, literature articles. n = not specified.

Source	Number of Participants	Trial Type	Duration of the Trial	Title of the Study
[16]	23,579	Randomized trial	2003–2013	Depression and Diabetes Mellitus Multimorbidity is Associated with Loss of Independence and Dementia Post-Stroke
[17]	46	Review	n	Aerobic Training and Mobilization Early Post-Stroke: Cautions and Consideration
[18]	n	Review	n	Effects of Angiotensin-II on Brain Endothelial Cell Permeability via PPAR-alpha Regulation of Para- and Trans-Cellular Pathways
[19]	n	Review	2011–2019	Dipeptidyl Peptidase-4 Inhibitors for the Potential Treatment of Brain Disorders: A Mini-Review with Special Focus on Linagliptin and Stroke
[20]	70	Randomized trial	14 days	Sleep and Cognitive Function in Chronic Stroke: A Comparative Cross-Sectional Study
[21]	58,265	Meta-analysis	n	Cerebral Vascular Injury in Diabetic Ischemia and Reperfusion
[22]	n	Review	n	Occupational Physical Activity in Young Adults and Stroke: Was It Due to My Job?
[21]	n	Article	n	Impact of microRNAs on Ischemic Stroke: From Pre- to Post-Disease
[23]	160	Trial	April–June 2014	Increased Expression of STIM1/Orai1 in Platelets of Stroke Patients Predictive of Poor Outcomes
[24]	n	Article	n	Stroke in Women: Risk Factors and Clinical Biomarkers
[25]	291	Study	2009–2013	Intake of Potassium- and Magnesium-Enriched Salt Improves Functional Outcome after Stroke: A Randomized, Multicenter, Double-Blind Controlled Trial
[26]	374	Study	January 2005–May 2010	Clinical and Imaging Correlates of Outcome after Intracerebral Hemorrhage
[27]	78	Clinical trial	12 weeks	Rationale and Design to Assess the Efficacy and Safety of HT047 in Patients with Acute Ischemic Stroke: A Multicenter, Randomized, Double-Blind, Placebo-Controlled, Parallel-Group, Phase II Trial
[28]	489	Trial	2004–2009	A New Prognostic Scale for the Early Prediction of Ischemic Stroke Recovery Mainly Based on Traditional Chinese Medicine Symptoms and NIHSS Score: A Retrospective Cohort Study
[29]	n	Review	n	Cerebral Neovascularization in Diabetes: Implications for Stroke Recovery and Beyond
[30]	10	Study	n	Resistive Training Improves Insulin Sensitivity after Stroke

Table 3. Diabetes and stroke research directions, determined by the six clusters.

Cluster	Conclusions	Supporting Document
1	The post-stroke motor function is improved by physical activity due to low levels of epinephrine and norepinephrine.	[22]
	Resistive training can decrease post-stroke glucose metabolism and thus increase post-stroke survival.	[29]
	Age is an important factor in post-stroke recovery: patients under the age of 56 are more likely to recover than those over the age of 56.	[27]
	The combination of diabetes mellitus (DM) and high blood pressure (HBP) entails a poorer recovery in the first 3 days after the stroke.	[27]
	Patients with diabetes and heart disease are more likely to have a predominantly ischemic stroke, while smokers and alcoholics are more likely to have a hemorrhagic stroke.	[31]
	Mobilization of post-stroke patients in the first 6 h exacerbates injuries, whereas after 24 h from stroke the mobilization has beneficial effects.	[32]
	Late recovery in special centers encounters difficulties in transporting patients to these centers and thus the sequelae can no longer recover	[32]
	DM decreases the effectiveness of thrombolysis and increases the risk of post-trobotic hemorrhage.	[33]
	DM does not affect motor and functional recovery in the acute and post-acute phase of stroke.	[34]
2	DM impairs cortical plasticity.	[35]
	DM affects post-stroke neovascularization, thus preventing post-stroke recovery.	[36]
	Diabetes impairs spatial memory and hippocampal neurogenesis in ischemic stroke.	[37]
	Diabetes increases the risk of dementia by 85% compared to non-diabetic people.	[16]
	The association of dementia with diabetes in stroke patients leads to poorer results in post-stroke recovery.	[38]
	The recovery rate was slower in patients with stroke and diabetes. DM exacerbated anxiety and cognitive decline.	[39]
	DM highly increases neurovascular damage and thus depreciates post-stroke recovery.	[37]
	Obesity with diabetes determines reduced neurogenesis and impaired neuroplasticity after stroke.	[40]
	Obesity induces a reduced post-stroke recovery.	[41]
	Atrial fibrillation appears to affect post-stroke recovery.	[42]
	Gender-related: Women appear to be more likely to have a stroke than men.	[43]
	In the first 3 months after the stroke, mortality is higher in the event of hemorrhagic stroke.	[31]
	Intake of N acetyl seryl aspartyl lysyl proline (AcSDKP) has led to improved neurological functional recovery in rats with diabetes.	[23]
	Thiazolidinedione treatment in diabetic stroke patients has intensified post-stroke functional recovery by decreasing infarct volume and vasodilation.	[44]
	Long-term administration of potassium and magnesium benefits post-stroke recovery.	[25]
	Sulfonylureas and metformin used in hemorrhagic stroke causes angiogenesis and has a high safety profile.	[37]
	Metformin mediates post-stroke recovery by increasing angiogenesis.	[29]

Table 3. *Cont.*

Cluster	Conclusions	Supporting Document
3	In the elderly, 40% have moderate functional post-stroke impairment, but people over 85 show slower rehabilitation.	[45]
	Angiogenesis in diabetic patients is greatly slowed down.	[46]
	Glycemic control prevents the decline of neovascularization and post-stroke recovery.	[29]
	Type 1 diabetes has a 4–6 times higher incidence of ischemic stroke occurrence.	[47]
	Post-stroke blood–brain barrier dysfunction (BHE) plays an important role in limiting functional recovery in diabetic patients.	[41]
	Angiotensin-II is a significant factor in increasing endothelial permeability in the brain and contributes to angiogenesis and neurogenesis.	[18]
4	Effects of DM treatment on post-stroke recovery: Antidiabetic medication such as DPP4 inhibitors, sulfonylurea. Glimepiride causes faster post-stroke recovery in obese diabetic patients.	[48,49]
	DPP4 does not decrease the risk of stroke but causes early recovery and rehabilitation in the first 3 days after a stroke.	[19]
	Obesity and diabetes worsen post-stroke recovery, and these effects are counteracted by the administration of DPP4/sulfonylurea at 3 days post-stroke, leading to early recovery.	[19,48]
	Stroke shows an increased number of neurons 6 weeks after the stroke. Diabetes causes neuroplasticity and thus this effect of increasing the number of neurons is abolished.	[48]
5	Innovative therapies in animal studies. Administration of C21 to a type 2 angiotensin-II receptor agonist on day 3 after a stroke resulted in a reduction in neuroinflammation in male animals with diabetes.	[50]
	Inhibition of TOLL-4 (TLR4) receptors in microvascular endothelial cells would reduce inflammation and improve post-stroke recovery in diabetics.	[46]
	miRNA assay would be a biomarker for the diagnosis of stroke and the evaluation of the effectiveness of stroke treatment.	[51]
6	White matter lesions in the brain occur in diabetic patients long before stroke occurrence.	[23]
	Patients with chronic kidney disease (CKD) more frequently suffer ischemic stroke than hemorrhagic stroke.	[52]
	In patients with BCR, uremic toxins cross the BHE and are thus involved in cognitive dysfunction and neurodegeneration.	[52]
	In stroke patients, the combination of CKD worsens recovery and limits the choice of therapies for stroke treatment.	[53,54]

4. Discussion

This article presented a new method of literature review, using scientometric methods. The method can be replicated by PhD students and researchers, in order to quickly obtain an image of any field researched. Studies in human models have shown that the therapeutic balance of diabetes is a very important factor in the rehabilitation of diabetic patients after stroke. The review of the articles and research directions led to conclusions that are important in the analysis of solutions for patients with diabetes and stroke. Articles studied in either animal or human models have highlighted several research directions for post-stroke rehabilitation programs.

The investigation of the articles led to different conclusions, which we present below in six directions of research.

1. Identification and possible modification of aggravating risk factors for stroke patients

Obesity associated with diabetes are factors that worsen the prognosis of recovery after stroke.

Ischemic strokes occur predominantly in diabetic patients with arrhythmias and acute coronary syndromes, while accidental hemorrhagic strokes occur predominantly in smokers and alcoholics [31].

2. The influence of mental disorders on post-stroke recovery

Depression increases the risk of readmission to recovery centers and may contribute to dementia. A diabetic patient-centered recovery strategy alleviates the risk of depression and multiple post-stroke metabolic complications [16].

3. Understanding post-stroke vascular remodeling processes

In young people, remodeling processes are active and long-lasting compared to the elderly, in whom remodeling is slowed down due to the presence of other comorbidities, such as diabetes, atherosclerosis, and dyslipidemia.

Glycemic variations in diabetics have negative effects on angiogenesis, so stimulating it in stroke patients is not currently an optimal solution for neurological recovery [29,54].

4. The role of physical activity on post-stroke recovery

Information on this issue is still controversial. The indications for physical exercise in the early post-stroke phases showed progress in recovery, but neither their intensity nor their optimal duration was established. The lack of data in the literature indicates that early post-stroke mobilization is limited [32].

5. The effects of drug therapies and dietary supplements

The effects of hypoglycemic medication on the risk of stroke are heterogeneous.

Sulfonylureas and metformin appear to have a potential protective effect in diabetic patients with hemorrhagic stroke [29].

Studies of DPP4 inhibitors in diabetic patients have shown a reduced risk of stroke. Complementary studies are needed to show possible effects of reducing brain damage in the case of stroke [19].

The use of long-term dietary supplements enriched with potassium and magnesium seems to be beneficial for post-stroke recovery, but the results require further studies to substantiate the indication [25].

The administration of micro-RNA biomarkers (miRNAs) in stroke therapy could be an alternative to long-term sequelae, but studies are still ongoing [21].

Another class of innovative antidiabetic drugs that inhibit SGLT2 with complex mechanisms of action do not increase the incidence of stroke. SGLT2 inhibitors produced a 50% decrease in hemorrhagic stroke compared to a placebo [55].

6. There are a number of factors that can cause patients to drop out of rehabilitation programs. These are related to transportation problems to the recovery centers, long distances to reach these centers, boring exercises, lack of a companion, and limited time.

Therefore, future studies should focus on ways to overcome these barriers to encourage the participation of these patients in rehabilitation programs.

5. Conclusions

Following the scientometric research and the analysis of the specialized literature, the authors wish to raise the awareness of the specialists regarding the following aspects.

Proper management of diabetes and its many complications, including stroke neurology, requires the rapid identification of research results in different types of therapies and their effectiveness applied to various categories of patients.

The recovery of the diabetic patient after stroke after hemodynamic stabilization is the same as in the case of nondiabetic patients, but involves a longer period of time and has poorer results.

SGLT2 inhibitors had no effect on overall cerebrovascular events; however, results for stroke after using them vary depending on the kind of stroke, with a potential benefit for

hemorrhagic stroke prevention. Further prospective trials comparing the effects of SGLT2 inhibitors on different stroke subtypes are needed.

Finally, we conclude that scientometric methods allow a rapid and efficient analysis of the research directions generated by scientific production in the field, making a decisive contribution to improving the approach to the disease and long-term treatments of patients with diabetes and stroke.

6. Limits of the Study

The scientometric analysis must be interpreted taking into account the limitations of the research. First, the results are limited to publications (articles and papers presented at conferences) published in 2011–2021 and indexed in the Web of Science database.

However, this scientometric analysis has allowed us to identify the main actors and research directions in the field in recent years. The results of the research show that many of the previous interests are still relevant today.

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Who's Getting Shots First? Dealing With the Ethical Responsibility for Prioritizing Population Groups in Vaccination

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Background: The current pandemic has raised several ethical dilemmas, related to conducting real-time trials for new treatments or vaccines or with decisions such as accessibility to vaccines.

Study Question: Should there be a prioritization of access to the vaccine based on ethical and objective criteria or should the access be done at random?

Study Design: To determine the ethics and reality of rationing the accessibility to anti-COVID vaccine according to the official strategies.

Data Sources: The study is based on the consultation of (1) scientific articles from international databases (Google Scholar, PubMed, ProQuest, and Clarivate), (2) public health documents, and (3) official information of various governments.

Results: The analyzed documents revealed that a few similarities can be observed in European countries when it comes to the first categories of people who have received the vaccine: people living in care facilities and medical staff; it can also be seen that the vaccination plan was adopted by each country for the needs and characteristics of its population, the prioritization being done in 2–14 stages; some of them divided, in their turn, into subsequent substages. Most of the states subject to the analysis assigned the medical staff in the first stage, followed by those in the sectors ensuring the maintenance of essential services, afterward by the elderly or people with comorbidities, only later to expand to other social categories.

Conclusions: Prioritization of vaccine administration is not only necessary, unavoidable, but also problematic both ethically and logistically, which should involve leaders in the field of public health, but also medical staff, regardless of their specialization. Prioritization of vaccination can not only have an impact on individual health (physical and emotional) but also on society from public health, economic, and sociocultural point of view.

Keywords: vaccination, COVID-19, public health, ethical dilemma, fair distribution

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INTRODUCTION

Pandemics have constantly raised a consequential problem as regards the public health measures to be taken worldwide.

In recent years, the opportunities of travel have much increased, and hence, the great number of people traveling to different continents has heightened the possibility of transmitting infections with interpersonal transmission.

Pandemics have been limited, over time, by means that we have rediscovered in the current pandemic: physical distancing, quarantine, lockdown measures, contact tracing, travel bans—if we refer to the preventive measures and the constant attempt to discover an effective treatment for those affected.

Quarantine has proven its effectiveness since ancient times, but the current pandemic has brought along a return to the well-known means of limiting the pandemic and the tendency to use certain terms that are at least difficult to understand and ethically controversial, such as that of social distancing.

In this context, more and more people have tried and are trying to limit the effects of this pandemic both in the economic area and in that of human interaction, defending the acute need to have readily available means of limiting the pandemic, such as the vaccine.

Vaccination is not a new method; in various forms, it has been used since antiquity. The right of each of us to benefit from any way of maintaining our health; the ethical dilemmas raised by the introduction of new methods in current practice; but also the need to respect, above all, life and human values, all these are aspects of an analysis that cannot be neglected even in the case of the introduction of new vaccines in the current activity.[1–3]

As shown by Ezekiel J Emanuel and all: "Three values are particularly relevant: benefiting people and limiting harm, prioritizing the disadvantaged and equal moral concern."[4]

Ethical values are not only a possibility of theoretical approach, but they must be understood and analyzed according to the current context, recognizing the need to move from the purely theoretical aspects of an analysis to a practical analysis.

Vaccination has always brought undeniable benefits: from the reduction of death toll to the diminution of disabilities, whether we are talking about smallpox, polio, whooping cough (pertussis), or hepatitis. The more deaths the disease causes, the more we want to have a means to prevent its occurrence, and vaccination has proven to be an effective means in this regard.

According to the World Health Organization (WHO), the Allocation Framework (and resulting Mechanisms, including the Algorithm) is a key component of an overall approach centered on equitable access to COVAX vaccines, adapted to be pragmatic, so that participants can have access to vaccines as soon as possible.[5,6]

The development of a vaccine to combat the current pandemic was achieved in a relatively short time, by various methods from a technical point of view, but the biggest problem came only later, when the capacity to produce vaccines worldwide has been and still is insufficient compared with the number of those who want to be vaccinated.

In this context, the fears about unfair decision must be discussed and analyzed, and the moral impact of our decision must be understood and assumed.

Study question

Should there be a prioritization of access to the vaccine based on ethical and objective criteria or should the access be done at random?

Study design

To determine the ethics and reality of rationing the accessibility to anti-COVID vaccine according to the official strategies.

Data sources

The study is based on the consultation of (1) scientific articles from international databases (Google Scholar, PubMed, ProQuest, and Clarivate), the main keywords in the search being: ethics + covid-19 vaccine to which + fair distribution, priority, rationing, health worker, or public health are sequentially added, (2) public health documents, and (3) official information of various governments.

RESULTS

Coronavirus in international publications

COVID-19 vaccine has been a common topic in all databases, with a large number of researchers and articles showing that there is a major concern in this direction.

We could even say, on analyzing the published articles, that there were a large number of articles on this topic.

Thus, from the beginning of the pandemic, 7911 articles on COVID vaccination were indexed in PubMed, of which 317 analyze the ethical aspects, and 137 articles refer to the allocation of vaccines.

In the period 2020–2021, 4103 articles on vaccination and COVID-19 were indexed in the Clarivate database, of which 62 address ethical issues, and only 3 refer to the ethical issue of vaccine allocation.

A simple mapping in Clarivate (Web of Science) and PubMed shows that the number of coronavirus-related articles is extremely high, as evidenced by the major concern in the last year related to the determination of the etiological agent or the treatments that can be administered.

If, in an article published in 2020,[7] Belli and all were already talking about 18,875 coronavirus-related articles indexed in Clarivate (Web of Science), an analysis we performed in February 2021 on the articles indexed in the same database shows that in 2020, 76,065 coronavirus-related articles were indexed; the dynamics revealing the major concern for this topic, with articles from very different fields, as shown in Figure 1.

Prioritizing vaccine administration in different countries

The analysis of documents developed globally or nationally shows that different strategies have been developed for the distribution and prioritization of COVID-19 vaccines.

Thus, when they aim to reduce the deaths caused by the virus, according to the WHO, the groups that should be given priority are primarily those at high risk of death or severe symptoms: older adults who are at risk due to age, adults who are at risk due to their living conditions, living in asylums, care institutions, or people with comorbidities. In this vein, priority is also given to people at high risk of infection: health workers or employees who have jobs where physical distance cannot be properly implemented.[8]

Moreover, strategies regarding vaccination were also elaborated and proposed at the level of the European Union. Thus, a technical report of the European Centre for Disease Prevention and Control presents 4 main strategies: vaccinating people at risk to have severe symptoms and outcomes, which includes people at risk due to their older age, people older than 60 years, and people at risk due to pre-existing diseases or conditions.[9]

The systematization of the information related to the staging of the administration of vaccines and the type of vaccine is presented in Figure 2.

In the context of the strategies used in prioritizing vaccine administration, to achieve the goals of the vaccination process, including minimizing mortality rate, as well as preventing or diminishing the economic damage generated by the pandemic, proactive planning is required and is also essential for the distribution of the vaccines in an ethical manner.[10]

Every country adapted and implemented its own vaccination policies, depending on the characteristics and needs of its citizens. In this regard, the policies adopted by various countries are essential because they highlight important aspects of the ethics of the vaccine prioritization process (Figure 3).

Thus, in the cases of European countries, similarities can be observed regarding the groups of people who had priority in receiving the vaccine: people who live in care facilities and health workers.

Most countries placed health workers first in the vaccination strategy; for some countries, the decision was made whether or not they were at the forefront of the fight against vaccines; for other countries, the staff prioritization was achieved in 2 stages. This strategy included countries such as the United States, Romania, or Italy[11,12]; others used a different

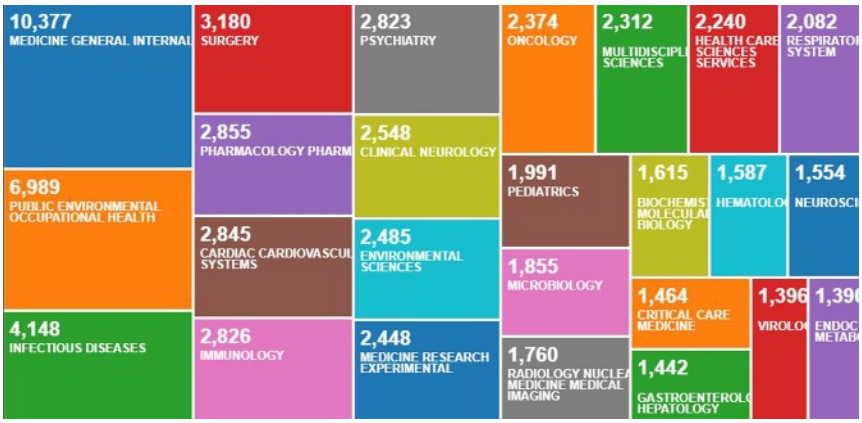

FIGURE 1. Coronavirus mapping about articles indexed in Web of Science.

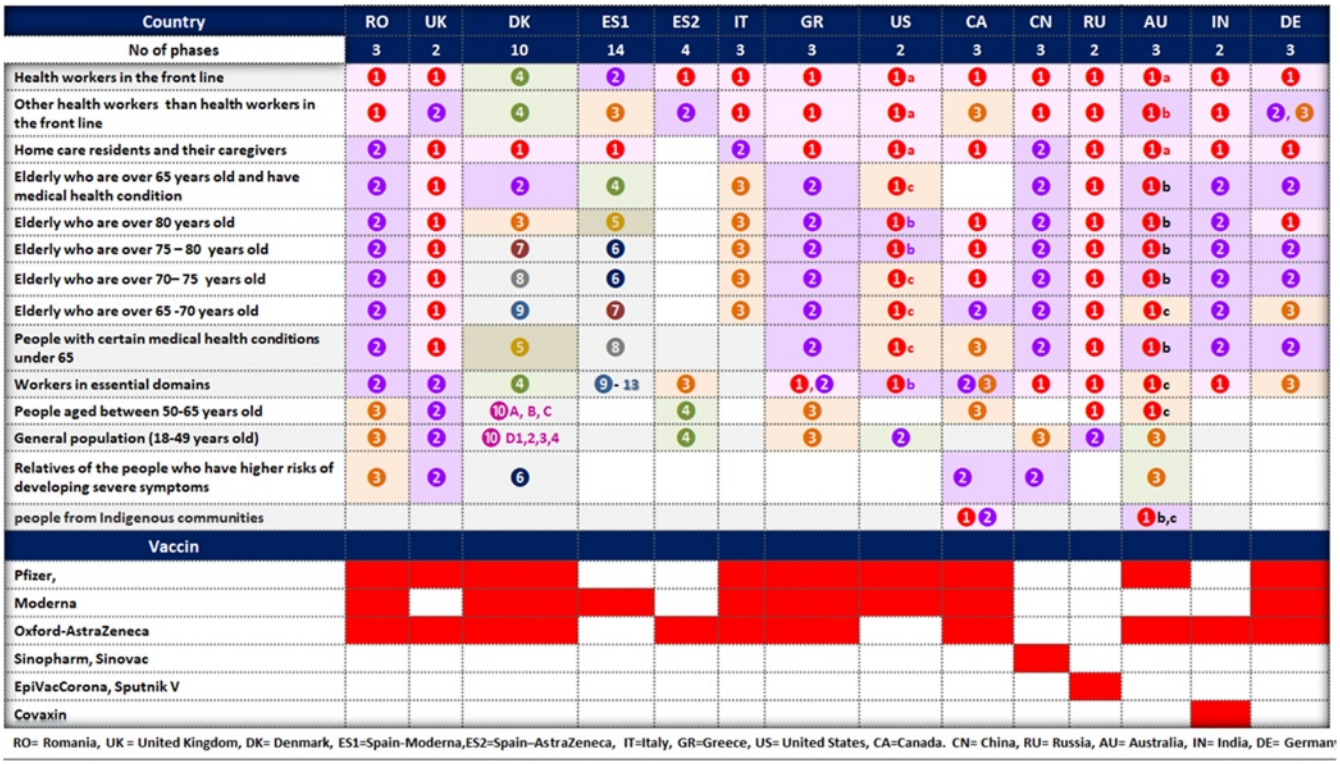

FIGURE 2. Systematization of the information related to the staging of the administration of vaccines.

Type of population	principle	Maintain the society function	More public health or individual benefit	Risk for infection	Risk for transmission	Risk to severe pathology	Motive for prioritization
Active population	utilitarian	+++	PH>I	+++	++++	++	Maintaining the company's long-term and medium-term functionality. High economic benefit
Community workers	utilitarian	+++++	PH>I	++++	++++	++	Maintain the society function and national security
Health care workers	utilitarian	+++++	PH>I	+++++	+++++	++++	Maintain the function of health care system protect people who promote the greatest good, in this case treating the most patients. Promoting social usefulness
	egalitarian						High risk of exposure at high level of contact with the virus
Home care residents	egalitarian	+	PH<I	+++	+++	+++++	High risk of infection and outbreaks
Home care caregivers	utilitarian	++++	PH>I	+++	+++++	+++	Maintain the home care open and assure the caregiving to the residence. High benefit to others, Promoting social usefulness
People from Indigenous communities	egalitarian	+++	PH<I	+++++	+++++	+++++	Lack of infrastructure and lack of health and education services
Pregnant women	egalitarian	+++	PH<I	+	+	+	High risk of infection and outbreaks;
Relatives of the people who have higher risks of developing severe symptoms	utilitarian	++	PH<I	+++	++++	+	High benefit to others
Retired people	egalitarian	+	PH<I	+++	++	++++	High risk of infection and outbreaks
People at risk due to various diseases/ sickest	utilitarian	+	PH<I	++++	++	+++++	Give priority to the worst off
Teachers	utilitarian	+++	PH>I	+++++	+++	+++	Maintaining the company's long-term and medium-term functionality
Workers in essential domains	utilitarian	+++++	PH>I	+++	+++++	+++	Maintaining the company's long-term and medium-term functionality High benefit to others
Worker from security	utilitarian	++++	PH>I	+++	+++	+	Maintain the society function and national security

FIGURE 3. Values framework for the allocation and prioritization of vaccination.

strategy, including in the first stage the people in care facilities and their caregivers, for instance, Denmark, whereas others developed a special strategy for indigenous communities, for example, Australia or Canada, or a mixed strategy like in the United Kingdom.[13,14] While in Romania and Spain, health workers were among the first people to receive the vaccine; in Denmark, conversely, health workers represent the fourth group of people who had vaccine priority, having been preceded by the people older than 65 years and older than 80 years, and also, in the United Kingdom, health workers were vaccinated after people older than 80 years.

The strategies developed by some countries were changed during the campaign, as was the case in Denmark, which established initially 12 groups that had priority in receiving the vaccine, that were later compressed into 10 groups.[15]

In other countries, different priorities have been set depending on the type of vaccine; in Spain, different groups of people are vaccinated at the same time, with a different prioritization of access to the Comirnaty vaccine (Pfizer/BioNTech) or the Oxford AstraZeneca ChAdOx1-S vaccine.[16]

Australia and Canada granted access for indigenous communities in the early stages of the vaccination campaign.[17,18]

Moreover, differences can be observed regarding the prioritization of essential workers, such as teachers, police officers, and firefighters. While in Romania and Spain, these people followed immediately after health workers, care facility residents, the elderly, or people with medical conditions; in Denmark instead, the initially adopted plan placed essential workers at the bottom of the line. However, in the updated vaccination plan of Denmark, it includes 10 priority groups, and the workers who have critical functions in society are included in the fourth group, next to health workers.

DISCUSSION

The guidelines of prioritization

The development of "guidelines" of prioritization seems to be, in some decision-makers' conception, the optimal solution for them not to be accused of subjectivism and of pursuing their own interests in the decision they make. However, the development of guidelines for ethical dilemmas is not entirely possible.

The development of a prioritization algorithm should take into account the balance between the number of those who want to be vaccinated and the number of available doses, but also other factors, as shown in Figure 4.

The problem of prioritization did not arise with the current pandemic, but there were concerns about understanding the issues related to prioritization of other vaccines as well, such as in the case of the flu pandemic.[19–21]

It is difficult to develop a nondiscriminatory mechanism for the distribution of vaccines in a situation such as that induced by the COVID-19 pandemic, starting from a few elements that need to be analyzed and quantified so that as many willing people as possible can be vaccinated.

The decision to prioritize the administration of the vaccine is often difficult to manage, which makes each

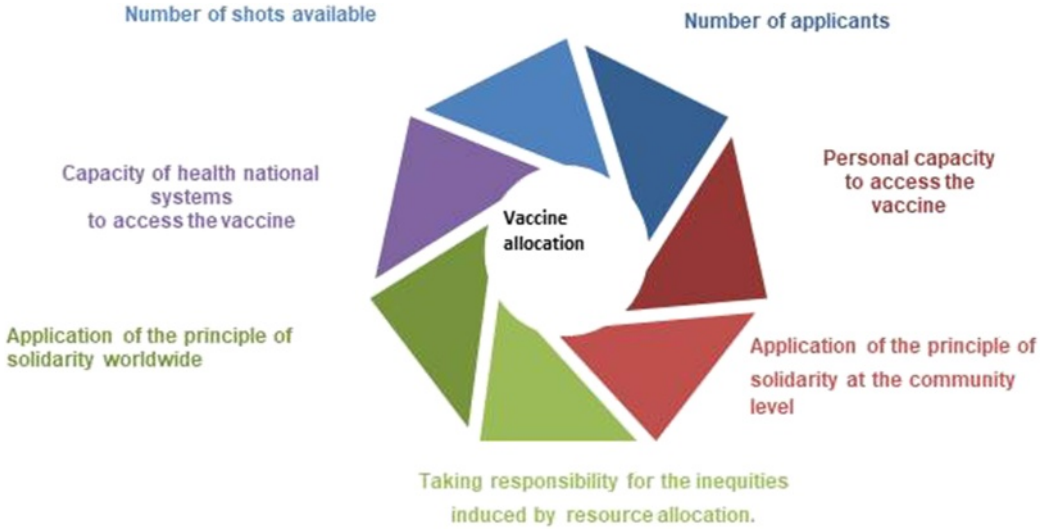

FIGURE 4. Factors influencing vaccine prioritization.

administration carry a certain moral burden on the shoulders not only of those who decided to prioritize the vaccine but also of those who administer it because each syringe involves, within the medical work, an assumption of ethical responsibility, too. The responsibility is even greater when we talk about vaccines in which there is a risk of losing valuable doses by packaging in multidose vials.

Medical staff is an important resource in a pandemic, which can be quickly depleted, unless measures are taken to protect them. Medical staff, whether doctors, nurses, or support staff, have been affected since the beginning of the pandemic; hence, a large number of both diseases and problems related to physical and/or mental exhaustion appeared very quickly.

Starting from the need to protect the medical staff so that they, in their turn, can care for patients; one of the main issues regarding the distribution of vaccines is related to the place of medical staff in the various stages of vaccine administration.[22]

The right to decide when and with what vaccine to get vaccinated—an individual right.

The individual's right to accept or not the administration of a vaccine must be seen as a fundamental right of any individual, similar to the right to accept or not any other treatment.

Prioritizing vaccine administration and adopting a fair and ethical process in this regard has become a challenge for many European and non-European countries. In this context, when addressing the subject of whom and under what circumstances is entitled to receive the vaccine to the detriment of other persons, several aspects can be highlighted.

Thus, 2 major objectives of the vaccination process can be observed: the former is a direct objective and refers to the protection of the individual against a future infestation, and the latter can be seen as an indirect objective that refers to the global protection of people, by reducing transmission and implicitly the risk of infection, even for the people who will not receive the vaccine.[23] Furthermore, the matter of transmission, regarding people who can more easily transmit the virus, can also be considered when elaborating policies for vaccine distribution or allocation. In this regard, attention can be paid to the fact that, if people who mostly benefit from the effects of the vaccine, such as the elderly, are prioritized, the number of lives that are saved may not be maximized; however, prioritizing the vaccination of those who are most likely to transmit the virus, such as children, "may offer more benefits to the population at large."[23] Nevertheless, in contrast to this affirmation, a study about vaccine prioritization, according to the age criteria, shows that vaccinating first the people aged 60 years or even older could actually minimize the number of deaths,[24] but it also reveals that, in the case of infection susceptibility by age, the vaccination of people younger than 20 years has a greater impact on diminishing cumulative cases than the vaccination of people between 20 and 49 years old.[24]

Special mention should be made of people with psychiatric pathology. As shown by Victor Mazereel and all, "Patients with severe mental illness experience barriers to immunization, including a lack of knowledge and awareness, accessibility problems, costs, fears about immunization, and often no recommendations from their primary care provider."[25]

Vaccination of children also raises some ethical issues starting from 2 aspects: a significantly lower number of serious cases due to SARS-CoV-2 infection registered among children, and their possibility of being a source of transmission of infection among both adults and children.[26]

While referring to the vaccination plans adopted by Australia and Canada, it can be observed that both governments decided that one of the priority groups in receiving the vaccine should be represented by Aboriginal, Torres Strait Islander adults, and people from other indigenous communities. In other words, aboriginals were considered a priority group in the process of vaccination, Australia and Canada offering them the opportunity to receive the vaccine in the first phase of the process.

One should consider, in this regard, the fact that aboriginals and people from indigenous communities are usually affected by discrimination.

Over time, aboriginals and indigenous people have experienced multiple types of discrimination. Discrimination can take many forms, one of the most common form being racism. Thus, there are multiple ways through which racism can be expressed, such as: stereotypes—beliefs about people who belong to a certain race and prejudices—which refer to racist emotions, but it can also be expressed through behavior and specific actions.[27] Even more, the concept of colonization is often used in relation with the concept of racism, for it is taught that colonization may be considered the initial event that favored and caused discrimination against indigenous and aboriginal people.[28]

The discrimination against aboriginals has been a subject of interest for many researchers, especially in the case of aboriginals from Canada and Australia.[29–32]

Just as in Australia, aboriginal people are discriminated in multiple life situations in Canada, too. For example, a study shows that Canadian aboriginal students mostly experience racism on school grounds, but also in public spaces, and that they are discriminated in more life situations than those students who do not belong to this racial group.[33]

Approaching the subject from the perspective of health and well-being, it is known that Australian aboriginals have poorer states of health and well-being than other populations.[34] Thus, both in Canada and Australia, aboriginals often experience discrimination when they access medical services. Thus, studies revealed that for indigenous people, hospitals and care facilities are unwelcoming that many health care workers do not take into account the external factors, which influences the aboriginals' health, and thus, they blame the aboriginals for their precarious health state, considering that their illness is mainly caused by the fact that they do not take care of themselves.[35]

Despite the numerous cases when aboriginals were discriminated before, in the context of the pandemic and of the vaccination programs implemented, both Australian and Canadian governments considered them priority groups for receiving the vaccine. In this regard, the Australian government stated that priority was given to aboriginals because of their higher risk of developing severe symptoms of the disease and of spreading the virus. This higher risk is caused by many factors such as pre-existent medical conditions, chronic illnesses, or living in shared spaces.[17]

However, in the light of the above-mentioned aspects, we argue that the aboriginals and people from indigenous communities may also be given vaccine priority to protect the cultural heritage of the countries because aboriginal groups are among the founders of the countries, but also to make up for the way aboriginals were treated over time and to show them that the countries respect them and take into consideration their needs, too.

Therefore, the various plans and strategies the European countries adopted to perform the vaccination process, as well as the moral and ethical implications of the act of prioritizing certain categories of people in this regard, can be approached from the perspective of utilitarianism.

In a broad way, utilitarianism refers to the action of "maximizing the overall benefits at the societal level."[6] Thus, we argue that the development of the COVID-19 vaccination process can fall within the practices implied by the utilitarianism theory due to the fact that in European countries, a tendency toward achieving a general state of well-being of the entire population and a tendency to obtain maximum benefits from the vaccination process can be observed.

Utilitarianism can be considered an ethical theory according to which the actions that are moral are the ones which "tend to promote the greatest good, for the greatest number,"[36] and the theory focuses on the idea that in any given situation, actions and measures must be performed with the purpose of helping each individual live its best life and achieve the highest level of welfare.[37]

The concepts and beliefs that utilitarianism comprises were firstly outlined by Jeremy Bentham, who introduced and presented the core principle of the theory: the principle of utility. In this regard, throughout their lives, humans have to deal with pain and pleasure, with right or wrong decisions, and utility is the principle upon which actions can be assessed, accepted, or rejected, depending on their tendency to increase or reduce happiness.[38]

Thus, an action is right and correct when it produces happiness in terms of pleasure and lack of pain, and it is wrong when it produces pain or sadness, pleasure and the absence of pain usually being the main outcomes that people seek.[39] In other words, in the context of this theory, an action should be described as being right or wrong by taking into account its potential of producing and creating happiness and of diminishing feelings of unhappiness.[40,41]

Even more, utilitarianism can be seen as the theory which postulates that the elements that give an activity or decision the nature of being right or wrong are merely represented by the consequences of that activity or decision.[42] In this regard, 2 types of utilitarianism were identified: act and rule utilitarianism. The former one states that a deed is right as long as its consequences are at least as good as the consequences of any other deed that could be an alternative to the deed performed.[43] Similarly, the rule utilitarianism states that the right and correct rule is the one that has the best possible consequences.[44]

Because utilitarianism falls within the category of ethical theories, morality is a focal point in this perspective. Thus, in the view of Hare (1981), there are 2 levels on which our moral thinking takes place: the intuitive and the critical level. In the context of a moral conflict, of situations characterized by the existence of conflicting responsibilities or tasks, in the case of intuitively thinking, people may consider that the situation cannot be resolved, but in the case of critical thinking, a solution must be found.[45] For example, adapting the idea previously mentioned to the health field, when doctors find themselves in the situation of treating multiple patients who suffered severe injuries, they must use critical thinking so as to establish the order in which they will handle the patients.[44]

Considering the concepts which the theory of utilitarianism focuses on, this theory has become a subject of interest for researchers in the context of public health in general and in public health crises in particular. Thus, in public health, decision making usually is related to the objective of utilitarianism, that of aiming to obtain a general state of well-being.[46]

As well as in other situations, when confronted with public health emergencies, in the view of utilitarianism, the moral duty of doing the right thing can be done by

maximizing utility,[47] by conducting activities that bring the most positive and beneficial results for the entire population. Transposing this belief in the context of performing the vaccination process against COVID-19, many countries established strategies and prioritized certain categories of people to achieve the best possible outcomes: prevent infection, reduce the transmission of the virus, and implicitly reduce the mortality rate. While pursuing these goals, it can be observed that in establishing the stages of the vaccination process and the people included in each stage, a general rule found in the utilitarian theory was adopted: the rule according to which people's quality of life is essential, meaning that not only how many years a person lives is important but how well that person lives.[44] In this regard, it can be inferred that the scope of the vaccination process was not only to prolong the life of different categories of people but also to ensure that people will be able to continue to live well and to not be deprived again of the things that the pandemic deprived them of.

Quality of life and people's life satisfaction are elements that must be considered in the process of achieving a general state of well-being, especially in cases of public health crises.[48] Thus, a popular model that is used to assess quality of life is the QALY model, which comprises both quality and quantity of life-years lived or gained based on the medical interventions that people had.[49] In other words, 1 QALY is represented by a year lived in a very well health state, and the value of the model decreases when the health of people is affected.[50] While referring to the COVID-19 pandemic, one medical intervention that can have the power to improve people's quality of life is vaccination. Because the first phases of the process involved vaccinating vulnerable people and people at higher risks of contacting the virus such as health workers, the tendency to improve the life of the entire population by first protecting those who protect us and those who cannot protect themselves can be observed.

The COVID-19 pandemic generated many ethical and deontological issues with respect to the decisions made to treat patients infected with the virus and with respect to the methods used to prevent the spread of the virus. Thus, when trying to decide upon the best response strategies to the pandemic, utilitarianism was the theory mostly considered and debated.[51] In this regard, although some researchers who approached this subject argue that responses to a crisis from a utilitarianism perspective must be supported by principles of justice and autonomy to ensure that decisions are taken in an equitable manner,[52] other researchers state that utilitarianism is indeed the best way to approach crisis situations and that during such situations, emphasis should be made on utilitarian principles.[53]

Hence, an approach of the pandemic and of the issue of vaccine prioritization from the utilitarianism perspective implies that ethic concerns at the individual level are surpassed by ethic principles that refer to the entire population and according to which the least restrictive measures are taken to achieve a general state of well-being.[52]

Therefore, taking into account the general response strategies of European countries during the COVID-19 pandemic, their vaccination programs, the ethical concerns of the decisions regarding who should have priority over who in receiving the vaccine, but also people's grievances and complaints, other questions appear: Is the utilitarianism perspective alone a suitable approach for making decisions in times of crisis, or should it be supported by other theories and principles? In the context of the ethical implications of the vaccination process, would a universal vaccination plan or strategy have been a better solution than allowing each country to decide on the best way to prioritize vaccination? Or would a unique solution bring other types of issue such as discrimination, or noncompliance with the right of each country to implement a suitable plan according to the characteristics of its population?

Vaccinating people at risk

One of the important issues related to the prioritization of vaccine administration is the dispute over the access of the population at risk, either due to age, or due to associated pathology, or due to difficult economic conditions.

The restricted access to vaccination may be due to inequities in the access to basic facilities, as is the case of disadvantaged groups (unemployed, migrants, homeless people, and people from poor communities or living in isolated areas without access to medical facilities).

CONCLUSIONS

Even before declaring pandemic with SARS-CoV-2, it became clear that a major public health problem was emerging, initially denied not only by local authorities but also by organizations such as WHO.

Prioritizing the access to vaccines is not an easy problem to solve, and certainly, any decision is subject to constant re-evaluation. The problems related to the development of a vaccine are only an intermediate stage in the analysis of the role and place of vaccination in combating a pandemic, knowing that subsequent logistical issues related to the production of the required number of doses but also to its distribution are equally important, regardless of whether we are talking about economically developed or less developed countries.

One year after the global outbreak of the pandemic, there are several lessons we should learn: developing a rapid and collaborative system of response to new diseases, rapidly setting targets and means of intervention, as well as establishing risk groups and prioritizing the public health measures as far as they are concerned, and especially improving the communication between the health system and the state medical and nonmedical institutions and the population.

The current pandemic should give us, in addition to a lesson on medical intervention, an ethics lesson that seems to convince us that bioethics is not and cannot be complementary in medical practice.

Prioritization of vaccine administration is not only necessary, unavoidable, but also problematic both ethically and logistically, which should involve the leaders in the field of public health, but also medical staff, regardless of their specialization.

Prioritization of vaccination can not only have an impact on individual health (physical and emotional) but also on society from the point of view of public health, as well as from the economic and sociocultural point of view.

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The Impact of and Adaptations Due to the COVID-19 Pandemic on the Histopathological Diagnosis of Skin Pathologies, Including Non-Melanocyte and Melanoma Skin Cancers—A Single-Center Study in Romania

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Abstract: *Background and Objectives*: The COVID-19 pandemic has globally affected health systems and services. Non-melanoma skin cancers (NMSCs) are the most common malignancies around the world. This study aimed to analyze the differences in the benign and malignant histopathological diagnoses performed on radical excision skin tumors and skin biopsies in the dermatopathology ward in Mures Clinical County Hospital, Targu Mures, Romania, 1 year prior to and during the COVID-19 pandemic, to emphasize the changes in the diagnostic process as per the new regulations. *Materials and Methods:* A total of 1168 histopathological diagnoses were included in the study—302 from the COVID-19 period and 866 from the non-COVID-19 period—considering the number, type, and frequency of the histopathological diagnoses as variables to be analyzed. *Results*: In the COVID-19 period, out of the 55 NMSC and melanoma histopathological diagnoses, 50.9% (n = 28) were BCCs, 20% (n = 11) were SCCs, 10.9% (n = 6) were basosquamous cell carcinomas, and 18.18% (n = 10) were melanoma cases. Regarding the non-COVID-19 period, out of the 173 NMSC and melanoma histopathological diagnoses, 46.82% (n = 81) were BCCs, 22.54% (n = 39) were SCCs, 7.51% (n = 13) were basosquamous cell carcinomas, and 23.12% (n = 40) were melanoma cases. *Conclusions:* During the COVID-19 pandemic, a decrease in histopathological diagnoses at the dermatopathology ward in our hospital was observed, for both benign and malignant pathologies, especially for NMSCs and melanomas, compared to the same period 1 year prior to the pandemic.

Keywords: COVID-19 pandemic; non-melanocytic cancers; histopathology

1. Introduction

Many patients globally suffered respiratory symptoms at the end of 2019 and needed intensive care. The identified virus, SARS-CoV-2, which is the cause of the COVID-19 disease, spread rapidly throughout the world [1]. In less than 2 months, most countries

were affected by the disease. The World Health Organization (WHO) declared the outbreak of COVID-19 a pandemic, which has caused major health challenges worldwide [2]. There were over 133 million confirmed cases and over 2.9 million COVID-19 deaths worldwide as of 5 April 2021 [3].

Cutaneous lesions can be divided into three main categories: benign lesions, malignant lesions, and premalignant lesions. In histopathology, lesions can be identified on the basis of the primary cell of origin of the lesion or which of the three components of the skin is most affected by the pathologic process (epidermis, dermis, and hypodermis). The majority of skin lesions are benign and, morphologically, they appear as various dermatitis, nodules, cystic lesions, keratotic lesions, or papules that grow slowly. Malignant tumors of the skin are solitary lesions with irregular and rapidly growing dimensions that may ulcerate. These tumors of the skin are able to metastasize and are able to appear as new lesions or from other pre-existing skin lesions, being divided into melanocytic and non-melanocytic skin cancers. Histopathological diagnostics and treatments must be in accordance with the clinical presentation and type of surgery: biopsy or elective surgery [4,5].

Non-melanocyte skin cancers (NMSCs), also known as keratinocyte cancers, are the most common and most frequently diagnosed types of cancer in humans. Various NMSCs have been reported in the literature, with various histologic versions that frequently cause important differential diagnoses with other cutaneous tumors [6,7]. Compared to other malignancies, non-melanocytic skin cancers express low metastatic potential and are typically associated with a favorable prognosis. While their metastatic potential is limited, these malignancies can be exceedingly destructive to local tissue, leading to some patients requiring complex excisional and reconstructive procedures [8]. Despite the appearance of novel nonsurgical treatment modalities, surgical resection remains the most common treatment method for NMSCs, with 4 mm clinical margins in low-risk basal cell carcinomas (BCCs) and 4–6 mm clinical margins in local low-risk squamous carcinomas (SCCs). Melanocytic skin cancer, also known as melanoma, is one of the most aggressive types of skin cancer and one of the leading causes of cancer-related mortality due to its metastatic power. The majority of patients with newly diagnosed melanoma are in the early stage of the disease. For these patients, surgical excision is the treatment of choice and is curative in the majority of cases. When diagnosed at an advanced stage, melanoma remains a lethal type of cancer [9].

The COVID-19 pandemic has highly diminished opportunities to perform elective surgery for skin cancers in plastic surgery wards. In order to establish a priority in performing elective surgery in surgical wards, some measures should be taken into consideration, with one of the most used measures today being the risk of worsening of the skin lesion within the next month. Additionally, patients who had their melanoma excised recently or patients with ulcerated and bleeding skin cancers should be taken into consideration and prioritized in order to benefit from urgent and adequate treatment during the COVID-19 period. Furthermore, elective surgery should be considered for rapidly growing lesions such as basal cell carcinomas or squamous cell carcinomas [10].

This study aimed to analyze the differences in the histopathological diagnoses established by H&E staining and immunohistochemistry performed on radically excised skin tumors and skin biopsies in the dermatopathology ward in Mures Clinical County Hospital, Romania, both benign and malignant, 1 year prior to and during the COVID-19 pandemic, to emphasize the changes in the diagnostic process and number of histopathological diagnoses as per the new regulations.

2. Materials and Methods

A cross-sectional study was performed by analyzing the diagnostic data from the dermatopathology ward in Mures Clinical County Hospital, Targu Mures, Romania, between April 2019 and February 2020 (before the COVID-19 pandemic) and between April 2020 and February 2021 (during the COVID-19 pandemic). The hospital in which the study was performed is a county university hospital, which has both clinical and surgical wards.

The samples came to our dermatopathology ward from the surgical units, especially the plastic and reconstructive surgical ward and the general surgery wards for patients who underwent radical excision, as well as from the dermatology clinic for patients who underwent skin punch biopsies. The two study periods were defined as the COVID-19 pandemic period (April 2020–February 2021) and the non-COVID-19 period (April 2019–February 2020). Diagnostic data were collected from the ward's database and included histopathological reports, especially histopathological diagnoses. After collecting the data, the number of histopathological diagnoses, the types of histopathological diagnoses, and the frequency of each histopathological diagnosis were analyzed and represented graphically using the Microsoft Office suite, specifically Microsoft Excel and Descriptive Statistics. The inclusion criteria comprised dermatopathology tissue samples that were sent from different surgical wards in our hospital that were analyzed and diagnosed during the study period. The exclusion criteria comprised cases with other histopathological diagnoses that were sent for consultation to the dermatopathology ward. According to these criteria, we included 1168 histopathological diagnoses and excluded nine histopathological diagnoses.

3. Results

Of the 1168 histopathological diagnoses included in the study, 302 were established during the COVID-19 period and 866 were established during the non-COVID-19 period. Figure 1 presents the monthly distribution of histopathological diagnoses by case numbers, which the dermatopathology ward established during the two periods.

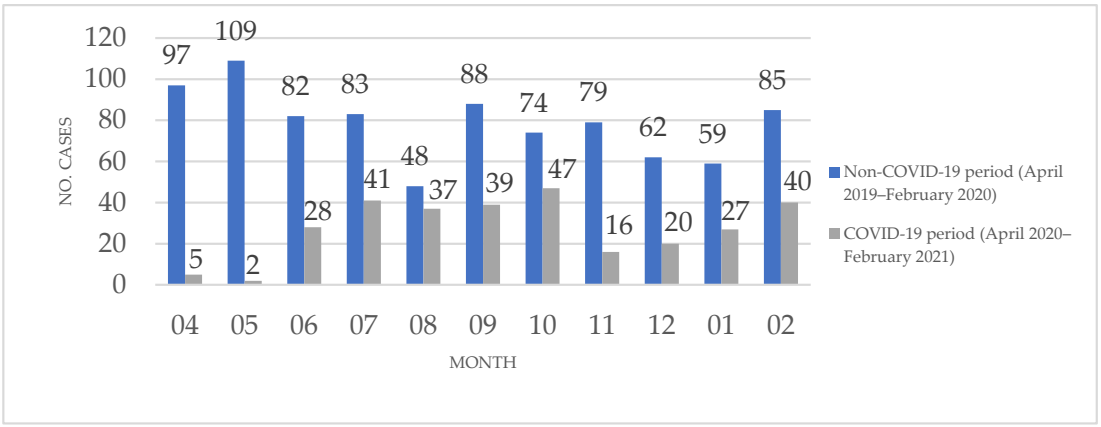


Figure 1. The monthly distribution of histopathological diagnoses established between April 2019 and February 2020 and between April 2020 and February 2021 in the dermatopathology ward of Mures Clinical County Hospital, Targu Mures, Romania.

The main histopathological diagnoses established during the COVID-19 period, based on the presence of benign, benign-appearing, and malignant lesions, as well as the number of cases for each pathology, are presented in Table 1.

A comparison between the benign and malignant lesions diagnosed during the COVID-19 and non-COVID-19 periods is presented in Figure 2. During the COVID-19 period, out of the 302 histopathological diagnoses, 80.13% (n = 242) of the cases presented with benign lesions and 19.87% (n = 60) presented with malignant lesions. In the non-COVID-19 period, out of the 866 histopathological diagnoses, 74.25% (n = 693) of the cases presented with benign lesions and 25.75% (n = 173) presented with malignant lesions.

Regarding the malignant histopathological diagnoses, Figure 3 presents the distribution of the NMSCs, comprising BCCs, SCCs, and basosquamous carcinomas, as well as melanocytic skin cancers, comprising melanomas, during the two studied periods. In the COVID-19 period, out of the 55 NMSC and melanoma histopathological diagnoses, 50.9% (n = 28) of the cases presented with BCCs, 20% (n = 11) presented with SCCs, 10.9% (n = 6) presented with basosquamous cell carcinomas, and 18.18% (n = 10) pre-

sented with melanoma. Regarding the non-COVID-19 period, out of the 173 NMSC and melanoma histopathological diagnoses, 46.82% (n = 81) of the cases presented with BCCs, 22.54% (n = 39) presented with SCCs, 7.51% (n = 13) presented with basosquamous cell carcinomas, and 23.12% (n = 40) presented with melanoma.

Table 1. The histopathological diagnoses established between April 2020 and February 2021 in the dermatopathology ward of Mures Clinical County Hospital, Targu Mures, Romania.

Benign Lesions			
Histopathological diagnosis	No. of Cases	Histopathological Diagnosis	No. of Cases
Chalazion	3	Lobular capillary hemangioma	1
Clavus	6	Mixed mesenchymal tumor	2
Dermal naevus	19	Mixed thrombus	1
Dermatofibroma	6	Normal skin	5
Dilated pore of winer	2	Pseudoepitheliomatous hyperplasia	1
Epidermoid cyst	33	Psoriasiform dermatitis	4
Fibrolipoma	2	Psoriasis	12
Fibroma	5	Pyoderma vegetans	1
Gigantocellular granuloma	4	Pyogenic granuloma	3
Granulation tissue	29	Squamous papilloma	16
Hyperkeratosis	1	Seborrheic keratosis	9
Incarnated nail	3	Sinusoidal hemangioma	7
Junctional naevus	21	Skin atrophy	2
Keloid scar	3	Spitz naevus	1
Keratoacanthoma	7	Sub-chronic dermatitis	1
Lentigo	1	Trichilemmal cyst	12
Lichen sclerosus	1	Trichoepithelioma	1
Lymphatic nodules	4	Verrucous naevus	4
Lipoma	10	Vesiculobullous lesion	1
Total Cases: 242			
Malignant Lesions			
Histopathological diagnosis	No. of Cases	Histopathological Diagnosis	No. of Cases
BCC	28	Porocarcinoma	1
Basosquamous carcinoma	6	Sarcoma	4
Melanoma	10	Squamous carcinoma (SCC)	11
Total Cases: 60			

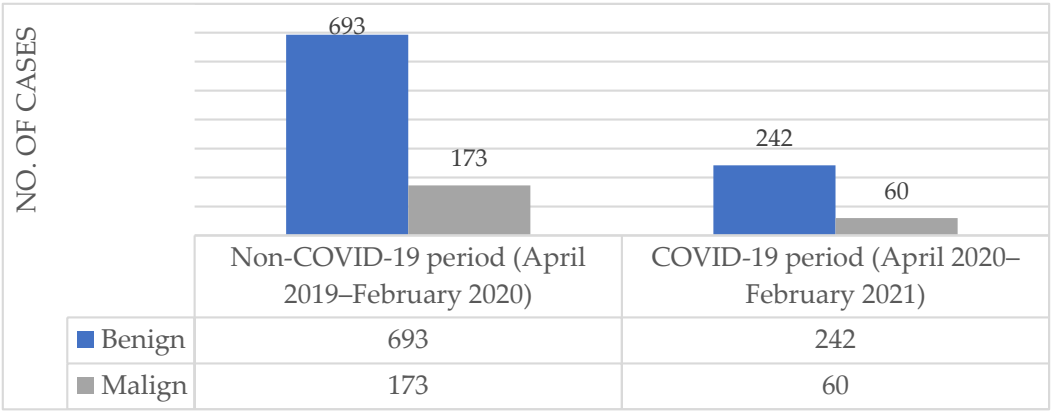
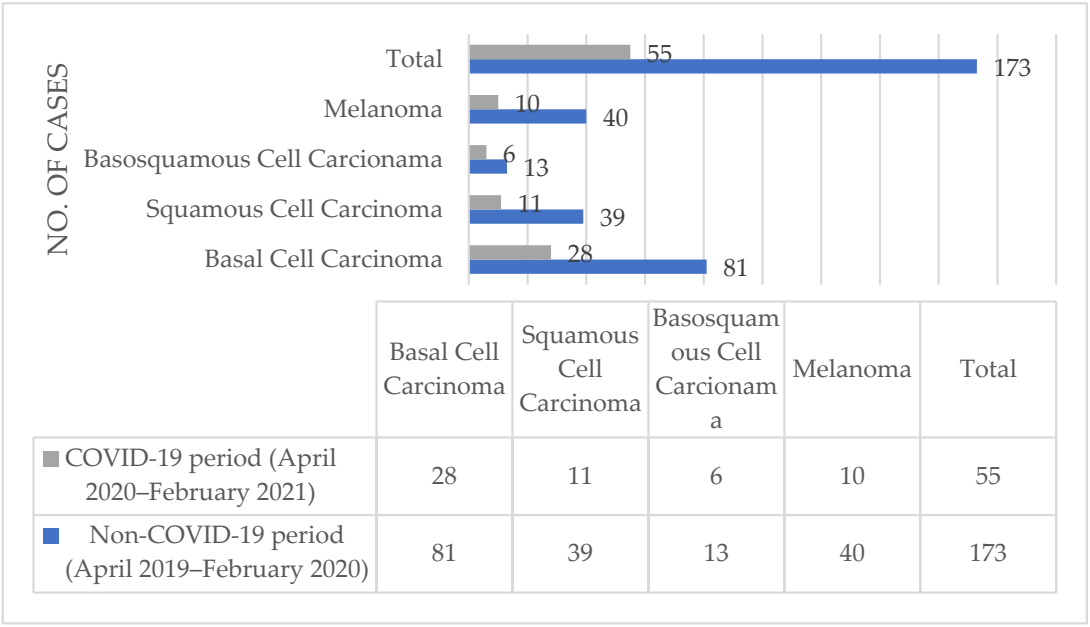


Figure 2. The counts of benign and malignant histopathological diagnoses established between April 2019 and February 2020 and between April 2020 and February 2021 in the dermatopathology ward of Mures Clinical County Hospital, Targu Mures, Romania.



	Basal Cell Carcinoma	Squamous Cell Carcinoma	Basosquamous Cell Carcionama	Melanoma	Total
COVID-19 period (April 2020–February 2021)	28	11	6	10	55
Non-COVID-19 period (April 2019–February 2020)	81	39	13	40	173

Figure 3. NMSC and melanoma counts from the histopathological diagnoses established between April 2019 and February 2020 and between April 2020 and February 2021 in the dermatopathology ward of Mures Clinical County Hospital, Targu Mures, Romania.

4. Discussion

The COVID-19 pandemic caused by the outbreak of SARS-CoV-2, i.e., the novel coronavirus, emerged suddenly and has become a global health problem, as declared by the WHO in March 2020. Every country has established new procedures and protocols for the treatment and diagnosis of various pathologies, dividing their national health facilities into COVID-19 and non-COVID-19 wards. Due to the sudden appearance of this epidemiological emergency in the form of a pandemic, the restrictions imposed due the characteristics of the epidemiological event (in this case, COVID-19, a disease with airborne transmission and a high risk of contagion), and the necessity to move almost the whole healthcare force toward providing COVID-19-related services, the clinical and surgical activities of many specialists have declined, influencing patients' access to high-quality medical services and decreasing patients' satisfaction with healthcare referrals, thus influencing the quality of life for patients diagnosed with skin cancers [11].

Pathology services include training and teaching for residents, autopsies, and quality assurance in the activity of the laboratory, combined with diagnostic activities. The COVID-19 pandemic has challenged all of these aspects. As a result of the reduction in surgical procedures and an almost complete cessation of aerosol-generating specimens and procedures, such as gastric or pulmonary endoscopy, the diagnostic workload and workflow have been dramatically affected in almost all histopathology services since the beginning of the COVID-19 pandemic [1,12].

Throughout the COVID-19 pandemic, a series of functioning rules have been implemented, not only internationally but also in our country. These proceedings had a much greater applicability at the level of clinical departments; however, some of the guidelines also applied to paraclinical departments. Diagnostic procedures in a pathology wards can be categorized as pre-analytical, analytical, and post-analytical. Regarding the possible transmission of SARS-CoV-2, the pre-analytical phase is the most favorable phase in which the virus can intervene. During this phase, fresh tissue is received in the pathology ward, and the tissue samples, biological materials, or formalin-fixed tissue and organs are processed. According to the guidelines from RCPath (Royal College of Pathologists), fresh tissue handling is discouraged [1,13]. The measures taken to minimize the possible SARS-CoV-2 transmission route while handling the tissue samples are aimed at increasing

the time of the formalin fixation process. Additionally, while handling the tissue samples, the laboratory personnel are advised to wear adequate protective equipment. Another applied measure involved establishing a program for receiving the biological samples arriving from the surgical departments. As in those cases reported in the literature, in our pathology service, there were no cases of infection with SARS-CoV-2 related to tissue sample handling [1,2]. The personnel from our ward are tested for COVID-19 frequently, and almost 95% were vaccinated with a COVID-19 approved vaccination scheme before the end of March 2021. The dermatopathology ward in Mures Clinical County Hospital has been strongly affected by these changes. The number of histopathological diagnoses significantly decreased from 866 histopathological diagnoses during the non-COVID-19 period to 302 during the COVID-19 period, as shown in Figure 1. Specifically, there was a large decrease in the first 2 months of the COVID-19 period (April and May 2020), with an increase in the subsequent months. Peaks were observed in August 2020 and October 2020, but they were not comparable to the COVID-19 period.

As seen in Table 1, the main histopathological diagnoses established in our ward were based on benign pathologies, most of which were epidermoid cysts, granulation tissues, and junctional and dermal nevi. Additionally, biopsies from the skin were used to establish diagnoses of psoriasis or psoriasiform dermatitis in a significant number of cases. Regarding the comparison between the benign and malignant diagnoses established in our ward, a significant decrease was observed in both categories. The malignant diagnoses during the COVID-19 period decreased to less than half of the diagnoses from the non-COVID-19 period. Even though the number of malignant diagnoses decreased, as seen in Figure 3, there was a slight difference in the percentages of BCC and SCC diagnoses out of the total malignant diagnoses. A greater difference was seen in the diagnoses of BCC between the two study periods, with an increase during the COVID-19 period. Melanoma diagnoses decreased to one-quarter during the COVID-19 pandemic compared to the diagnoses established during the non-COVID-19 period. Moreover, the histopathological diagnoses of NMSCs were negatively impacted during the COVID-19 period, with fewer cases diagnosed during this period.

During the COVID-19 pandemic, several multidisciplinary recommendations were developed regarding the local treatment of skin cancer patients. Regarding NMSCs, specifically, BCCs and SCCs, the main recommendations were as follows: to delay the treatment for a period of 2 or 3 months, unless the patient is highly symptomatic or immunosuppressed, or to prioritize those with an advanced stage of disease ($\geq$T2b). Concerning those patients suffering from Merkel cell carcinoma, the main recommendations suggest the prioritization of treatment or the possibility of deferral for those with a favorable stage of disease (T1b) who are at a high risk of experiencing COVID-19 complications. Regarding cutaneous melanoma, the recommendations suggest delaying treatment for patients with T0–T1 stages of the disease for 3 months if no macroscopic residual disease is found when a biopsy is performed. For those patients with disease beyond stage T2, a 3 month delay is recommended if the biopsy margins are declared negative [14,15].

Considering the specific area in which pathology services work, a series of protective measures have been taken. Due to the new social distancing regulations, the working schedule has been adapted in order to respect the national regulations. Residents in training have changed their schedule according to their supervisor. If there is a case of direct contact with a SARS-CoV-2-positive person, social distancing is immediately implemented and the national regulations for testing and quarantine are applied. The "working from home" concept has been adopted by many domains during the pandemic. In the pathology ward, face-to-face meetings are only available for the pre-analytical phase while handling fresh tissue, as well as the analytical phase, in which slides are analyzed using a double-header or multi-header microscope that can be set in order to establish a pathologist–resident connection and that allows for focusing on hands-on training. The post-analytical phase is performed mostly from home, via online training and meetings [1,16].

In our pathology service, there was less experience with working from home before the COVID-19 pandemic. For this reason, we implemented a secure connection in order to be able to assure that the residents' training and personal training were in accordance with the new regulations by using dedicated e-learning platforms and online communication software [1,17].

The COVID-19 pandemic has had a negative effect on those patients who needed surgery, especially the oncological patients. Overcoming the fear of infection and, most importantly, returning to a normal life will allow patients to receive medical consultation and go to the dermatologist. We expect to observe an increase in the number of dermatological cases and, particularly, an increase in the number of NMSC and melanoma cases in the near future. We are also expecting to notice cases in much more advanced stages due to the period in which patients did not receive medical consultation regarding their skin pathology.

5. Conclusions

During the COVID-19 pandemic, a decrease in histopathological diagnoses at the dermatopathology ward in our hospital was observed, for both benign and malignant pathologies, especially NMSCs and melanomas, compared to the same period 1 year prior to the pandemic. The COVID-19 pandemic has been a challenge for histopathology services. In terms of maintaining the high quality of histopathological diagnostics, keeping a social distance, and adapting the patterns and processes of work, a restructuring of the activity in every part of the laboratory was needed. By using the new regulations, we were able to limit the transmission of SARS-CoV-2 without changing the quality of the histopathological diagnoses established in our ward, thus maintaining the patients' satisfaction regarding the medical services that they need for diagnostics.

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Toxicological Evaluation of Novel Cyclohexenone Derivative in an Animal Model through Histopathological and Biochemical Techniques

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Abstract: Toxicity studies were conducted to provide safety data of potential drug candidates by determining lethal and toxic doses. This study was designed for pre-clinical evaluation of novel cyclohexenone derivative with respect to the acute and sub-acute toxicity along with the diabetogenic potential. Acute and sub-acute toxicity were assessed after intraperitoneal (i.p) injection of the investigational compound through selected doses for 21 days. This was followed by assessment of isolated body organs (liver, kidney, heart and pancreas) via biochemical indicators and histopathological techniques. No signs of toxicity were revealed in the study of acute toxicity. Similarly, a sub-acute toxicity study showed no significant difference in biochemical indicators on 11th and 21st days between treated and control groups. However, in blood urea nitrogen (BUN) and random blood glucose/sugar (RBS) values, significant differences were recorded. Histopathological evaluation of liver, kidney, pancreas and heart tissues revealed mild to severe changes in the form of steatosis, inflammation, fibrosis, necrosis and myofibrillary damages on 11th and 21st days of treatment. In conclusion, the median lethal dose of the tested compound was expected to be greater than 500 mg/kg. No significant change occurred in selected biomarkers, except BUN and RBS levels, but a histopathological study showed moderate toxic effect on liver, kidney, pancreas and heart tissues by the cyclohexenone derivative.

Keywords: cyclohexenone derivative; toxic effects; liver; kidney; pancreas; heart

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1. Introduction

In the process of drug discovery, pre-clinical testing of novel chemical entities involves assessment of both safety and efficacy. It is carried out in selected animal models through international standardized protocols. Broadly, it includes assessment of safety and efficacy through pharmacological studies with reference to the dose, frequency and route of administration. Promising outcomes at these steps can provide justified bases to proceed further

into drug development [1]. Apart from pharmacological activity, toxicological assessment remained an integral part of the preclinical investigation. Toxicological evaluation is useful to provide data proving that the potential drug candidate is safe enough for next the phase of study i-e clinical testing. Such studies of toxicity testing have multiple utilities, such as an approximation of safe doses for humans and prediction of toxicity in susceptible organs. These provide scientific evidence regarding the safety profile, nature, variety and severity of adverse effects in association with its potential efficacy in usable doses, strength and concentration [2]. Cyclohexenones are cyclohexane derivatives that represent a carbonyl functional group and a double bond at positions C-1 and C-2, respectively. Their significant role is well known, including potential anti-inflammatory activity [3–5]. Furthermore, these compounds also have considerable antifungal, antibacterial, antiviral, anticancer, antiplasmodial and/or antimalarial activities [6–12]. Among these, one of the cyclohexenone derivatives is ethyl 6-(4-methoxyphenyl)-2-oxo-4-phenylcyclohexe-3-ene-carboxylate (Figure 1) [13].

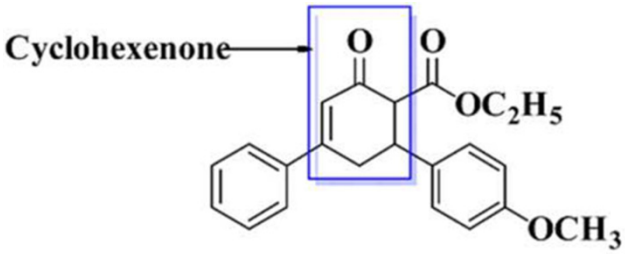

Figure 1. Ethyl 6-(4-methoxyphenyl)-2-oxo-4-phenylcyclohexe-3-ene-carboxylate.

Preliminary studies show that this derivative has a molecular formula of $C_{22}H_{22}O_4$, melting point = 92–95 °C, relative front = 0.51 n-hexane/ethyl acetate (7:3). Infrared (KBr) spectrum shows υ_{max} cm^{-1}: 3077 (Ar-H), 1689 (ketone C=O), 1735 (Ester C=O) and 2870 (Aliphatic C-H). ^{1}H-NMR ($CDCl_3$, 400 MHz) shows δ: 6.9–7.5 (m, Ar-H), 5.2 (s, ^{1}H ethylene), 3.4 (s, ^{3}H OCH3), 3.05 (d, ^{2}H, *J* = 2.3), 2.9 (t, ^{1}H *J* = 5.0, C-3) and 2.6–2.8 (q, 5H, CH_2CH_3, *J* = 7.0), and ^{13}C-NMR (100 MHz, $CDCL_3$) shows δ: 199.0 (C=O), 125–130 (Ar-CH), 112 (C-6), 40.2 (OCH_3), 159.0 (C-19) and 44.39 (C-3). Similarly, EI-MS showed; m/z (rel. int.%) 351 (M+), CHN Anal. Calculated for: C, 75.41; H, 6.33; O, 18.26. Found: C, 74.81; H, 6.38. Furthermore, molecular docking studies revealed a strong affinity toward cyclooxygenase I and II (Cox-I and Cox-II) enzymes. It was observed in studies conducted in our laboratory that this derivative attenuated the pain aroused by vincristine in the rodent model, due to its possible antinociceptive and antioxidant effect [14]. To integrate its potential pharmacological activities with safety profile, toxicological studies are required to be conducted in an animal model.

Various studies have reported that Nonsteroidal anti-inflammatory drugs (NSAIDs) can induce renal toxicity, including acute renal failure [15–17]. Renal prostaglandin production is mediated mainly by activities of Cox-I and Cox-II enzymes. Since prostaglandins are not only produced during inflammation but also act as modulators of physiological functions i.e. when the volume of blood is compromised, prostaglandins play a role in the renal circulation (vasodilatation, renin secretion and Na^+/H_2O excretion). Hindering of prostaglandin formation by the consumption of NSAIDs in these situations can possibly lead to the development of a number of disorders of altered renal function [18,19]. In addition to the above adverse effects, liver toxicity has also been reported in the literature [20–22]. NSAIDs have evident toxicological potential regarding cardio-toxicities. This is mediated by Cox-II inhibition in the kidney, leading to fluid retention by decreased prostaglandins production. This results in a reduction of the glomerular filtration rate. Excess sodium and water retention lead to elevated blood pressure and thus develops cardiac complications. Increase in the production of reactive oxygen species in cardiac cells due to these drugs is also related to the initiation of damage to the myocardium [23–25]. Additionally, NSAIDs have possible membrane destabilizing effects on the pancreas, which may

contribute to pancreatic toxicity [26–28]. Thus, this novel compound with affinity for Cox enzymes may be anticipated to disturb renal, hepatic, pancreatic and cardiac functioning.

For this reason, if the potential drug candidate is supposed to be used for anti-inflammatory and anti-nociceptive activities, its safety profile needs to be assessed via toxicological evaluation at selected body organs through histopathological and biochemical indicators. Therefore, this study is designed to evaluate the acute and subacute toxic effects of this compound on isolated body organs of the liver, kidney, heart and pancreas in an animal model through histological and biochemical indicating parameters.

2. Materials and Methods

2.1. Animals Breeding and Ethical Approval

Animals (mice) kept in a light and dark sequence (12 h each) at a suitable temperature (22 ± 2 °C) in cages, fed with water and laboratory standardized food, were raised in an animal house in the Department of Pharmacy, University of Peshawar (UoP). Specified age and weight range animals were selected for experimental studies. Ethical approval of preclinical studies on cyclohexenone derivative (CHD) was granted by the ethical committee of the Department of Pharmacy, UoP, via endorsement testament number 01/EC-18/PHARM; dated 16 October 2018. Experimental studies on mice were executed in congruence with UK Animals (Scientific Procedures) Act 1986.

2.2. Procedures

2.2.1. Selection of Dose(s) and Route of Administration

The selection of dose(s) of this novel compound for its potential toxicity was based on previous studies by Jawad et al., conducted in the laboratory of the Department of Pharmacy, University of Peshawar, where he reported that this derivative had attenuated the pain aroused by vincristine in rodent model at doses of 30–45 mg/kg. The acute studies were performed on four groups constituting three treatment groups at doses of 250 mg/kg in group I, 350 mg/kg in group II and 500 mg/kg in group III, and a control group IV was used to understand the dose-dependency of the effect. Keeping in view the efficacy of doses, the maximum reported dose (45 mg/kg) of the said compound was taken as a starting dose for sub-acute toxicity study [14]. Similarly, Jawad et al. also provided information of LD_{50} value as beyond 240 mg/kg. To find the exact LD_{50} value, a higher dosage level of 250 mg/kg, 350 mg/kg and 500 mg/kg were selected to determine the possible lethal value of the dose [14].

Furthermore, the intraperitoneal (i.p) route of administration was selected, looking at the efficacy studies conducted by the same investigator. The researcher observed that this derivative had reduced the pain aroused by vincristine in an animal model using the same i.p route [14]. His finding was associated with the i.p route of administration; therefore, it was decided to correlate the toxicological effects with the pharmacological indication (analgesia) using the same route of administration. As its pharmacokinetic profile is not well established yet, and the researcher could not anticipate its physicochemical stability, site and rate of absorption in the gastrointestinal tract of the included animals; therefore, i.p route was considered to be more appropriate. It also provided better bioavailability with least exposure to unwanted physiological environment inside body.

2.2.2. Acute Toxicity

A total of 24 mice (BALB/c), irrespective of their sex, having permissible weights of 20–30 gm, 8–12 weeks old, and kept in a light and dark sequence (12 h each) at an appropriate temperature (22 ± 2 °C), were used in the acute toxicity experiment. Toxicity studies were performed on both genders of the animals under study, aiming to assess the toxic effects in both sexes and to minimize the chances of inter-gender variation related to the said study. Keeping in view these facts, mice were selected irrespective of their gender [29,30]. Animals were equally divided into 4 groups i–e (n = 6); the groups treated with the compound were (groups I, II and III) and the control-normal saline treated group

(group IV). Acute toxicity was assessed after intraperitoneal (i.p) injection of compound at the doses of 250 mg/kg to group I, 350 mg/kg to group II, 500 mg/kg to group III and saline to group IV. The adverse effects of the novel compound were noted at 30 to 60 min, then at 24, 48 and 72 h intervals and daily there-after, for a period of 14 days [30,31].

2.2.3. Sub-Acute Toxicity

A total of 24 mice (BALB/c) irrespective of their sex, having acceptable body weights (20–30 gm), 8–12 weeks old and kept in a light and dark sequence (12 h each) at a suitable temperature (22 ± 2 °C), were tested in the sub-acute toxicity experiment. As stated earlier, the toxicity studies were performed on both the genders of the used animal, aiming to assess the toxic effects on both the sexes and to minimize the chances of inter-gender variation related to the said study [29,30]. Animals were categorized into group I (compound treated, n = 12) and group II (control/normal-saline treated, n = 12) [31–33]. Animal's groups were treated in such a way that group I received an intraperitoneal (i.p) injection of the compound at a dose of 45 mg/kg/day for 21 days and group II received the vehicle and served as a control group.

2.2.4. Pre-Clinical Observations and Survival

Signs of pre-clinical toxicity and mortality were noted. Pre-clinical observations included alteration in body weights, gait, posture and environmental interactions (cage–mates interactions and building nest). These observations were conducted weekly throughout the course of the acute as well as sub-acute study [34,35].

2.2.5. Biochemical Assessment

During the course of treatment, blood samples were collected on 11th day (6 animals) and on 21st day (6 animals) from each group (sub-acute toxicity study groups). After centrifugation at 3000 rpm for 10–15 min, the separated serum was kept at 4 °C until analysis. The biochemical indicators evaluated were comprised of analysis of serum glutamic pyruvic transaminase (SGPT, also known as ALT) and serum glutamic oxaloacetic transaminase (SGOT, also known as AST) by utilizing analytical kits (ALT, AST; GO F400CH, Chema Diagnostica, Monsano, Italy); serum creatinine by using CR 0500CH, Chema Diagnostica, Monsano, Italy; serum blood urea nitrogen (BUN) using blood urea kits (Zone Industrille 61800 SEES, Normandy, France); random blood glucose level test using Abbott Glucometer; amylase tests through analytical kit by Randox Laboratories, London, UK; and troponin I and creatinine kinase-myocardial band (CK-MB) by utilizing standardized instrumental analytical technique through Finecare^{TM} Florescence Immunoassay analyzer. Data obtained from these tests were used to correlate with nature and level of toxicities in the hepatic, kidney, pancreatic and cardiac tissues [36].

2.2.6. Histological Evaluation

For histological evaluation, on 11th and 21st days, 6 animals (on respective duration) were euthanized and their selected body organs (kidney, liver, heart and pancreas) were isolated for assessment of subacute toxicity. The selected organs/tissues being isolated were placed in 10% Formalin solution (neutrally buffered) for 6 to 48 hours. After fixation, they were cut into pieces of a proper thickness (5 µm) and placed in paraffin to form blocks. Different size sections were made through microtome and stained using H and E staining technique [37]. After staining, slides were examined using camera equipped light microscope (LABOMED LX400, iVu 3100, Auburn Court Fremont, CA, USA). The images acquired were assessed for any alterations. Those changes included hyperemia, necrosis, inflammatory cell aggregation, steatosis, fibrosis, interstitial edema, hemorrhage, degeneration of myocytes, glomerular injury and ectasia/tubular injury. These were subjectively assessed for scoring by a pathologist as none, minimum, mild, moderate and severe [38–40].

2.3. Statistical Analysis

Descriptive statistics and suitable t-test were performed using SPSS Version 22.0 (IBM, Armonk, NY, USA) and Graph-Pad Prism Software Version 5.01 (Graph-Pad Software Inc., San Diego, CA, USA). Effects during acute toxicity were assessed by applying one-way ANOVA (followed by Bonferroni multiple comparison). For parametric tests, the assumption of normality was calculated using the Skewness and Kurtosis analysis, followed by determination of z-values. Similarly, for homogeneity of variance, Levene's test was applied (see Tables 1 and 2). *p*-values less than 0.05 were considered as statistically significant.

Table 1. Parametric tests, the assumption of normality by using the Skewness and Kurtosis analysis, followed by z-values.

Groups	Days of Treatment	Biochemical Tests	*n*	Mean	Skewness		z-Value	Kurtosis		z-Value
			Statistic	Statistic	Statistic	Std. Error		Statistic	Std. Error	
Control	11th day	Blood Urea Nitrogen (mg/dL)	6	13.33	0.248	0.845	0.293	−0.014	1.741	−0.008
		Serum creatinine (mg/dL)	6	0.517	−0.313	0.845	−0.370	−0.104	1.741	−0.059
		Alanine aminotransferase (ALT) U/L	6	94.50	−0.574	0.845	−0.679	−1.132	1.741	−0.650
		Aspartate aminotransferase (AST) U/L	6	24.00	0.433	0.845	0.512	−1.175	1.741	−0.674
		Valid N (listwise)	6							
	21st day	Blood Urea Nitrogen (mg/dL)	6	15.17	0.319	0.845	0.377	−1.171	1.741	−0.672
		Serum creatinine (mg/dL)	6	0.483	0.313	0.845	0.370	−0.104	1.741	−0.059
		Alanine aminotransferase (ALT) U/L	6	92.17	−0.036	0.845	−0.042	0.428	1.741	0.245
		Aspartate aminotransferase (AST) U/L	6	24.67	−0.224	0.845	−0.265	−1.864	1.741	−1.070
		Valid N (listwise)	6							
Treatment	11th day	Blood Urea Nitrogen (mg/dL)	6	26.67	0.435	0.845	0.514	0.586	1.741	0.336
		Serum creatinine (mg/dL)	6	0.483	0.313	0.845	0.370	−0.104	1.741	−0.059
		Alanine aminotransferase (ALT) U/L	6	98.00	−0.515	.845	−0.609	0.729	1.741	0.418
		Aspartate aminotransferase (AST) U/L	6	25.50	0.461	0.845	0.545	−1.260	1.741	−0.723
		Valid N (listwise)	6							
	21st day	Blood Urea Nitrogen (mg/dL)	6	18.50	0.000	0.845	0	−1.200	1.741	−0.689
		Serum creatinine (mg/dL)	6	0.500	0.000	0.845	0	−1.875	1.741	−1.076
		Alanine aminotransferase (ALT) U/L	6	94.50	0.401	0.845	0.474	1.635	1.741	0.939
		Aspartate aminotransferase (AST) U/L	6	24.50	0.255	0.845	0.301	−1.312	1.741	−0.753
		Valid N (listwise)	6							

Table 2. Parametric test for homogeneity of variance using Levene's test.

	Levene's Test for Equality of Variances—11th Day		Levene's Test for Equality of Variances—21st Day	
	F	Sig.	F	Sig.
Blood urea Nitrogen (mg/dL)	0.741	0.410	1.250	0.290
Serum creatinine (mg/dL)	0.000	1.000	0.160	0.698
Alanine aminotransferase (ALT) U/L	0.877	0.371	2.094	0.178
Aspartate aminotransferase (AST) U/L	0.030	0.865	1.042	0.331

3. Results

3.1. Acute Toxicity

Acute toxicity was evaluated using separated mice in groups, namely, group I (250 mg/kg), group II (350 mg/kg), group III (500 mg/kg) and group IV (control). After intraperitoneal injection (i.p) of selected doses of the cyclohexenone derivative (250 mg/kg, 350 mg/kg, 500 mg/kg) to respective groups, no signs of pre-clinical toxicity and mortality/death were observed in any treated group of mice up to a dose of 500 mg/kg. Therefore, lethal dose is expected to be greater than 500 mg/kg. Applying one-way ANOVA (followed by Bonferroni multiple comparison) revealed no significant change in the body weights of the group treated with compound as compared to the control group.

3.2. Sub-Acute Toxicity

3.2.1. Body Weights

The mean values of body weights of mice at baseline, 7th and 11th day of treatment were 26.67, 27.17 and 26.83 g for control group and 26.5, 26.67 and 26.17 g for treated group, respectively. Applying independent sample t-test revealed no significant difference ($p > 0.05$) in body weights of treated and control groups.

The mean values of body weights at baseline, 1st, 2nd and 3rd week of treatment were 26.33, 26.83, 27.33 and 27.5 g for control group and 25.67, 26, 26.17 and 26.17 g for treated group, respectively. Applying independent sample t-test revealed no significant difference ($p > 0.05$) in body weights of treated and control groups.

3.2.2. Biochemical Assessment

On 11th and 21st days, blood samples from treated and control groups were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum creatinine, blood urea nitrogen (BUN), random blood glucose, serum amylase, cardiac troponin-I (cTn-I) and creatinine kinase-myocardial band (CK-MB). Results of these indicators are mentioned in the following headings and tables.

Liver

On 11th day of treatment, the mean value for ALT was 94.5 U/L in control and 98 U/L in treated group. Similarly, mean value for AST was 24 U/L in control and 25.5 U/L in treated group. By application of independent sample t-test, the *p*-value for ALT was 0.205 and for AST the *p*-value was 0.447. Thus, showing no significant difference of biochemical indicators values (ALT and AST) between treated and control groups on 11th day of treatment.

On 21st day of treatment, the mean value for ALT was 92.17 U/L in control and 94.5 U/L in treated group. Similarly, mean value for AST was 24.67 U/L in control and 24.5 U/L in treated group. Sample t-test revealed the *p*-value for ALT was 0.738, and for AST the *p*-value was 0.933, thus reflecting no significant difference in terms of biochemical indicators values between the treated and control groups on 21st day of treatment. All these values are depicted in Table 3.

Kidney

On 11th day of treatment, the mean value for BUN was 13.33 mg/dL in control and 26.67 mg/dL in treated group. Similarly mean value for serum creatinine was 0.517 mg/dL in control and 0.483 mg/dL in treated group. When independent sample t-test was aplied, *p*-values for BUN and serum creatinine were 0.00 and 0.461, respectively, thus revealing no significant difference in case of serum creatinine values but a significant difference occurs in BUN values between treated and control groups.

Table 3. Biochemical indicators: showing number of animals (*n*); mean ± standard deviation; and maximum, minimum and *p*-value of ALT and AST on 11th and 21st days of treatment.

Group	Statistical Parameter	Alanine Aminotransferase (ALT, 11th day)	Alanine Aminotransferase (ALT, 21st day)	Aspartate Aminotransferase (AST, 11th day)	Aspartate Aminotransferase (AST, 21st day)
Control (*n* = 6)	Mean ± SD	94.50 ± 5.089	92.17 ± 5.456	24.00 ± 3.464	24.67 ± 2.805
	Minimum	87	84	20	21
	Maximum	100	100	29	28
Treatment (*n* = 6)	Mean ± SD	98 ± 3.742	94.50 ± 15.681	25.50 ± 3.082	24.50 ± 3.834
	Minimum	92	72	22	20
	Maximum	103	120	30	30
Treated vs. control	*p*-value	0.205	0.738	0.447	0.933
Treated (11th day) vs. treated (21st day)	*p*-value	0.606		0.629	

On 21st day of treatment, the mean value for BUN was 15.17 mg/dL in control and 18.5 mg/dL in treated group. Similarly, mean value for serum creatinine was 0.483 mg/dL in control and 0.5 mg/dL in treated group. By the application of independent sample t-test, significant difference was observed in values of BUN between control and treated group (*p*-value 0.03), but no significant difference occurred in values of serum creatinine between treated and control groups (*p*-value 0.734).

When biochemical indicators of treated group on 11th day were compared with treated group on 21st day, no significant difference was recorded between the said results of treated group (ALT *p*-value = 0.606, AST *p*-value = 0.629 and serum creatinine *p*-value = 0.734). However, in BUN values, a significant difference was observed between treated groups on 11th and 21st days (see Table 4).

Table 4. Biochemical indicators: showing number of animals (*n*); mean ± standard deviation; and maximum, minimum and *p*-value of BUN and creatinine of treated and control groups on 11th and 21st days of treatment.

Group	Statistical Parameter	Blood Urea Nitrogen (BUN, 11th Day)	Blood Urea Nitrogen (BUN, 21st Day)	Serum Creatinine (11th Day)	Serum Creatinine (21st Day)
Control	Mean ± SD	13.33 ± 1.75	18.50 ± 1.871	0.517 ± 0.075	0.500 ± 0.0894
	Minimum	11	16	0.4	0.4
	Maximum	16	21	0.6	0.6
Treatment	Mean ± SD	26.67 ± 2.73	15.17 ± 2.639	0.483 ± 0.075	0.483 ±0.0753
	Minimum	23	12	0.4	0.4
	Maximum	31	19	0.6	0.6
Treated vs. control	*p*-value	0	0.03	0.461	0.734
Treated (11th vs. 21st day)	*p*-value	0		0.734	

Pancreas

On 11th day, half of the animals in control group were assessed for their amylase level, presenting mean values of 2683.17 U/L (in the treated group it was 2680.00 U/L). The same group of animals, when assessed for random blood glucose levels, showed means values of 83.83 mg/dL in control group and 47.83 mg/dL in treated group. Independent sample t-test showed a *p*-value of 0.831 for amylase level, showing no significant difference, but the random blood glucose has shown a *p*-value less than 0.05, thus showing a significant difference between the control and treated groups.

On 21st day, blood sample of the animals in the control group showed mean value of 2680.00 U/L; when evaluated for amylase level and in the treated group, it was

2672.00 U/L. The mean value of random blood glucose obtained in control group animals was 69.33 mg/dL, and in treated group of animals, the value was 56.67 mg/dL. The *p*–value obtained after sample t-test for amylase level was 0.515, thus showing no significant difference. The *p*-value obtained for random blood sugar level was 0.018, which is less than 0.05, thus showing a significant difference between control and treated groups. All these results are shown in Tables 5 and 6.

Table 5. Biochemical indicators: showing number of animals (*n*); mean ± standard deviation; maximum, minimum and p-value of serum amylase; and random blood glucose level on 11th and 21st days of treatment.

Group	Statistical Parameter	Serum Amylase (U/L) (11th Day)	Serum Amylase (U/L) (21st Day)	Random Blood Glucose (mg/dL) (11th Day)	Random Blood Glucose (mg/dL) (21st Day)
Control (*n* = 6)	Mean ± SD Minimum Maximum	2683.17 ± 26.649 2649 2720	2680 ± 23.421 2655 2700	83.83 ± 7.35 73 90	69.33 ± 9.07 60 85
Treatment (*n* = 6)	Mean ± SD Minimum Maximum	2680 ± 23.45 2655 2710	2672 ± 17.07 2650 2700	43.66 ± 13.89 23 65	56.67 ± 6.18 48 65
Treated vs. control	*p*-value	0.831	0.515	0.000	0.018
Treated (11th day) vs. treated (21st day)	*p*-value	0.184		0.508	

Table 6. Biochemical indicators: showing number of animals (*n*); mean ± standard deviation; and maximum, minimum and *p*-value of random blood glucose level weekly up to 21 days.

Group	Statistical Parameter	Random Blood Glucose (mg/dL)	Random Blood Glucose (mg/dL)	Random Blood Glucose (mg/dL)	Random Blood Glucose (mg/dL)
Time Period		**Baseline**	**7th Day**	**11th Day**	**-**
Control (*n* = 6)	Mean ± SD Minimum Maximum	83.57 ± 7.232 74 92	82.67 ± 4.179 79 90	83.83 ± 7.360 73 91	- - -
Treatment (*n* = 6)	Mean ± SD Minimum Maximum	82.17 ± 4.535 78 90	60.33 ± 3.011 572 66	47.83 ± 9.517 39 65	- - -
Treated vs. control	*p*-value	0.710	0.000	0.000	-
Time Period		**Baseline**	**7th Day**	**14th Day**	**21st Day**
Control (*n* = 6)	Mean ± SD Minimum Maximum	72.50 ± 4.722 70 69	75.50 ± 6.979 69 85	75.83 ± 6.555 68 85	69.33 ± 9.070 60 85
Treatment (*n* = 6)	Mean ± SD Minimum Maximum	80.83 ± 9.042 74 92	60.00 ± 7.563 79 90	56.17 ± 4.355 73 91	56.67 ± 6.186 48 65
Treated vs. control	*p*-value	0.073	0.004	0.000	0.018

Heart

On 11th day, half of the animals in control group, when assessed for their cardiac troponin-I levels, showed mean values of 0.29667 ng/dL and in the treated group 0.3 mg/dL (Table 7). Same animals when assessed for their CK-MB levels; the mean values of 11 U/L in control group and 13.83 U/L in treated group were recorded. Independent sample t-test showed *p*-value of 0.916 for cTn-I, thus, showing no significant difference and

for CK-MB it was 0.672, also showing no significant difference between the control and treated groups on 11th day.

Table 7. Biochemical indicators: showing number of animals (*n*); mean ± standard deviation; and maximum, minimum and *p*-value of cTn -I and CK-MB on 11th and 21st days of treatment.

Group	Statistical Parameter	Cardiac Troponin-I (ng/dL) (cTn-I, 11th Day)	Cardiac Troponin-I (ng/dL) (cTn-I, 21st Day)	Creatinine Kinase-Myocardial Band (U/L) (CK-MB, 11th Day)	Creatinine Kinase-Myocardial Band (U/L) (CK-MB, 21st Day)
Control (n = 6)	Mean ± SD	0.29667 ± 0.055	0.25833 ± 0.02483	11 ± 2.098	13.83 ± 1.871
	Minimum	0.25	0.23	9	8
	Maximum	0.4	0.27	14	12
Treatment (n = 6)	Mean ± SD	0.3 ± 0.0522	0.26167 ± 0.0286	13.83 ± 2.14	13.67 ± 2.160
	Minimum	0.26	0.22	12	11
	Maximum	0.4	0.3	16	17
Treated vs. control	*p*-value	0.916	0.834	0.672	0.896
Treated (11th day) vs. treated (21st day)	*p*-value	0.497		0.786	

On 21st day, blood sample was assessed for cardiac troponin-I in control group of animals, for which the mean value of 0.2583 mg/dL and in treated group, mean value of 0.26167 ng/dL were recorded. The mean value of CK-MB obtained in control group animals was 13.83 U/L, and in treated group of animals, the value was 13.67 U/L. The *p*-value obtained after the sample t-test for cTn-I was 0.834, thus showing no significant difference. The *p*-value obtained for CK-MB level was 0.896, which is greater than 0.05, thus showing no significant difference between control and treatment groups.

When treated groups of animals were compared for their biochemical indicators on 11th and 21st days, no significant difference was observed. The *p*-value for amylase was 0.184, for random blood glucose level was 0.508, for cTn-I was 0.497 and for CK-MB was 0.786. However, random blood glucose level was significantly decreased by the end of 11th and 21st day of treatment, which presents no significant difference in the random blood glucose lowering effect in both treated groups of animals on 11th and 21st days (all these outcomes are shown in Table 7).

3.2.3. Histopathological Evaluation

Effect on Liver

On 11th day, histopathological assessment of liver tissues revealed mild steatosis in all treated group animals. Mild hyperemia was observed in two animals, while mild fibrosis and inflammatory cell aggregation were observed only in one animal. No such pathological changes were observed in control group animals. Similarly, histopathological evaluation of liver tissues on 21st day revealed mild inflammatory cell aggregation in all treated group animals. Mild fibrosis and necrosis were observed in three and one treated group animal, respectively. Steatosis was observed in all animals ranging from mild to severe type. No such changes were observed in control group animals.

Images of liver tissue of mice on 11th day of treatment with normal saline have shown no signs of inflammation, hyperemia, steatosis and necrosis. However, images of liver tissue of mice on 11th day of treatment with cyclohexenone derivative (CHD) have shown some histopathological changes. Steatosis in hepatocytes was observed. Hyperemia and necrosis were also seen.

Figure 2A,B shows representative images of liver tissue of mice on 21st day of treatment with normal saline. Normal liver tissue architecture can be seen in these figures.

Normal hepatocytes were observed. Central vein and hepatic portal triad can be seen. No inflammation, hyperemia, steatosis and necrosis were observed.

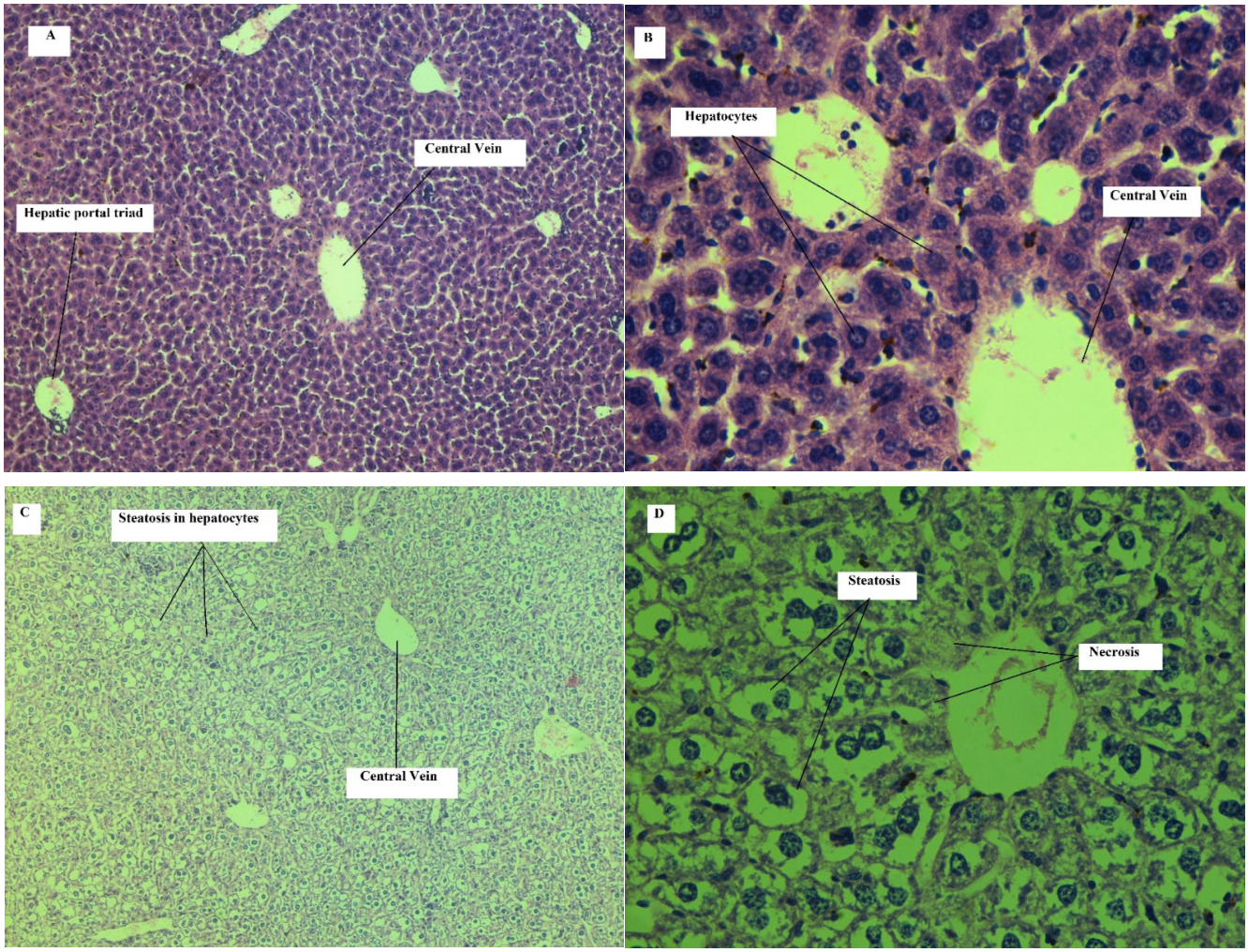

Figure 2. Liver of mouse treated with normal saline (H and E, 5µm, **A**: 100×, **B**: 400×): representative images of the liver of mouse treated with normal saline (i.p) for 21 days showing normal central vein, hepatocytes and hepatic portal triad. Liver of mouse treated with CHD (H and E, 5 µm, **C**: 100×, **D**: 400×): representative image of the liver of mouse treated with CHD (45 mg/kg/day, i.p) for 21 days. Steatosis and necrosis can be seen.

In Figure 2C,D, representative images of liver tissue of mice on 21st day of treatment with cyclohexenone derivative (CHD) are shown. Architecture changes were observed. Steatosis and necrosis in hepatocytes can also be seen. Those induced changes, present on 11th day, became more severe on 21st day of treatment. Further interpretation of the slides/images can be found in Table 8.

Effect on Kidney

On 11th day, histopathological assessment of kidney tissues revealed mild glomerular and tubular injury in all animals of treated group. In three animals, mild steatosis was observed. Mild hyperemia and inflammatory cell aggregation were detected only in one animal. These effects were observed in the animals of control group. Similarly, on 21st day, histopathological assessment has shown mild to moderate glomerular and tubular injury in treated group animals. Mild steatosis observed in five animals. In two animals of the treated group, mild fibrosis and necrosis were observed. Mild to moderate inflammatory cell aggregation was also seen in 4 animals of the treated group. Hyperemia was observed

in one animal of the treated group. However, the animals of the control group did not show such pathological changes when assessed on the same day.

Table 8. Histopathological evaluation scores of kidney and liver tissue slides of mice on 21st day of treatment.

Histopathological Findings	Animal Group-I (Control)						Animal Group-II (Treated)					
	1	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Kidney												
Hyperemia	0	0	0	0	0	0	0	0	0	0	1	0
Necrosis	0	0	0	0	0	0	1	0	0	0	0	1
Inflammatory cell aggregation	0	0	0	0	0	0	1	0	0	1	2	1
Fibrosis	0	0	0	0	0	0	0	0	1	1	0	0
Glomerular injury	0	0	0	0	0	0	1	1	2	1	2	1
Steatosis	0	0	0	0	0	0	0	1	1	1	1	1
Ectasia/tubular injury	0	0	0	0	0	0	1	1	1	2	2	2
Liver												
Hyperemia	0	0	0	0	0	0	0	0	0	0	0	0
Necrosis	0	0	0	0	0	0	0	0	0	0	0	1
Inflammatory cell aggregation	0	0	0	0	0	0	1	1	1	1	1	1
Fibrosis	0	0	0	0	0	0	1	0	0	1	0	1
Steatosis	0	0	0	0	0	0	2	1	3	2	2	2

0 none; 1 mild; 2 moderate; and 3 severe.

Kidney tissue of mice on 11th day of treatment with normal saline has shown normal renal tissue architecture, glomeruli, Bowman's capsules and convoluted tubules. It was observed that glomeruli were normal and with intact structure. Similarly, it was found that normal Bowman's capsule with endothelium was intact. No signs of inflammation, fibrosis, hyperemia and tubular injury were seen, but on 11th day of treatment with cyclohexenone derivative (CHD), the images of kidney tissue show multiple injured glomeruli with reduced size, inflammatory cells infiltration and increased blood flow (hyperemia) with tubular injury.

Figure 3A,B shows representative images of kidney tissue of mice on 21st day of treatment with normal saline. Normal renal tissue architecture can be seen in these images. Glomeruli, Bowman's capsule and convoluted tubules are visible. Glomeruli were normal, and intact structure was observed. Similarly, Bowman's capsules were normal and with intact endothelium. No inflammation, fibrosis, hyperemia and tubular injury were detected.

In Figure 3C,D, representative images of kidney tissue of mice on 21st day of treatment with cyclohexenone derivative (CHD) are shown. Multiple injured glomeruli with reduced size can be seen. Inflammatory cell infiltration and injured tubules are visible. Necrosis and steatosis can be seen in figures. The architectural changes presented on 11th day of treatment have shown an increase in frequency and severity on 21st day of treatment. Numerical scoring of the histopathological changes can be seen in Table 8.

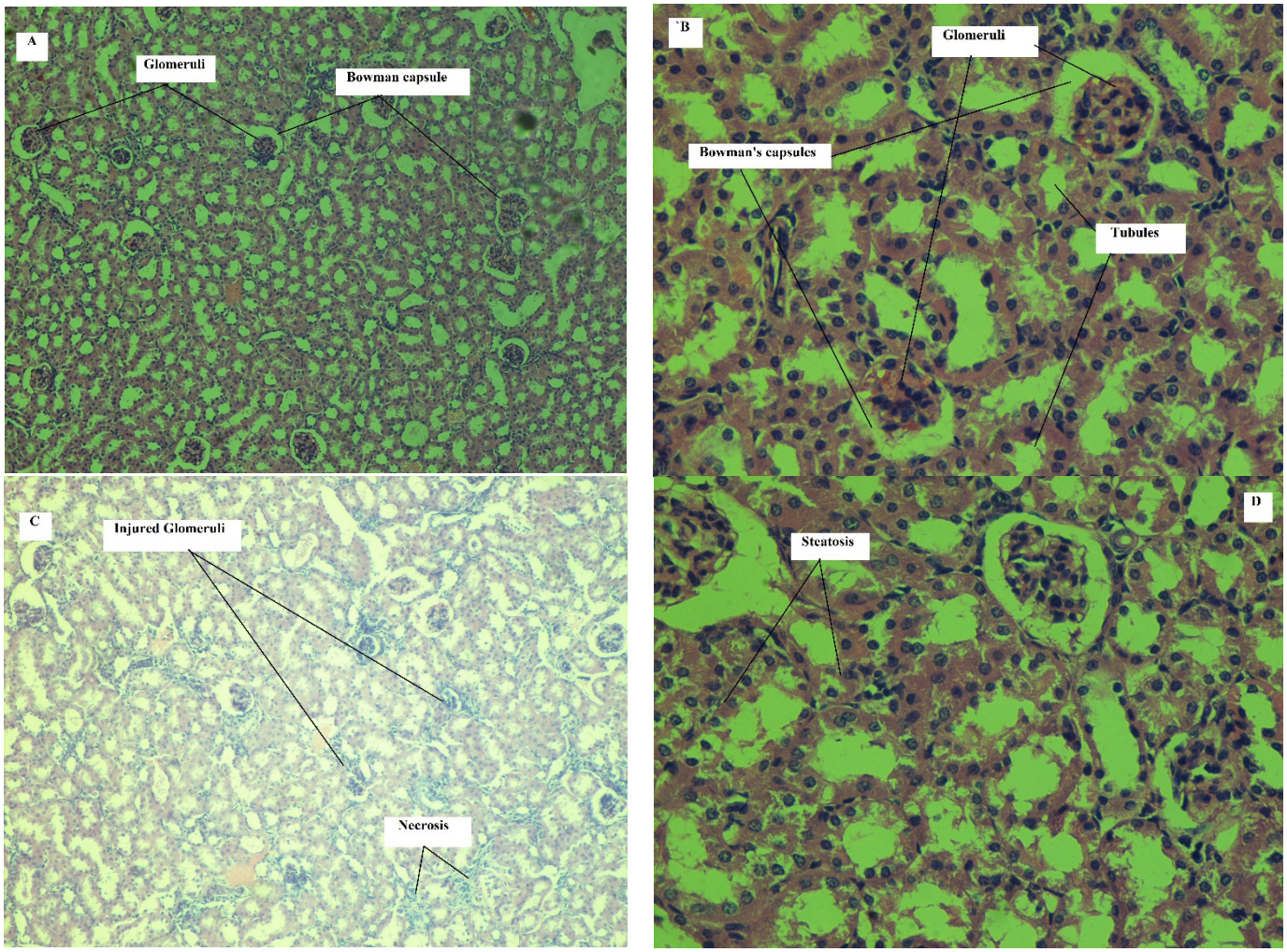

Figure 3. Kidney of mouse treated with normal saline (H and E, 5 µm, **A**: 100×, **B**: 400×): representative images of the kidney of mouse treated with normal saline (i.p) for 21 days showing glomeruli and Bowman's capsules. Kidney of mouse treated with CHD (H and E, 5 µm, **C**: 100×, **D**: 400×): Representative images of the kidney of mouse treated with CHD (45 mg/kg/day, i.p) for 21 days showing injured glomeruli, steatosis and necrosis.

Effect on Pancreas

Histopathological assessment on 11th day of treatment in mice has presented mild edema in their tissues in all animals of treated group. Necrosis was observed mildly in four animals. Mild inflammation was found in all animals except one, in which moderate inflammation was observed. Treated animals did not show hemorrhage and fat necrosis. These changes were not observed in control group animals. The results of assessment of pancreatic tissue on 21st day showed moderate edema in half of the animals in treated group and marked to severe edema in the remaining animals of the same group. There was mild necrosis in half of the group of treated animals and moderate in the remaining half with mild to moderate inflammation. No hemorrhage and fat necrosis were observed. In control group of animals, no such variations were observed on 21st day.

Histology of the pancreas has shown normal architecture in control group of animals after treatment with normal saline on 11th day of the treatment. Acinar cells were intact with endothelium with normal intra lobular ducts. No signs of edema, inflammation and necrosis were seen. However, histology of pancreas after treatment with cyclohexenone derivative (CHD) in treated group of animals, demonstrated alterations in normal histology on 11th day of treatment. Acinar cells were mildly necrotized in half of the animals. Mild edema and inflammation were observed in tissues surrounding acinar cells.

Figure 4A,B represents histology of pancreas after treatment with normal saline in control group of animals on 21st day of treatment. The architecture of pancreas has presented normal histology. Acinar cells were intact with endothelium with normal intralobular ducts. No signs of edema, inflammation or necrosis can be seen in the figure.

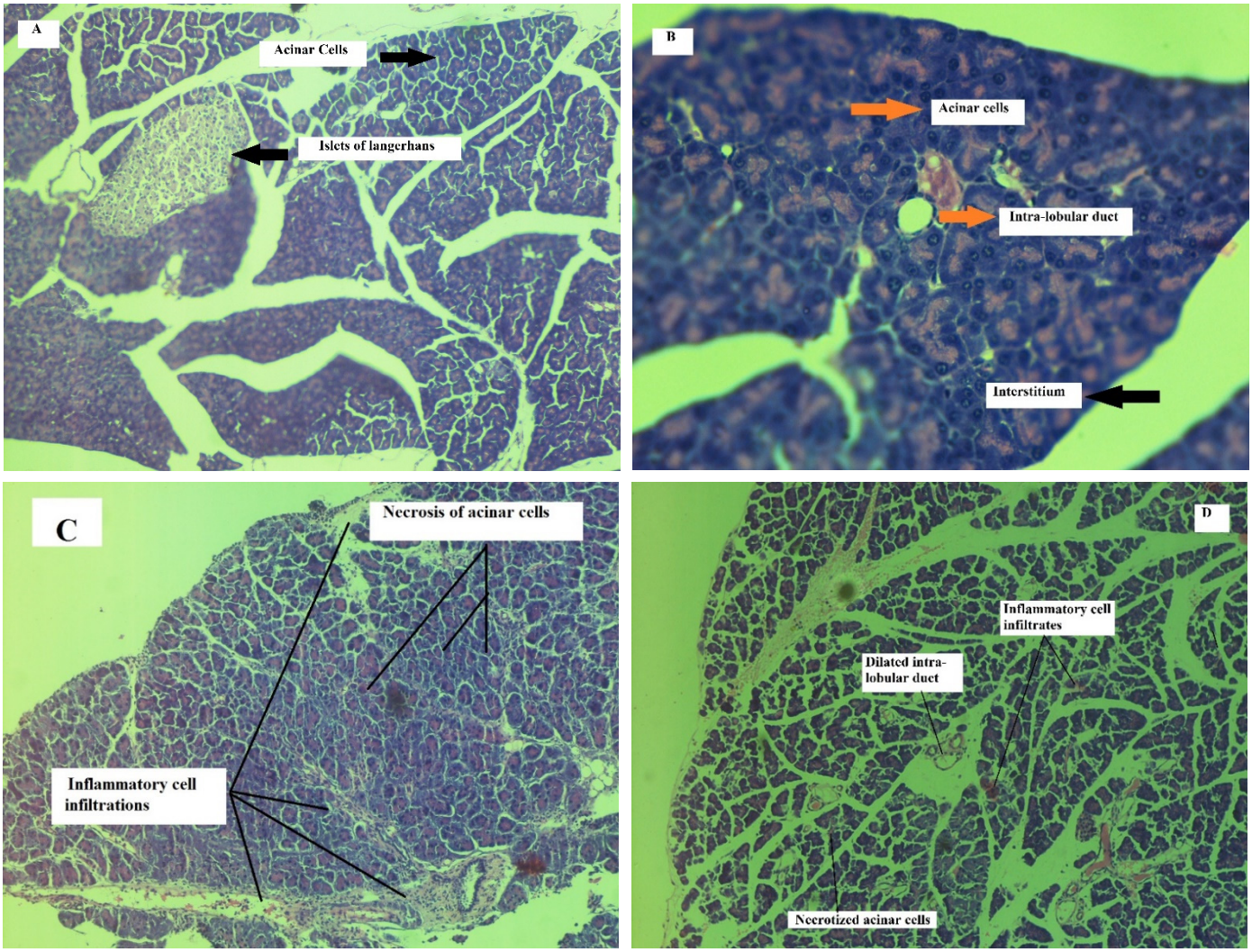

Figure 4. Normal saline treated pancreas of mouse (H and E stained, 5µm, **A**:100×, **B**: 400×): Representative image of pancreas of mouse treated with normal saline for 21 days showing normal intact acinar cells, islet of Langerhans with normal inter and intra lobular ducts. Pancreas of mouse treated with CHD (H and E stain, 5µm, **C**:100×, **D**: 400×): representative images of pancreas of mouse treated with CHD (45mg/kg/day, i.p) for 21 days showing moderately necrotized acinar cells with interstitial edema and inflammation.

Figure 4C,D signifies histology of pancreas after treatment with cyclohexenone derivative (CHD) in treated group of animals on 21st day of treatment. The architecture of pancreas has shown alteration in normal histology. Acinar cells were moderately necrotized in half of the animals of the treated group. Edema and inflammation was observed in tissues surrounding acinar cells. The alterations brought were increased in intensity on 21st day of treatment with CHD, as evident from the data shown in Table 9.

Table 9. Histopathological scoring of pancreatic tissues of control and treated groups of mice during 11th and 21st days of treatment.

Scoring of Pancreas Tissue Slides												
on 11th Day	**Control Animals**						**Treated Animals**					
Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Edema	0	0	0	0	0	0	1	1	1	1	1	1
Necrosis	0	0	0	0	0	0	1	1	1	0	1	0
Inflammation	0	0	0	0	0	0	1	1	2	1	1	1
Hemorrhage	0	0	0	0	0	0	0	0	0	0	0	0
Fat necrosis	0	0	0	0	0	0	0	0	0	0	0	0
on 21st Day	**Control Animals**						**Treated Animals**					
Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Edema	0	0	0	0	0	0	2	2	3	3	2	4
Necrosis	0	0	0	0	0	0	2	1	2	1	1	2
Inflammation	0	0	0	0	0	0	1	1	2	2	1	3
Hemorrhage	0	0	0	0	0	0	0	0	0	0	0	0
Fat necrosis	0	0	0	0	0	0	0	0	0	0	0	0

0 none; 1 mild; 2 moderate; 3 marked; and 4 severe.

Effect on Heart

Histopathological assessment of heart in mice on 11th day of treatment with CHD revealed mild to moderate focal and multi focal damages. Minimum to mild inflammation was observed in half of the animals, but no myofibrillary damage and necrosis was observed. Any such changes were not observed in control group of animals. Assessment of heart tissue in treated group of animals showed moderate focal damages on 21st day. Multifocal damages were also observed in all animals. Moderate inflammation was found in the heart tissue of all animals. Mild myofibrillary damage was observed in half of the animals, except in one animal where moderate damage was found in myofibrils. No necrosis was found in treated group animals. No such detrimental changes were found in heart tissues of the control group animals on 21st day of treatment.

Histology of heart after treatment with normal saline in control group of animals on 11th day showed normal cardiac myocytes. Heart cells represented normal histology with mono or bi-nucleate muscle fiber. No signs of focal or multi focal damage were observed. No sign of inflammation and necrosis can be seen in the figure.

Heart cells in animals after treatment with cyclohexenone derivative (CHD) showed alterations in normal histology on 11th day of treatment. Cardiac myocytes also showed minimum focal damages. Multi-focal degenerations were observed with mild inflammation. However, no necrosis or myofibrillary damage was observed.

Figure 5A,B represents histology of the heart after treatment with normal saline in control group of animals on 21st day of treatment. The architecture of the heart presents normal cardiac myocytes. Heart cells represent normal histology with mono or bi-nucleate muscle fiber. No signs of focal or multi focal damage were observed. No sign of inflammation and necrosis can be seen in the figure.

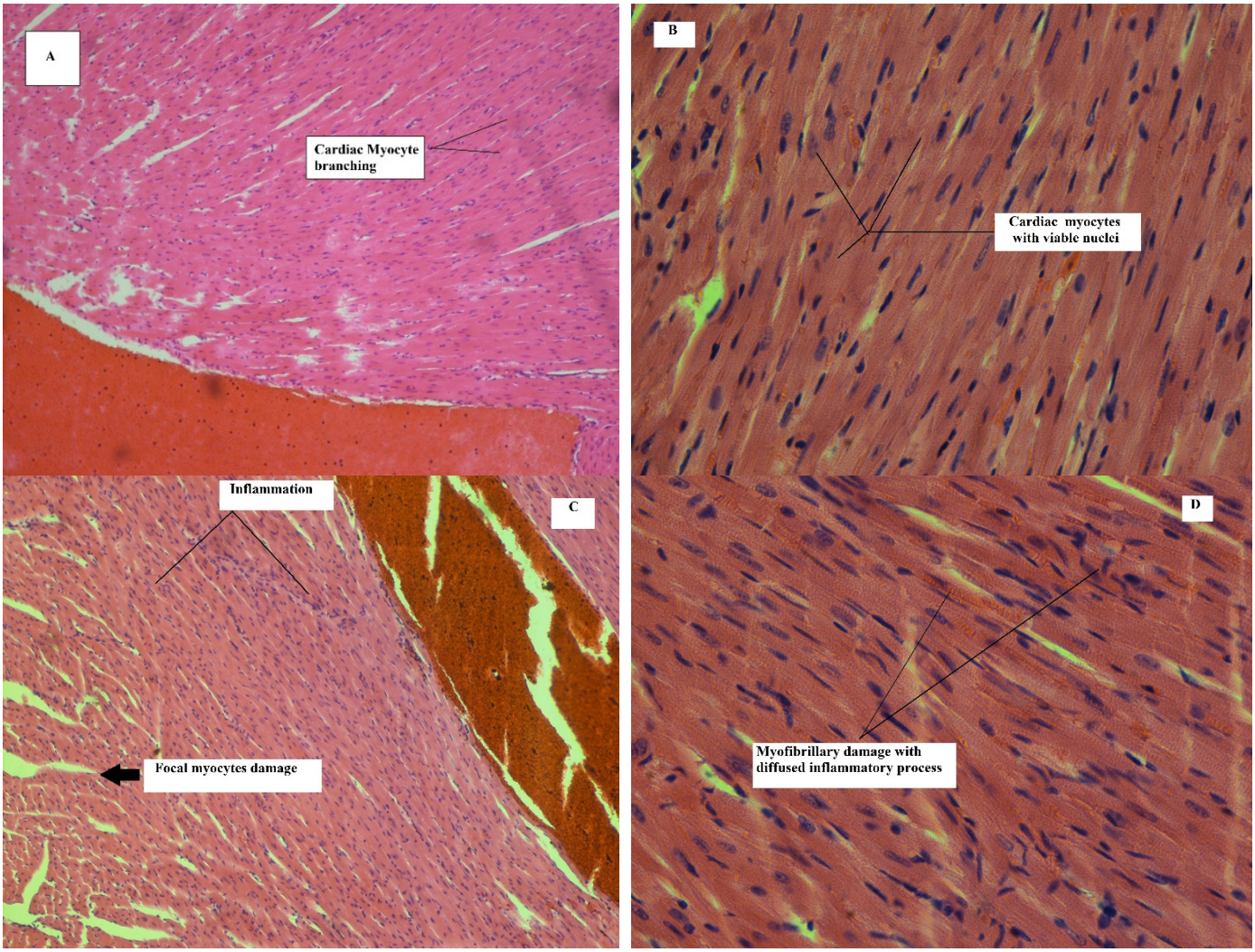

Figure 5. Normal saline treated heart tissue of mouse (H and E stained, 5 µm, **A**:100×, **B**: 400×): representative image ofheart tissue of mouse treated with normal saline for 21 days showing normal cardiac myocytes with mono or bi-nucleate muscle fiber. Heart of mouse treated with CHD (H and E stain, 5 µm, **C**:100×, **D**: 400×): representative images of heart tissue of mouse treated with CHD (45 mg/kg/day, i.p) for 21 days showing moderate focal myocytes damages with inflammatory cell infiltrations and myofibrillary damage with inflammatory process.

Figure 5C,D represents histology of heart in animals, after treatment with cyclohexenone derivative (CHD) on 21st day of the treatment. Architecture of heart shows alterations in normal histology. Moderate focal damages can be seen in cardiac myocytes. Moderate multi-focal degenerations were observed. No necrosis and myofibrillary damage were observed. The alterations were increased in intensity on 21st day of treatment with CHD. Further details of the above-mentioned findings/outcomes are given in Table 10.

Table 10. Histopathological scoring of heart tissues of control and treated group of mice during 11th and 21st days of treatment.

Scoring of Heart Tissue Slides												
on 11th Day	**Control Animals**						**Treated Animals**					
Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Focal myocytes damage	0	0	0	0	0	0	2	2	3	2	2	2
Multifocal degeneration	0	0	0	0	0	0	2	2	2	2	2	2
Multifocal degeneration with inflammatory process	0	0	0	0	0	0	0	2	2	0	1	1
Myofibrillar degeneration/diffuse inflammatory process	0	0	0	0	0	0	0	0	0	0	0	0
Necrosis with diffuse inflammatory process	0	0	0	0	0	0	0	0	0	0	0	0
on 21st Day	**Control Animals**						**Treated Animals**					
Scoring Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Focal myocytes damage	0	0	0	0	0	0	3	3	3	3	3	3
Multifocal degeneration	0	0	0	0	0	0	3	3	3	3	3	3
Multifocal degeneration with inflammatory process	0	0	0	0	0	0	1	2	3	3	3	2
Myofibrillar degeneration/diffuse inflammatory process	0	0	0	0	0	0	0	0	2	3	2	2
Necrosis with diffuse inflammatory process	0	0	0	0	0	0	0	0	0	0	0	0

0 none; 1 minimum; 2 mild; 3 moderate; and 4 severe.

4. Discussion

The toxicological assessment of cyclohexenone derivative (CHD) for liver through biochemical indicators (ALT, AST) revealed no significant difference between treated and control groups. However, on 11th day of treatment, the mean values of corresponding indicators (ALT and AST) were higher in treated as compared to control group. On 21st day of treatment, the mean value of ALT was higher in treated group than the control group. However, no signs of any considerable difference in mean values of AST in control and treated group were found (approx. 25 for both treated and control groups).

In the light of the above, when liver injury occurs, the ALT leaks out into general circulation where its half-life is approx. 42 h. Thus, the value of this biochemical indicator was raised in blood but was not detectable beyond its half-life [41]. In the current study, the difference of mean values of ALT of control and treated group became minimal when the mean values of 11th and 21st day of treatment were compared. This could have been related to its short half-life, where it was released into general circulation due to damaged hepatocytes. Thus, the difference of mean values of ALT in control and treated group decreased on 21st day as compared to 11th day of treatment. Similarly, AST has shorter half-life than ALT. Thus, comparing the difference in mean values of control and treated group of AST indicated a decrease in difference on 11th and 21st days of treatment. The ALT and AST values of treated group were not significantly different from control group. Although histopathological changes in liver were observed, this may have been due to the hepatocytes. Cell membrane remained mostly intact, and significant damage was not observed. Consequently, the cytosolic enzymes (ALT, AST) were not leaking out to general circulation, and thus no significant difference were observed in biochemical tests used for liver function assessment. In the light of this, previous toxicity studies have also shown that the presence of histopathological changes does not correlate better with biochemical indicators [42,43], although in other studies histopathological and biochemical indicator changes occur concurrently. In such studies, the biochemical indicators increased significantly due the reason that notable hemorrhage and necrosis were observed in treated group. This led to the release of enzymes from hepatocytes cytoplasm, which caused a significant increase in these biochemical indicators in blood [44–46].

Although mean values of biochemical indicators (ALT and AST) were higher in treated group than the control group, the difference was statistically insignificant. With reference to the previous studies, the known hepatotoxic-drug (CCl_4)-treated rodents showed mean values of ALT and AST to be more than 200 U/L and 30 U/L, respectively. This alteration by carbon-tetrachloride was majorly associated with liver injury through its metabolites that were generated during metabolism by cytochrome P_{450} [47–50]. In our current study, both on 11th and 21st days of treatment, the mean values of ALT were less than 100 U/L and the mean values of AST were less than 30 U/L. This shows that the said cyclohexenone derivative in a given dose and duration does not need to be very toxic to alter the ALT and AST values as compared to a known hepatotoxic drug. Drugs causing liver injury can be assessed through biochemical indicators, but their clinical significance is not clear. An increase of aminotransferase level > 3 upper limits of normal and jaundice (>3mg/dL) can be regarded as a risk of developing liver injury [51]. As in the current study, there was a slight increase in aminotransferase level, which may not indicate clinical significance.

Toxicological assessments of slides obtained from liver tissues revealed that mild steatosis occurred in treated animals on 11th day of treatment. Fibrosis and inflammatory cell aggregation occur in one treated animal, while hyperemia occurred in two treated animals. Such changes have shown increase in severity and frequency on 21st day of treatment, showing possible toxic effects of the administered compound. Biochemical indicators of liver have shown that the mean values of ALT in treated group were higher than control group. This increase may possibly be due to the fact that histopathological changes were observed in treated group animals, although ALT values of treated group were not significantly different from control group.

In connection to the above histological results of the liver, steatosis observed in liver tissue of mice in treated group could be related to β oxidation. Aspirin is a known cyclooxygenase inhibitor, and its induced steatosis in the liver is related to β-oxidation [52]. The molecular docking study of administered cyclohexenone derivative (CHD) is known to have affinity for cyclooxygenase enzyme that might be causing steatosis in liver tissue due to β-oxidation. Biochemical indicators of liver function (ALT and AST) on 11th day showed increase in mean values in treated group as compared to control group. This could be related to architectural damage that occurred on 11th day of treatment but without significant difference for the mentioned duration.

Toxicological assessment of cyclohexenone derivative for kidney through biochemical indicators (BUN and serum creatinine) revealed no significant difference in serum creatinine values of the treated and control groups. However, the BUN value has shown a significant difference between treated and control groups both on 11th and 21st days of treatment. In this connection, previous studies reported that in rodents treated with a known nephrotoxic agent, serum creatinine was more than 1mg/dL and BUN value more than 60 mg/dL. In those studies, in addition to increase in biochemical indicators values in treated group as compared to control group, histopathological evaluation revealed marked damage to kidney tissue. Architectural damage in those studies included necrosis, tubular injury, inflammation and vacuolization [47,53–55]. The results of current study showed that the mean values of serum creatinine, both on 11th and 21st days of treatment, were equal or less than 0.5 mg/dL. Similarly, the BUN mean values of treated group on 11th and 21st days were less than 60 mg/dL. This shows that the said cyclohexenone derivative (CHD) did not alter the biochemical indicators of kidney function as compared to known nephrotoxic agent.

Toxicological evaluation of cyclohexenone derivative through histopathological evaluation of slides from kidney tissues revealed that on 11th day of treatment, the glomerular and tubular injuries were mild in treated animals. These changes become moderate on 21st day of treatment, showing possible toxic effects of the administered compound, especially if used for extended period of time. Similarly, inflammation and fibrosis also appeared after 21st day of treatment. These effects support the increase in values of BUN in treated group as compared to control group. Some previous studies also demonstrated a significant

increase in BUN values with histopathological changes in kidneys [46,56]. Similarly, with reference to the above, the decrease in size of glomeruli can be related to treatment with cyclohexenone derivative (CHD). Molecular docking studies indicated that this compound has affinity for cyclooxygenase enzymes. By blocking prostaglandins (PGs) synthesis, they block the vasodilation effect of PGs leading to vasoconstriction in the glomerulus. This ultimately resulted in reduced size of glomeruli and consequent damage [57]. Such histoarchitectural changes were also observed in our current study.

Toxicological assessment of cyclohexenone derivative for pancreas through biochemical indicators (amylase and random blood glucose) showed no significant changes in amylase level during treatment with CHD up to 21 days as compared to the control group. The amylase level when compared on 11th day and 21st day, between the control and the treated group of mice, indicated *p*-values that were not significant: 0.831 and 0.515, respectively. Treatment with CHD induced significant changes in random blood glucose levels in mice as compared to the control group animals on 11th and 21st days with *p*-values of less than 0.05 and 0.018 on respective days. The random glucose level was lowered during treatment up to 21 days in an animal model. The difference in the mean values of control group mice was minimal, but in the treatment group, the difference in the mean values at baseline, 11th and 21st day was greater, showing lower blood glucose level on 11th day and 21st day as compared to baseline. In treatment group, the glucose level was higher at the initial day of experiment and decreased up to the end of treatment. The random blood glucose values, when compared on 11th day of treatment and baseline, showed a $p < 0.05$, which is statistically significant. The *p*-value for random blood glucose level, when compared between the groups on the 21st day, gave a *p*-value that was statistically significant, i.e., 0.018. At baseline, the value was not significant with a *p*-value of 0.073.

With reference to the above, physiologically, glucose uptake by beta cells causes increase in ATP production, thus closing the ATP sensitive potassium levels. At the membrane potential of 50 mv, influx of calcium inside beta cells takes place and thus mediating the release of insulin. Studies found that NSAIDs like meclofenamic acid, which is also a Cox inhibitor, has been found to alter the blood glucose levels by altering the ATP sensitive potassium-channel. Its glucose lowering effect is brought by insulin release by inhibiting the ATP sensitive-potassium channels, which produces an increase in the potassium level inside beta cells of Langerhans and hence causes insulin release, triggering the hypoglycemic effect [26,58,59]. Acetylsalicylic acid and flufenamic acid also produce hypoglycemia due to increased insulin secretion and decreased gluconeogenesis by liver and reduced insulin clearance. In the present study, the glucose levels were not affected at baseline but showed marked hypoglycemia reaching at the end of sub-acute studies [26,60]. Opioids, such as tramadol and methadone, have also been found to produce hypoglycemia. It has been found that opioids are directly linked with mu-receptor agonism, resulting in direct uptake of glucose by hepatocyte and skeletal muscle cells. Some studies have found that the blood glucose lowering effect of tramadol and methadone was inhibited by naloxone. This revealed involvement of mu-receptors in the blood glucose lowering effect by opioids, with blood glucose levels monitored as low as 20 mg/dL [61–63]. In present case, toxicological investigation for cyclohexenone derivative showed that it has no effect on amylase activity but significantly lowered blood glucose levels up to hypoglycemic range in treated group of animals.

Histopathological assessment of pancreatic tissue slides revealed mild alterations on 11th day of treatment with cyclohexenone derivative. On 21st day, the histopathological parameters were intensified regarding induced changes. On 11th day, there was mild edema, necrosis and inflammation found in treated group of animals. No such changes were observed in control group of animals. These changes became moderate in intensity on 21st day, showing possible toxic effects due to treatment with CHD.

Furthermore, cyclohexenone derivative, being the inhibitor of cyclooxygenase enzyme, has been shown to increase hydrogen peroxide inside cellular compartments. This usually leads to cellular damage, releases cytokines and thus attracts inflammatory infiltrates due

to degeneration of lipid, protein and nucleic acids. This may lead to cell apoptosis and necrosis and various pathological conditions [23]. The random blood glucose level in cyclohexenone-treated group showed a gradual decrease up to 21 days as compared to the baseline values, whereas the diabetogenic agents are required to raise the blood glucose levels beyond 300 mg/dL or 16.7 mmol/L. The blood tests for this agent in treated animals did not show random blood glucose levels above 300 mg/dL [64]. These results revealed that the test compound did not contain significant potential to induce diabetes for the said treatment.

The biochemical indicator of the heart (cTn-I and CK-MB) did not show any significant changes in the control and treated group by CHD for 21 days. When cTn-I level was compared between control and treated groups, no significant difference was observed with *p*-values of 0.916 and 0.834 on 11th and 21st days of treatment, respectively. Similarly, the value of CK-MB in control and treated group animals was found to be non-significant, with *p*-values of 0.672 and 0.896 on 11th and 21st days of dosing, respectively.

Nevertheless, cyclohexenone derivative, having affinity for cyclooxygenase, may induce cardio-toxicity by elevating the normal blood pressure. This effect is due to inhibition of Cox-II in kidney, which causes increased sodium and fluid retention and thus generates unwanted workload on heart due to elevated blood pressure, thus leading to elevated CK-MB and cardiac troponin-I levels due to any injury to the heart tissues [23]. Another mechanism causing increased levels of cTn-I and CK-MB is high level of reactive oxygen species in cardiac tissues. In normal physiological condition, reactive oxygen species are produced in mitochondria, which lead to synthesis of hydrogen peroxide via superoxide dismutase. This can be further converted to water molecules by glutathione peroxidase. NSAIDs have been shown to increase hydrogen peroxide species, which leads to cellular damage in myocytes due to degeneration of lipid, protein and nucleic acids. All these events lead to cell apoptosis and necrosis of the cardiac cells [23].

Histopathological assessment of heart tissues revealed that the level of cardiac troponin-I was less than 0.4 ng/mL, and for CK-MB blood test, the level was between 5–25 IU/L. The results showed that cyclohexenone derivative (CHD) did not induce any significant toxic effect on the biochemical indicators of the heart of mice as compared to toxicity producing agents, but it did alter the normal histology of the cardiac myocytes. As compared to control group, the treated group animals produced an alteration after CHD treatment. On 11th day, the heart tissue showed mild cardiac myocyte damage with inflammatory cell infiltrates, while on day 21, the heart tissue revealed mild damage with moderate adverse effects. Mild myofibrillary damage without necrosis was also observed. The damage to cardiac myocytes could be related to the affinity of the compound (CHD) to cyclooxygenase enzyme in kidneys, which, by inhibiting Cox-II enzyme, discontinues the vasodilation by prostaglandins (PGs). These processes result in sodium and water retention and elevate blood pressure, causing damage to heart tissues. Other possible mechanisms include increased production of hydrogen peroxide species, which can induce cellular damage to cardiac myocytes [23]. The histopathological alterations induced in tissues of heart suggested the possible toxic effects of this novel compound used for the treatment [65–67].

5. Conclusions

The acute toxicity studies of the novel compound have shown that the median lethal dose of the said compound is expected to be greater than 500 mg/kg, as no signs of toxicity and mortality were observed at this dose. Similarly, sub-acute toxicity revealed no significant changes in selected biomarkers except BUN and RBS levels. However, histopathological evaluation has shown mild to moderate toxic effects on liver, kidney, pancreas and heart tissues. These findings show that the novel compound may be toxic to selected body organs if used for sub-chronic or chronic duration.

6. Study Limitations and Future Plan

Findings of this study would have been more evident if the observed toxic effects had been correlated with the pharmacokinetic profile of the cyclohexenone derivative. Furthermore, it will be more beneficial to conduct toxicological studies of this novel compound on some additional vital body organs (like CNS and GIT) and for a longer duration (sub-chronic and/or chronic treatment) in an animal model. Therefore, we are planning to perform the assessment of pharmacokinetic parameters and to further extend the toxicological evaluation of the tested compound in an animal model.

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sustainability

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Review

The Implications and Effects of Medical Waste on Development of Sustainable Society—A Brief Review of the Literature

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Abstract: The sustainable development of humanity imposes precise norms regarding the management of natural resources, their extraction, use, and the introduction in a complex, innovative circuit of the waste resulting from exploitation. The paper deals with some aspects related to the sustainable management of general medical waste on the one hand and the medical waste specific to the COVID-19 pandemic, on the other hand. Medical waste requires special treatment given its impact on the environment and on humanity. The management of activities related to its storage, transport, destruction is an important point in the sustainable development of mankind, especially in the current context of the pandemic. Medical waste is in a continuous increase in quantity and involves many effects in various activity fields. Through a scientometric study in the Web of Science—WOS database, the authors identify clusters of keywords, analyze the articles identified in the WOS and identify the main research directions and existing concepts. Corroborating and interpreting the results obtained, three significant trends of approach to medical waste are identified: M—management (1); E—exposure (2); and D—distribution (3). An extensive map of the concepts is made, a narrow map of the concepts used, and a theoretical map of the concepts. The link between medical waste and the development of a sustainable society is demonstrated, and it is possible to open new research directions. The scientometric research undertaken on 1192 WOS articles that were published in 2020 led to the selection of 32, focused on issues related to hazardous medical waste, especially of COVID-19 patients. Following this approach, the authors were able to see, by comparison, the different forms of management of this waste in different countries, thus being able to contribute to the creation of procedures for the collection, storage, and destruction of this hazardous waste, with direct influence on the environment.

Keywords: sustainability; medical waste; higher education institutions; impact; economic aspects

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1. Introduction

COVID-19: What is to be done now? This pandemic has affected all areas of activity. The most devastating effects were on health, the environment, and education. Medical waste has exploded in terms of quantity and influenced the reorganization of their management in hospitals, the public domain, and the environmental approach.

Waste remains a complicated issue for human society due to its health and environmental, economic, and social impacts. In particular, medical waste negatively influences the health of the population and the environment, so it must be managed with great care.

The influence of hazardous medical waste on society and the environment, as a result of previous research, leads to a question: What is more critical in the issue of

medical waste? Management—by creating and implementing sorting procedures, expenses/bed/patient/day, material and staff costs, especially that in these times of pandemic the need for staff has increased considerably; Exposure—by creating specific procedures related to the transport of waste from hospitals to collection/incineration points, expenses related to equipment, personnel, fuel, maintenance; Distribution—procedures regarding a superior recovery of medical waste, the possibility of using combustion residues, so as not to affect at any time the quality of the environment.

This study aims to probe the literature by scientometric methods, the content of the literature published in the Web of Science—WOS database, the influence of COVID-19 on the approach to medical waste and environmental sustainability, and their influence on society. The scientometric analysis made possible the highlighting of the study deficit on this topic, the empirical studies were analyzed, and the needs and effects on the scientific elements to be developed were highlighted, the need to promote the results through teaching/education.

The purpose of the article is to find the strong points resulting from the analysis of the literature on medical waste from the beginning of the COVID-19 pandemic and to see in which direction most research is heading related to the sustainable management of hazardous medical waste. Also, this study gives a general image of waste management in different countries, and contributes to creating specific procedures in the way to optimize collection, transport, storage, destruction/incineration, useful in Romania, but also to disseminate this experience to other countries.

This paper is divided into the following sections, which have been considered key to achieving the proposed objectives. Section 1 is the introduction; Section 2 defines the research method, research questions, and results analysis. Following the scientometric analysis, 32 articles have been selected that deal with the urgent and exciting research directions during COVID-19. Three significant research directions in medical waste were identified, M—Management, E—Exposure, D—Distribution. Section 3 presents discussions on the three approaches, M, E, D. Section 4 develops the paper's development conclusions.

2. Research Method

2.1. Literature Review by Scientometric Methods

A scientometric study was performed. The research was conducted on the Web of Science database. Articles on the subject of Medical Waste were searched. The 2020 papers were included in the study. We imposed this limitation to analyze the literature that appeared during the COVID-19 pandemic.

The database, downloaded from the Web of Science, was analyzed using the VOS Viewer software (VOSviewer version 1.6.16, free software from https://www.vosviewer.com/ (accessed on 11 January 2021)).

We limited the options to analyze the authors' terms in the whole article and the articles' abstracts. VOS viewer software allowed us to view the map of these concepts and divide these terms into clusters. We analyzed these clusters and determined the research directions for this field during COVID-19.

The following three research questions were defined:

RQ1: What are the most critical research directions addressed in the articles on medical waste?

RQ2: What is the impact of medical waste on a sustainable society with the advent of COVID-19?

RQ3: How is the impact of medical waste assessed?

2.2. Stages of the Scientometric Study

The realization of the scientometric study included seven stages according to Table 1, starting from the formulation of the problem to the establishment of protocols and research criteria, data extraction, which were subsequently analyzed, synthesized, and discussed.

Table 1. Scientometric study's stages.

No.	Stages	Description
1	Problem Formulation	Mapping and bibliometric analysis of publications using descriptors
2	Research Protocol	"Medical waste"
3	Research Database	Claryvate analytics, WEB OF SCIENCE—WOS 7/09/2020
4	Eligibility Criteria	Refined by: STRING 1: publication years: (2020) and web of science categories: ("medical waste") As results: 5112 articles Refined by: STRING 2: publication years: (2020) and web of science categories: (environmental science) and web of science categories: (public environmental occupation health) and (engineering environmental) and document types: (article or proceedings paper) As results: 1265 articles
5	Data Extraction	Bitext format
6	Analysis And Synthesis Of Results	Qualitative (descriptive) and quantitative (bibliometrics) using VOS Viewer
7	Discussion Of Results	Exhaustive analysis and verification of data obtained

In the research stage, which consisted of STRING 1, which included all the requirements/criteria established as necessary for the study, it resulted in 5112 articles, then STRING 2, which resulted in 1265 items. This methodological procedure is represented in Figure 1.

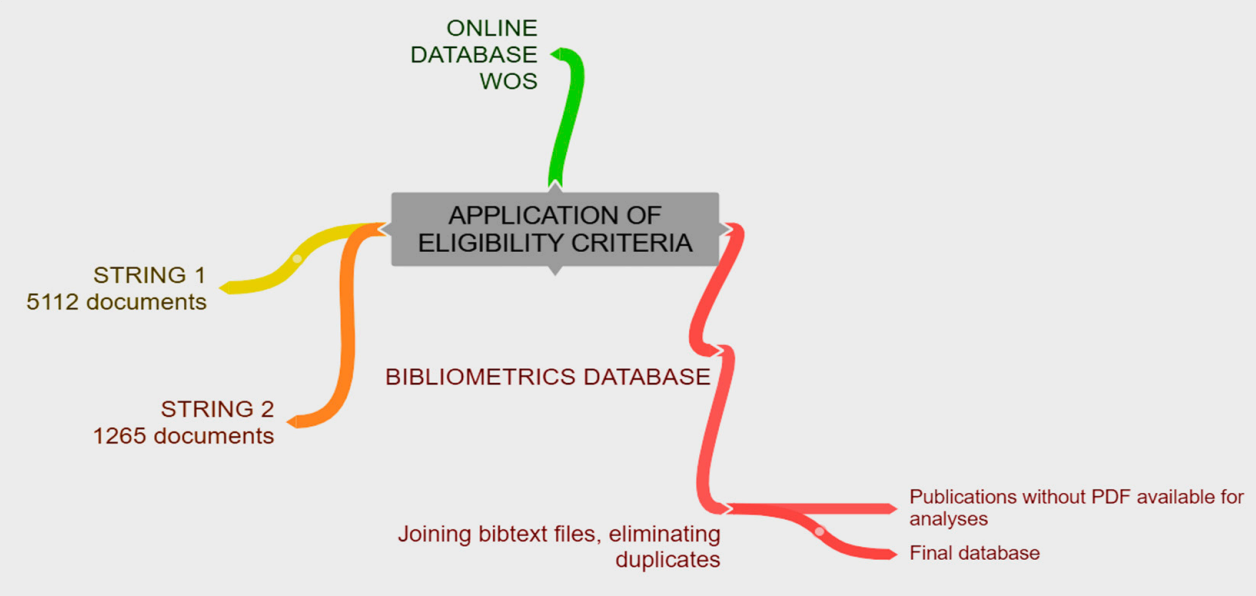

Figure 1. Research methodology scheme.

Using VOS Viewer, the database was analyzed in STRING 2.

VOS Viewer is software used for bibliometric analysis using databases extracted from WOS.

We limited the research to descriptors that appear in the database at least three times. Of the 4818 terms, 371 met the threshold. For each of the 371 words, VOS Viewer calculated a relevance score, and 60% of the most relevant terms were selected.

Analysis of the descriptors used in the title and summary was performed (Figure 2). We obtained three clusters of descriptors.

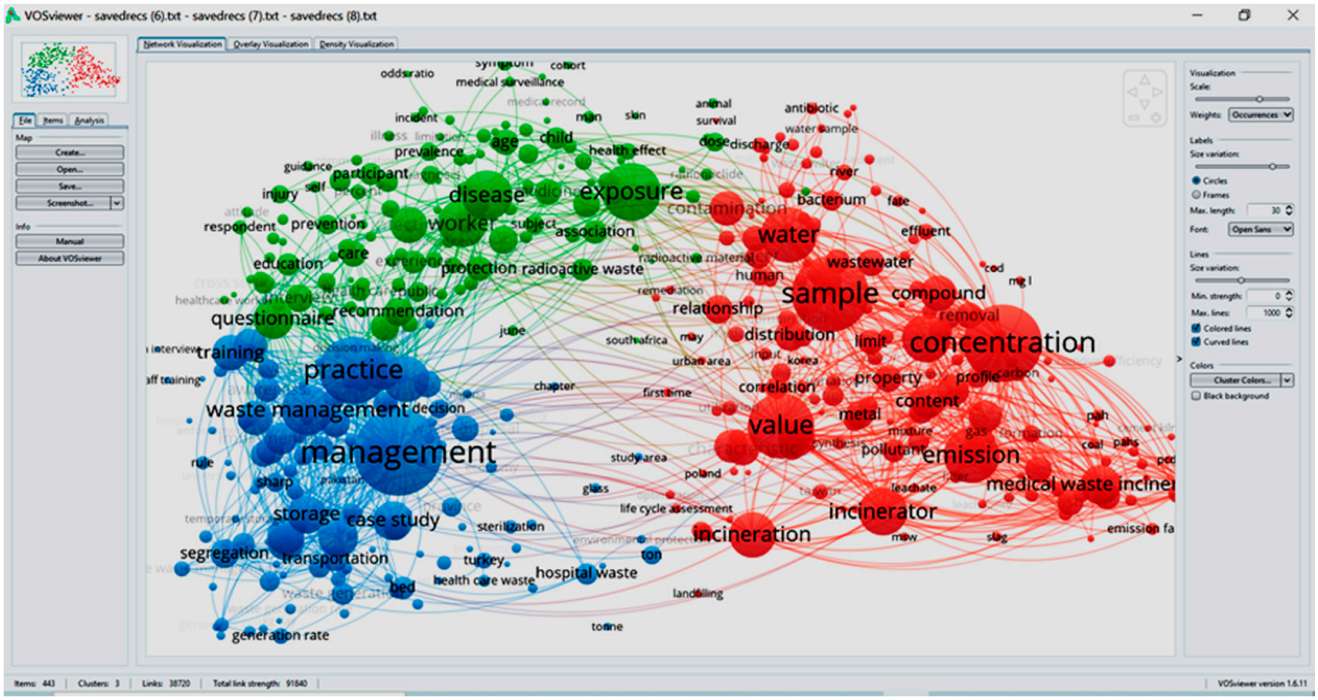

Figure 2. Generating clusters in "waste management" research using VOS Viewer.

The concepts map resulting from STRING 2 is presented in Figure 3.

Figure 3. Concept's map resulted from STRING 2.

2.3. Results Analyses

Three research directions were identified in the studies undertaken related to Medical Waste (MW): Management—M, Exposure—E, Distribution—D.

These were extracted from the analyzed articles, downloaded from the database, and classified in three directions. Since the onset of the pandemic and a significant increase in MW, there has been a growing interest in the MW approach.

Figure 4 summarizes the identified items in STRING 2, limited to items dealing with MW in the three directions. There are 32 items.

The authors analyzed the content of the 32 articles in the three directions: M, E, and D.

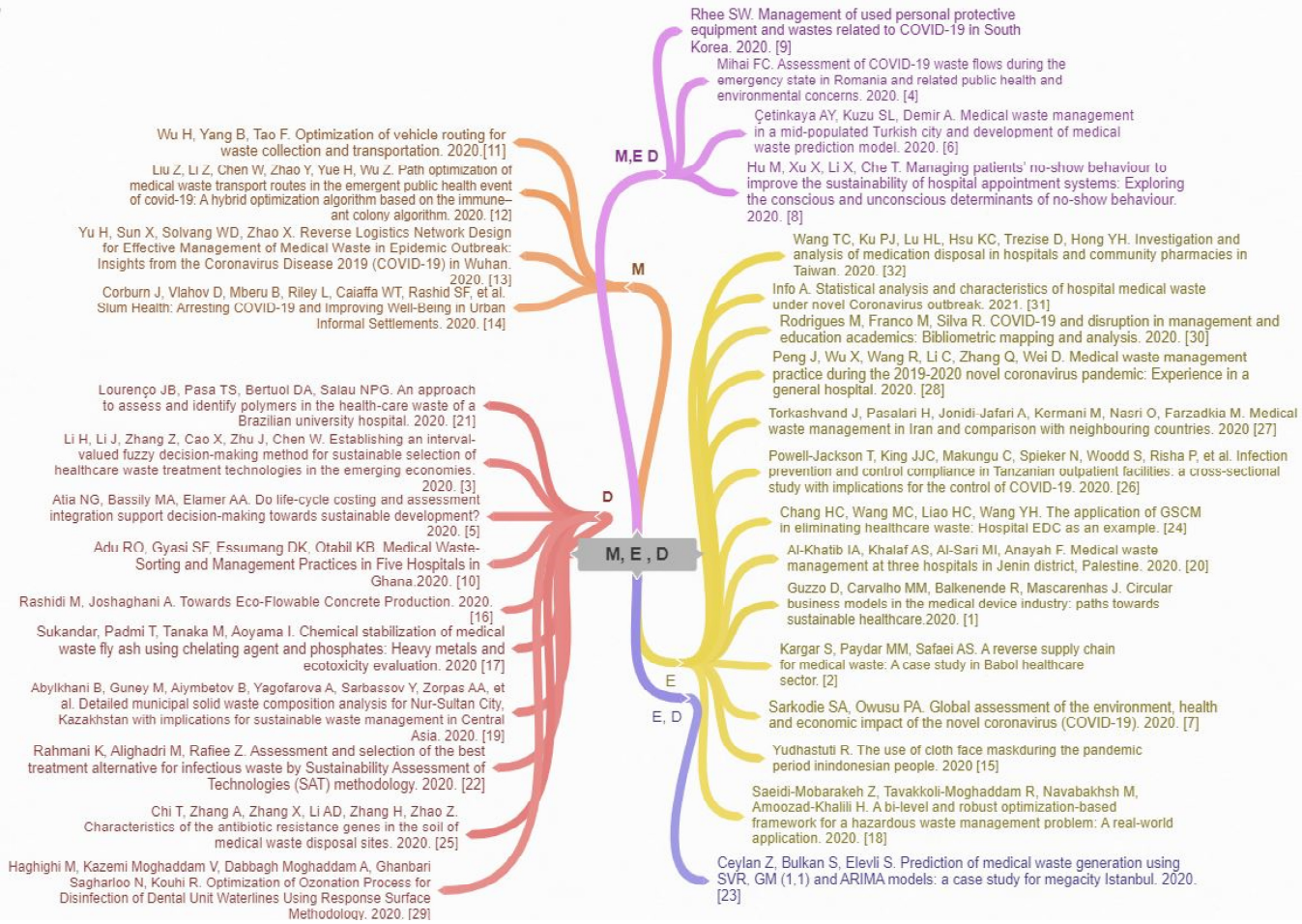

Figure 4. Content analysis taking into account authors and type of study [1–31].

2.3.1. M—Management

The management of COVID-19 in Romania, in a state of emergency, between 16 March and 14 May 2020, imposed a national blockade with restrictive measures and social distancing, with a tremendously strict restriction on population mobility, only justified and minimal travel and having only activities reduced economic, and the population entering technological unemployment [4]. A rapid assessment method of potentially infectious waste flow, the importance of medical and municipal waste management systems, and services in combating the COVID-19 virus in the community was necessary [4].

Cetinkaya et al. [6] present general data, statistics concerning medical waste per daybed in the USA, UK, France, Turkey, and a fair comparison with data from Chapter 18 of the Europe Union Catalogue. In conformity with the Medical Waste Control Legislation, the wastes from healthcare units are classified into six categories: general wastes, infectious wastes, genotoxic waste, pathological wastes, sharps waste, and hazardous wastes. According to this classification, the authors created a model for waste management.

Since the first appearance of the virus in the Wuhan food market in December 2020, appearing in the form of "pneumonia with unknown etiology", on 11 March, it was declared a universal pandemic and treated as such, making a comparison with SARS (2002–2003), Avian flu (2003–2009), or Ebola (2014–2016). Sarkodie et al. [7] analyze the impact of COVID-19 on humanity under different aspects: economic, environmental, and social impact, expressing opinions on policies worthy of consideration, monetary and health policies necessary to improve the living conditions of the population.

In this paper [8], Hu M et al. establish a model to optimize the flow of patients to hospitals to reduce waiting time and especially the discomfort created by the accuracy of

the patient scheduling process. Hypotheses to be considered from a multistage perspective were tested. Lack or delay in scheduling medical examinations is dependent on: waiting time, days or weeks, age, the language of conversation, living environment (urban, rural), schooling level, etc.

Liu and Li [3] establish an algorithm useful to optimize urban waste storage sites. They use an ant colony–tabu hybrid algorithm, taking into account the actual situation of COVID-19, for a fair and real analysis of environmental impact.

Also, the authors made a selection of temporary storage stations and realistic transportation demands. Using their algorithm, an efficient transport model of medical waste between hospitals and temporary storage stations can be created.

A multi-objective program, realized by Hao [13], is established for a longer time period, for a fixed number of data entries. This program is based on the medical waste transport network, and as a result, determining the best temporary storage locations and transport strategies. It is known that in a brief period, in this COVID-19 time, medical waste has exponentially grown, which needs measures for efficient management [13].

Any critical health situation creates economic, social, and environmental problems. Jason Corburn et al. [14] analyzes the way to improve and protect people, to improve their lives, and well-being, for people with modest incomes, low, on the verge of existence, migrants and proposes economic, social, and psychical measures (maybe major psychic influences leading to suicide).

The study by Maria Rashidi [16] is focused on integrating expired plastic syringes into the matrix of flowable concrete mortar, looking like a fine aggregate. This can be used in other applications and is well accepted by the environment. The study aimed to determine the properties of concrete forms obtained from used plastic syringes, especially hardness and durability.

Chrisanthi Vavva et al. [32] created a procedure for the stabilization of fly ash produced by a Greek MW incinerator. In the beginning, they present a detailed characterization of the fly ash, based on the European standard leaching test EN 12457/2 (2002), and according to the results, they studied the concentration of Pb, Total Dissolved Solids (TDS), the legal limit values for pH, density, particle size dimension, conductivity, chloride, and fluoride concentration, etc.

The real problem is to estimate the amount of waste to be generated in the next years. The authors analyzed the evolution of MW (Medical Waste) in Istanbul, the biggest city in Turkey. This can be critical for the evaluation of existing waste treatment service capacities. This study was realized to evaluate the performance of various mathematical modeling methods to anticipate medical waste generation [23]. These were used to estimate annual medical waste Autoregressive Integrated Moving Average (ARIMA), Support Vector Regression (SVR), Grey Modeling (1,1), and to conduct Linear Regression (LR) analysis, in the period 2018 to 2023. All databases collected from 1995 to 2017, provided from the Istanbul Metropolitan, were utilized to examine methods' forecasting accuracy.

The problem addressed by the authors is to eliminate medical waste in order to improve the quality of the treatment process of patients and to create a support system of management named green supply chain management (GSCM). This study used a hospital's emergency department crowding (EDC) to illustrate how to establish an emergency medical service (EMS), based on the Taguchi model, creating a simulation system to obtain a concrete parameter for solving hospitals' EDC and waste problems and increasing healthcare quality [24].

The influence of incinerated waste on soil quality is the subject of Ting Chi's research [25]. Antibiotic resistance genes (ARG) were determined for 45 different soil samples containing medical waste. There were physical and chemical analyses (i.e., dry matter content, pH value, and metal content). The genomes of microorganisms from the soil were extracted for high-throughput sequencing and big data analytics.

Research by Haghighi [29] is oriented on microbial contamination in dental unit waterlines (DUWLs). There is a potential risk of infections in immunocompromised

patients and medical staff who are usually exposed to contaminated water and aerosols generated from DUWLs.

2.3.2. E—Exposure to Medical Waste—Risks

The sources of COVID-19 medical waste are identified: hospitalized patients, people in quarantine, people in isolation at home. There is an increase in the amount of medical waste during the emergency and alert periods caused by the COVID-19 pandemic. Deficiencies are identified in collecting waste from quarantine and isolation sites—mixing and disposal together with household waste. A COVID-19 waste monitoring model is proposed in [4].

In South Korea, face protection masks and general protective equipment (PPE) used by medical teams are classified as medical waste. Rhee et al. identified that personal protective equipment used by hospital medical staff can lead to indirect infection of other people without contact with infected patients, and also the increase in the volume of medical waste represented by the personal protective equipment used [9].

Adu et al. identified deficiencies in waste collection in hospitals—due to the lack of a unitary color coding and labeling system, they identified deficiencies in the behavior of medical waste sorting among medical staff and recommended the organization of regular training courses for health workers [10].

Yudhastuti says that the use of cloth masks as an alternative protection measure during the COVID-19 pandemic, along with other protection measures (handwashing, physical distance, avoidance of crowds) reduces the amount of medical waste [15].

The source of infection can be effectively controlled by MW management. Improper management of medical waste can increase the spread of COVID-19. Classification of medical and household waste as waste in direct causal connection with COVID-19 and their management should be carried out along with the establishment of multidisciplinary teams for infection control and MW in hospitals [28].

2.3.3. D—Distribution

It was proposed to study an efficient and reliable supply chain of waste from the medical industry. A multi-criteria and multi-period model with three objective functions was proposed: the first of these minimizes total costs; the second refers to the selection of scaling technology, and the latter reduces the total stored medical waste. Saeidi–Mobarakeh created a "tri-objective MILP" model. This model refers to the optimization of medical waste management [18], very relevant being the appreciation of waste treatment technologies from the medical industry in emerging economies in a sustainable way.

Alternative waste treatment technologies in the medical industry are being studied by Li H et al., including their incineration, steam sterilization, microwave or ultraviolet heating systems as well as disposal in landfills; the results indicate that the established method is useful to help prioritize waste treatment technology in the medical industry. The selection of the most sustainable waste treatment technology in the medical sector in today's economies takes place according to the decision-makers who simultaneously consider various criteria [3]. A study shows that "integrating life cycle costs and life cycle assessment through the value chain reduces costs, improves environmental performance and improves economic and environmental efficiency in strategic decision-making". It examines whether the integration of life cycle cost (LCC) and life cycle assessment (LCA) "helps decision-making in a sustainable way".

In his study, Ati presents a challenge to the public sector by exploring the rules of waste management in the medical industry. The results obtained presented the importance of the classification stages in terms of reducing processing costs. The burning of waste from the medical industry is the most common method of treatment, which influences the total costs of the system but, most importantly, the impact on the environment [5].

According to Rhee et al. medical waste management flow should follow the steps: "Disposal in the medical waste container"; "Storage in the designated installation"; "Vehicle transport for medical waste"; and "Incineration treatment" [9].

Efficient programming of one or more routes of waste collection vehicles from the medical industry would be an optimal way to reduce collection and transport costs. At the same time, once this "correct and efficient" way is applied, the negative effect of specific waste would be automatically reduced and, at the same time, we would not be unsustainable in relation to the environment [11].

A two-tier optimization model is developed by Saeidi–Mobarakeh for decision-making on the hazardous waste management framework. A strategic problem of reverse network design is considered to be the issue of waste collection as a lower level of the decision hierarchy [18]. They selected the most effective alternative to treating infectious waste resulting from the medical industry by assessing the "sustainability of technologies" (SAT). This methodology contains three essential steps, such as "screening, scope and detailed evaluation". All the information needed to obtain the data is obtained by applying a questionnaire to medical and support staff; this questionnaire is based on questions about the rate of waste generation, active beds, average waste, waste management, treatment of infectious diseases, and a field visit. Depending on certain factors, each aspect is noted accordingly, multiplier factors (MF) were calculated by multiplying the weight taken into account for each criterion by the score of each aspect divided by the maximum score for each environmental, technical, economic, and social aspect of different "infectious waste treatment technologies," the most important criteria in selecting the best technology are "environmental" and "economic" [22]. In another study, "statistical analyzes are performed to evaluate the rates of generation and composition of medical waste generated during the treatment of the coronavirus pandemic" with reference to a care hospital from Jordan.

An average of the resulting medical waste is "3.95 kg/bed/day", a value 10 times higher than the "generation rate in the hospital during regular daily surgery". This high value of medical waste is largely attributed to the medical staff of the hospital using disposable personal protective equipment [31].

It is assumed that pharmaceutical waste also has an influence on the generation of medical waste; this may be partly due to patients who do not use the drugs properly; also, another cause would be the wrong prescription of drugs. This issue is currently being studied, but further investigation is needed [33].

3. Discussion

After the bibliographic research on the subject of interest and after the directions of its separation in M, E, and D were determined, we proceeded to the systematic, critical analysis of the valuable ideas, the role of this analysis being to create a central concept to which this scientific research by the authors directs.

There was significant interest from researchers from various countries such as China, Palestine, Iran, Turkey, Taiwan, Tanzania, Brazil, Indonesia, Ghana, South Korea, Romania in research aimed to establish sustainable management of medical waste resulting from the COVID-19 pandemic. These populations being in various situations: state of emergency, state of alert, quarantine at home.

3.1. M—Management

The management of COVID-19 in Romania, in a state of emergency, between 16 March and 14 May 2020, imposed a national blockade with restrictive measures and social distancing, with a great strict restriction on population mobility, only justified and minimal travel and having only reduced economic activities, the population entering technological unemployment [4]. A rapid assessment method of potentially infectious waste flow, the importance of medical and municipal waste management systems, and services in combating the community's COVID-19 virus were necessary [4]. In our opinion, the Romanian situation in waste management was a challenge, considering the novelty of the pandemic and the waste flow being influenced by the government experience in establishing procedures.

The authors of [6] express the importance of the accuracy of the data in the territory in order to have a future image regarding the estimation of the variation of these values. If in 2017 the amount of medical waste was 280 t, almost double compared to 2011, obviously this value is different now, especially in the context of the COVID-19 pandemic. The authors also analyze the amount of medical waste by age category and GDP% per capita (from $11.2 in 2011 to $10.5 in 2017). The EKC model (environmental Kuznets curve) gives a good appreciation of medical waste quantity per year, per bed-day, per capita, and also for an appreciation of GDP% to spend/year [6]. The model created is based on: the number of patients, bed occupancy rate, medical waste amount, waste amount establishes by the bed, person, day. It is a good experience of what the costs of waste management are in Turkey in comparison with other countries.

As for impact [34] on the environment, it can be noticed that measures of social distancing, isolation, quarantine, alertness, and urgency were quickly taken. In this way, it was observed that carbon emissions in the environment decreased significantly due to the decrease in cars, motorcycles, air transport, etc. The impact on health has materialized in the form of the increase in health, salary, sanitation, and cleaning costs when comparing various countries that are hard hit.

The economic impact consists of the allocation of additional funds for health, restored monetary policies, procurement priorities, the creation of social assistance programs, and limiting unemployment. As a result, it is necessary to determine the losses caused by COVID-19 and return to economic growth, environmental, and social conditions in the post-COVID-19 period. The model created by the authors HU L et al. shows us that age and sex are not very important in no-show behavior. It can be appreciated that the comfort aspect has particular importance. The channels for establishing the hospital–patient connection are not to be neglected. Finally, the authors added a no-show habit as an unconscious determinant. Our research has implications for managing patients' no-show behavior, improving the hospitals' management, and establishing a communication system between hospitals and patients.

The results of the research suggest the need for immediate installation of temporary incinerators, which may be an effective solution for managing the increase of medical waste during the COVID-19 outbreak in Wuhan, but the location selection of these temporary incinerators is a critical problem. The question is: Where to install storage points and incinerators? Due to the limitation on available data and knowledge at the present stage, more real-world information is needed to assess the effectiveness of the current solution [13].

Jason Corburn et al. [14] propose some measures for ways to protect residents of urban settlements, poor people, those who are living in poor conditions, and the population exposed to COVID-19:

- institute informal settlements/slum emergency planning committees in every informal urban settlement;
- apply an immediate moratorium on evictions;
- provide an immediate guarantee of payments to the poor;
- immediately train and deploy community health workers;
- immediately meet Sphere Humanitarian standards for water, sanitation, and hygiene;
- provide immediate food assistance;
- develop and implement a solid waste collection strategy; and
- implement a plan for mobility and health care immediately. Lessons have been learned from earlier pandemics such as HIV and epidemics such as Ebola" [14]. It is very known that at the beginning COVID-19 was treated as a respiratory affection, or one with a symptomatology near to Ebola, with medical researches on patients/posology.

The role of research is to show that used sanitary products can be reintegrated into other products through recycling, for example, syringes and other dangerous medical articles made of plastic or glass. This has been demonstrated (concrete forms added with plastic/glass have a hardness 30–50% higher than those without plastic) and leads to the

idea of informing decision-making bodies in developing procedures to be used by recycling hazardous medical materials [16].

The authors, following laboratory analyses undertaken through a generous program, established a new treatment method for flying ash resulting from incineration. The scaling method consisted of two processes; phosphoric acid treatment followed by washing with water, which is able to stabilize the flying ash.

This method has been successfully applied for the stabilization of Municipal Solid Waste Incineration (MSWI) flying ash and was used for Medical Waste Incineration (MWI) fly ash treatment [32].

The study presented in [23] is useful to estimate the amount of MW in the next period in order to design the flux of waste, future treatment, storage facilities, incineration capacities. The ARIMA (0, 1, 2) model with the lowest RMSE (763.6852), MAD (588.4712), and MAPE (11.7595) values and the highest R2 (0.9888) value showed a superior prediction performance compared to SVR, Grey Modeling (1,1), and LR analysis.

The results obtained from the models indicated that the total amount of annual medical waste to be generated would increase from about 26,400 tons in 2017 to 35,600 tons in 2023 [23].

The study presented in [24] makes two significant contributions. First, the study introduces the GSCM, an innovative green concept to healthcare, very useful for hospitals. Second, the study applied the dynamic Taguchi method to the EMS and neural network (NN) to construct a model revealing the relationship between factors—cause—and performance—effect [24]. Results obtained by researchers show that medical waste is an accumulation of Cu, Cr, Pb, and As in the tested soil samples. Compared to the controls, the samples collected from different areas containing medical waste were significantly enriched with ARGs annotated as sulfonamide and multidrug resistance genes, and in particular, in two subtypes. The comparatively higher abundance and diversity of ARGs in contaminated soil poses a potential risk to human health [25].

Based on achieved results, ozone was highly influential on microbial decontamination compared to peracetic acid and NaOCl disinfectant and can be used for the disinfection of DUWLs [29].

3.2. E—Exposure to Medical Waste—RISKS

According to Mihai, a COVID-19 waste monitoring model can reduce the risks of contamination of medical staff and the environment [4].

Equivalence and management of PPE used as infectious waste have an essential role in preventing indirect COVID-19 infection, according to the WHO (World Health Organization) guidelines. This establishes the flow of medical waste in direct causal connection to COVID-19. The principles of COVID-19 waste management are sustainability, transparency, and safety. Masks used in households should be disposed of separately from other waste and incinerated or disposed of in landfills [9].

A study recommends the organization of regular training courses for health workers. An interconnected approach is needed in medical waste management (hospitals, Ministry of Health, companies engaged in the collection, transport, and disposal of medical waste) [10].

Waste management of individual cloth masks should be done in the same way as standard operating procedures for medical waste. Proper management of individual masks decreases the potential danger of SARS-COV-2 spread [15].

Standardized management of medical waste related to COVID-19 can decrease the danger of disease in hospitals [28]. This standardized management can be realized based on experience from different countries to harmonize the medical and social politics, thus being useful to people affected by COVID-19. Psychically, people are influenced during the different periods, lockdown, quarantine, alert estate, and this can be a real and huge problem for medium and long periods.

This medical waste, incorrectly managed, poses a risk to medical staff and public health. A study identifies the increase in medical waste during COVID-19 with the pandemic's evolution [35].

3.3. D-Distribution

The management of uncertain parameters is done with a new programming approach called "RPP", also applying the "fuzzy objectives" method. Therefore, for "uncertain amounts of medical waste, the optimized number and location of facilities and types of technologies in treatment centers are determined" [2].

Steam sterilization is considered the best technology for treating waste generated by the medical industry, among the four sustainable options. Microwave or ultraviolet heating systems are the second option, incineration and storage being the least sustainable [3].

The waste generated following this pandemic of COVID-19 requires the collaboration of all parts of a management system. Disposable masks, gloves and coveralls, and other hospital equipment should be disposed of separately from other waste by guided workers with special equipment with the red symbol [9].

4. Conclusions

There are few types of research related to hazardous waste management directly associated with COVID-19, since the onset of this pandemic. Examples were found from several countries worldwide, such as China, Taiwan, Turkey, Iran, Jordan, Tanzania, Brazil, Palestine, Kazakhstan, Indonesia, Ghana, South Korea, Romania, which address the various issues presented in the paper. An investigation into the experience of some Western European states, as attacked by the virus, the USA or Japan, would have been useful. The examples would have helped create a procedure as simple as possible, economical, and with impact effects on humanity being as friendly as possible.

The risk of becoming ill with SARS-COV-2 can be reduced through the correct management of medical waste resulting from both specific activities in hospitals and the use of masks in quarantine and isolation locations as well as in daily activities, depending on the legal regulations in each country. Additional studies are required to guide the proper management of COVID-19 medical waste from households and non-medical institutions.

Selective waste collection is a topical issue with a significant influence on both humanity and the environment, having the most significant role in the process of disposal. Because the COVID-19 pandemic has generated a huge amount of hazardous waste, it is necessary to replace the current disposal methods, for example, the incineration method, which pollutes the air by releasing many toxic compounds into the atmosphere or the storage method, a real danger because the toxic compound remains in the groundwater for a long time. The technique that would have helped a lot during this pandemic is the steam sterilization of hazardous medical waste, so the process would have been controlled and carried out under certain conditions, preventing the dispersion of hazardous compounds.

Because existing studies have shown the resistance of the COVID-19 virus over time on various surfaces, a real problem is still the disposable masks used, the management of this problem is still under development. Despite being a source of this virus, their collection in specially arranged spaces and in due time is done with great difficulty, especially in Romania, where, unfortunately, these potentially hazardous wastes end up in the same place as the rest of the municipal waste.

Even if the financial effort of each country is considerable, for the protection of people and the environment, it would be useful to develop clear transport procedures, protected routes, intermediate collection points (if applicable), and short storage in destruction areas (the concept of "destroyed waste, zero waste" is useful).

Therefore, a joint human effort is needed to develop COVID-19 hazardous waste management procedures, to ensure transport security, to simplify material and human costs, to develop ways to dispose of this waste, and to use it in a sustainable, innovative way, so that the environment is not adversely affected

The limitations of this study are determined by the fact that only the Web of Science database was researched.

The authors provide a critical study on the new approach to medical waste during COVID-19. This study can help develop the following research directions in this field, creating international interdisciplinary teams based on which researchers can contribute to improve and prevent the implications of medical waste in society, medicine, and industry. Researchers may use the same research method for other topics as well. The article can also be beneficial for Ph.D. students and might be of some interest to practitioners.

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Full-Length Genome and Partial Viral Genes Phylogenetic and Geographical Analysis of Dengue Serotype 3 Isolates

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Abstract: Dengue fever is among the most common vector-borne diseases. Dengue virus (DENV), responsible for dengue fever as well as dengue hemorrhagic fever, belongs to the genus flavivirus and family Flaviviridae. Flaviviruses infect various vertebrate species and arthropods and are also responsible for diseases in birds, wild animals, and primates. DENV consists of a single-stranded, positive-sense RNA genome ~11 kb in size. Complete genome and partial gene sequences of geographically distinct DENV-3 strains were retrieved from the GenBank database. The evolutionary divergence of the 33 whole-genome and individual gene sequences of the nucleotides and amino acids of DENV-3 strains were generated with the maximum likelihood (ML) and Bayesian phylogenetic study (BEAST) methods using the MEGA 7 software. The genome size varied from 10,484 to 10,724 nucleotides among the strains with distinct geographical backgrounds belonging to Central America, South-Central Asia, and Eastern Asia. A phylogenetic analysis of the nucleotide and amino acid sequences of these DENV-3 isolates revealed extensive differences in the topologies due to PrM/M, NS1, NS2B, and NS3 genes. These results suggest substantial variation in the evolutionary pathways of the studied genes and genomes.

Keywords: dengue virus; dengue hemorrhagic fever; phylogenetic analysis; maximum likelihood

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1. Introduction

Dengue fever (DF) is one of the most common vector-borne diseases in the world, mostly found in tropical as well as in subtropical regions. Dengue virus (DENV), the causative microorganism of DF and dengue hemorrhagic fever (DHF), is a member of the Flavivirus genus and belongs to the Flaviviridae family. All flaviviruses contain single-stranded, positive-sense RNA and mostly infect hematophagous arthropods (ticks or mosquitoes), which complements its natural horizontal transmission cycle [1]. Infection with flaviviruses varies from asymptomatic to lethal, and more than 50% of viruses may cause human diseases. Influenza-like illness with sudden onset of fever, arthralgia, myalgia, retro-orbital headaches, maculopapular rash, leukopenia, vascular leakage, and encephalitis are mostly associated with flaviviruses infections [2]. The genus flavivirus also contains several important disease-causing viruses such as yellow fever virus (YFV), West Nile virus (WNV), and Japanese encephalitis virus (JEV), as well as tick-borne encephalitis virus (TBEV), associated with tick-borne diseases [3].

DENV serotypes have been classified into DENV 1–4, each possessing distinct antibody epitopes specific to the serotype [4]. Previous studies show that these four DENV serotypes evolved from their common ancestor in separate ecological niches. Each ecological niche was different, with a specific geographic location, distinct primate host populations, and different vector species [5]. The dengue virus genome is ~11 kb. It comprises an open reading frame (ORF), which codes for ten mature proteins, of which three are structural proteins (capsid (C), premembrane or membrane (prM/M), envelope (E)) and seven are nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), many of which play important roles in replication of the virus. The most immunologically significant protein is envelope (E) glycoprotein [6].

In around 1780, the first DHF epidemics were reported in Asian, African, and North American regions. For approximately the next 200 years, DHF epidemics remained localized in these three continents, albeit the epidemics seemed to be concentrated and more frequent in the tropics [7]. DENV-1 and DENV-2 were isolated during the Second World War in Japanese and American patients. DENV-3 and DENV-4 were isolated in the 1950s during the outbreaks in the Philippines and Thailand, respectively [8]. Infection caused by DENV may be clinical or subclinical, mild or acute, or afebrile or febrile, which may become severe and result in hemorrhagic disease [9]. In most cases, it causes DF, with symptoms including frontal headache, fever, myalgias, nausea, vomiting, rash, and often arthralgias.

DHF and dengue shock syndrome (DSS), a vascular leak disease initiated by the overwhelming of the immune system by the cells of the monocytic descent, are two of the most severe forms of DENV infection. In the most severe cases, infection with DENV will cause substantial hemorrhages, organ failure, and neural disorder that impersonate viral encephalitis [10]. The treatment of dengue fever is symptomatic, while DSS and DHF need fluid resuscitation treatment [6]. Based on genomic diversity, DENV serotypes are further classified into various subtypes/genotypes [11]. DENV-3 is further divided into genotypes 1 to 5 [12]. Most DENV-3 infections are caused by genotypes 1 and 3, with most of the DF and DHF outbreaks in the Indian subcontinent, the Americas, Southeast Asia, East Africa, and the South Pacific being due to this genotype, whereas genotypes 4 and 5 were not implicated with DHF epidemics [13].

The most commonly used technique for DENV genotyping employs the phylogenetic study of gene structures, specifically the E gene [14]. Previous phylogenetic studies in each of the four different DENV serotypes revealed genetic subtypes that differ up to 12% in the nucleotide sequence in the E gene, which is known to be the most antigenic part of the virus [15]. In the current study, we performed a comparative phylogeographic and phylogenetic analysis of 33 full genomes as well as individual gene sequences of DENV-3 strains to identify genetic variations and their evolutionary patterns over time.

2. Materials and Methods

2.1. Sequence Analysis and Phylogenetic Hierarchy

The complete genomes of 33 DENV-3 strains were downloaded from GenBank (table). These genomes were selected from a distinct topographical background surrounding the Central American, South-Central Asian, and Eastern Asian regions, where dengue outbreaks have previously been reported. Complete genome sequences from each strain were manually separated into 12 fragments. These fragments include 5′ and 3′ untranslated regions, three structural genes (capsid (C), premembrane or membrane (prM/M), envelope (E)), and seven nonstructural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Nucleotide sequences of all partial genes, 5′ and 3′ untranslated regions (UTRs), and complete genome nucleotide as well as amino acid sequences were aligned using the Clustal W program [16]. Phylogenies were generated using the MEGA7 program based on general time-reversible (GTR)/GTR + I + G nucleotide substitution models of maximum likelihood (ML). The strengths of phylogenies were evaluated by resampling with 1000 bootstrap replications.

2.2. Evolutionary Length among DENV-3 Strains

After the sequences of complete genomes or partial genes were aligned and analyzed, the same was further analyzed to determine the comprehensive evolutionary length between the DENV-3 strains. This was also carried out using the MEGA7 program [17]. The bootstrap resampling review was achieved via 1000 repetitions (Table 1).

Table 1. Locations and names of the DENV-3 isolates collected worldwide.

Country	Name of Isolate	Genome (bp)	Year	Nucleotide Accession	Protein Accession	Reference
Anguilla	AI/BID-V2976/2001	10,663	2001	FJ898462	ACQ44501	Direct Submission
Australia	Cairns 2008	10,707	2008	JN406515	AFN80339	Direct Submission
Cambodia	KH/BID-V2053/2008	10,648	2008	FJ639715	ACL99233	Direct Submission
Colombia	CO/BID-V3405/2007	10,659	2007	GQ868578	ACW83006	Direct Submission
East Timor	Hu/TL018NIID/2005	10,707	2005	AB214879	BAE48725	Direct Submission
Ecuador	EC/BID-V2975/2000	10,663	2000	FJ898457	ACQ44496	Direct Submission
French Polynesia	PF96/150296-46183	10,671	1994	JQ920479	AFY10043	Direct Submission
India	DEL-72	10,680	2008	GQ466079	ADM63678	Direct Submission
Mexico	MX/BID-V2989/2007	10,663	2007	FJ898442	ACQ44481	Direct Submission
Viet Nam	VN/BID-V1911/2008	10,637	2008	FJ547066	ACL98983	Direct Submission
Mozambique	MZ/BID-V2418/1985	10,663	1985	FJ882575	ACQ44384	Direct Submission
Paraguay	PAR 5532-07	10,707	2007	HQ235027	AEF65939	Direct Submission
Saint Lucia	LC/BID-V2979/2001	10,660	2001	FJ898463	ACQ44502	Direct Submission
Singapore	SGEHI(D3)0040Y09	10,250	2009/01	GU370052	ADC92353	Direct Submission
Sri Lanka	LK/BID-V2409/1997	10,682	1997	GQ252674	ACS32036	Direct Submission
Taiwan	99TW628	10,707	1999	DQ675533	ABG73599	Direct Submission
Thailand	TH/BID-V2318/2001	10,629	2001	FJ687448	ACN42695	Direct Submission
Trinidad and Tobago	TT/BID-V2982/2002	10,663	2002	FJ898459	ACQ44498	Direct Submission
USA	US/BID-V1620/2005	10,648	2005	FJ182010	ACH99657	Direct Submission
Venezuela	VE/BID-V2267/2008,	10,654	2008	FJ639826	ACL99113	Direct Submission
Samoa	WS/BID-V2973/1995	10,663	1995	FJ898456	ACQ44495	Direct Submission
Wallis and Futuna	WF95/090595-2448	10,671	9/5/1995	JQ920489	AFY10053	Direct Submission
Cook Islands	CK/BID-V2972/1991	10,663	1991	FJ898455	ACQ44494	Direct Submission
Brazil	BR/AL95/2009	10,707	2009	JF808120	AFK83755	Direct Submission
China	YN01	10,707	2013	KF824902	AHI17474	Direct Submission
Grenada	GD/BID-V3930/2002	10,653	2002	KF955505	AHG23270	Direct Submission
Indonesia	MKS-WS79b	10,707	29/03/2010	KC762693	AHG06377	Direct Submission
Nicaragua	NI/BID-V7658/2012	10,569	4/7/1905	KF973480	AHC98451	Direct Submission
Pakistan	Pakistan/56/2008	10,675	2008	KF041254	AHC72426	Direct Submission
Peru	PE/BID-V7289/2008	10,693	2008	KJ189301	AHI43684	Direct Submission
Philippines	VIROAF7	10,267	1964	KM190937	AIG60036	Direct Submission
Puerto Rico	PR/BID-V1728/2006	10,645	2006	KF955456	AHG23221	Direct Submission
Saudi Arabia	Jeddah-2014	10,635	26/01/2014	KJ830751	AIH13925	Direct Submission

3. Results

The 33 complete genome DENV-3 strains ranged in size from 10,484 to 10,724 nucleotides with various topographical backgrounds, including Central America, South Central Asia, and Eastern Asia. However, the length of the open reading frame (ORF) at ~10,240 was similar across the DENV-3 strains (Table 1). The key variance in the genome lengths was found in the 5′ or 3′ UTRs rather than in the ORFs.

3.1. Phylogeneses and Phylogeographic Presentation

The evolutionary study of DENV viral and topographical genes showed the processes by which the four serotypes (DENV1-4) differed and independently entered the human population, along with the locations in which the viral populations persisted for the four serotypes. This model, which depicted DENV phylogenetic antiquities, was built from a set of observed viral structures by employing maximum likelihood and Bayesian evolutionary analysis.

Our study model, which was used to reconstruct the evolutionary history of gene sequences, relies on sequence similarity in the form of a phylogenetic tree with individual nodes. Each of these individual nodes represents the most conjectural, contemporary common ancestor of the observed gene structures located at the tips of the trees. The maximum likelihood method chooses the tree that best explains the evolutionary pathway of the provided sequence data.

Our analysis, performed in MEGA7, revealed the presence of five clusters, namely Oceania, Southeastern Asia, Central America, South-Central Asia, and Eastern Asia, which are part of two main clusters: Oceania and the rest of the world (Figure 1). The Oceania cluster consists of Southeastern Asia, whereas the Philippines (Accession No.: KM190937) was found away from both of these clusters.

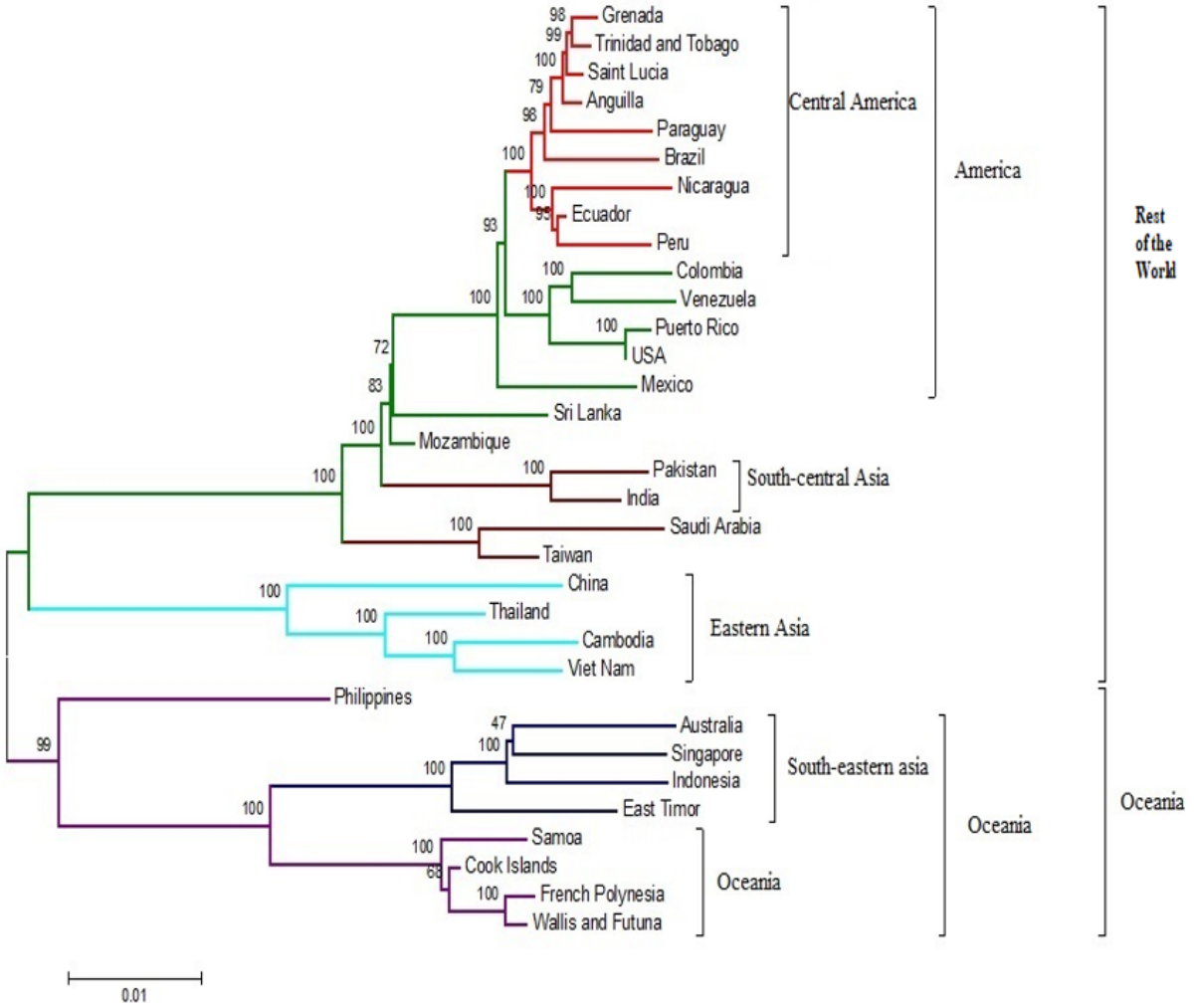

Figure 1. Phylogenetic maximum-likelihood tree of the DENV-3 full-genome nucleotide sequences. Trees were constructed using the MEGA v7 software with the bootstrap support of 1000 replicates. All nucleotide sequences were downloaded from the GenBank database for analysis as well as their respective DENV-3 isolates with their accession numbers listed.

3.2. Bayesian Evolutionary Analysis (BEAST) of Whole-Genome DENV-3

Figure 2 describes the same main clusters as mentioned in Figure 1. The first subcluster consists of Australia (Accession No.: JN406515), Singapore (Accession No.: GU370052), Indonesia (Accession No.: KC762693), and East Timor (Accession No.: AB214879). The second subcluster consists of Samoa (Accession No.: FJ898456), Cook Island (Accession No.: FJ898455), French Polynesia (Accession No.: JQ920479), Wallis and Futuna (Accession No.: JQ920489), and the Philippines (Accession No.: KM190937).

The second main cluster comprises China (Accession No.: KF824902), Mexico (Accession No.: FJ898442), and the USA (Accession No.: FJ182010), up to Grenada (Accession No.: KF955505). The origin of Pakistan (Accession No.: KF041254) and India (Accession No.: GQ466079) is rooted at the same node in this cluster.

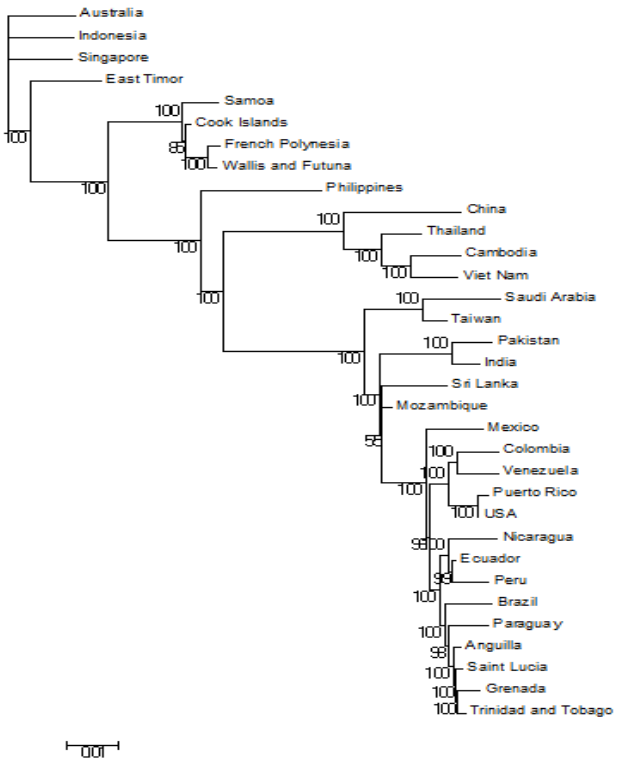

Figure 2. Bayesian evolutionary analysis (BEAST) of whole-genome DENV-3.

3.3. Phylogenetic Analysis Based on Amino Acids

Phylogenetic analysis based on amino acids was applied using the ML approach with reference to the JTT matrix-based pattern with the highest log likelihood (-13480.8871) (Figure 3). In this analysis, Eastern Asia (China (Accession No.: KF824902), Vietnam (Accession No.: FJ547066), Thailand (Accession No.: FJ687448), and Cambodia (Accession No.: FJ639715)) moved towards the Oceania cluster. This was a key difference between the whole-genome nucleotide analysis (Figure 1) and amino acid phylogenetic presentation (Figure 3).

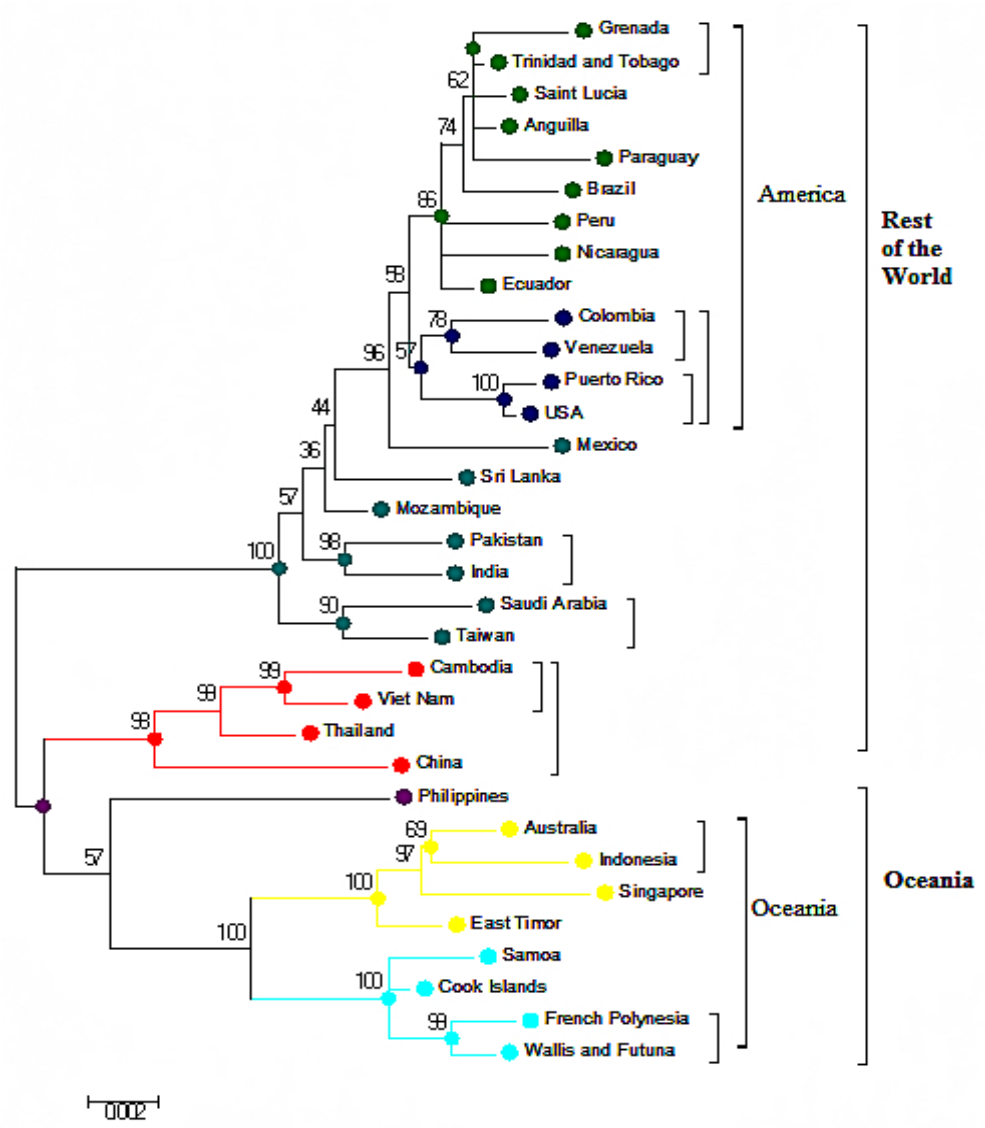

Figure 3. Phylogenetic maximum-likelihood tree of the DENV-3 full-genome amino acid sequences. Maximum-likelihood (ML) trees were constructed using the MEGA v7 software with the bootstrap support of 1000 replicates. All amino acid sequences were downloaded from the GenBank database for analysis with their accession numbers.

3.4. Phylogenetic Analysis of Capsid (C) and Envelope (E) protein

Changes in the amino acid sequences of the C protein moved from the Philippines strain (Accession No.: AIG60036) into the Eastern Asian cluster, whereas E protein changes moved from the China strain (Accession No.: AHI17474) near the Philippines strain (Accession No.: AIG60036) and away from the East Asian cluster (Figures 2, 3, 4 and 5A).

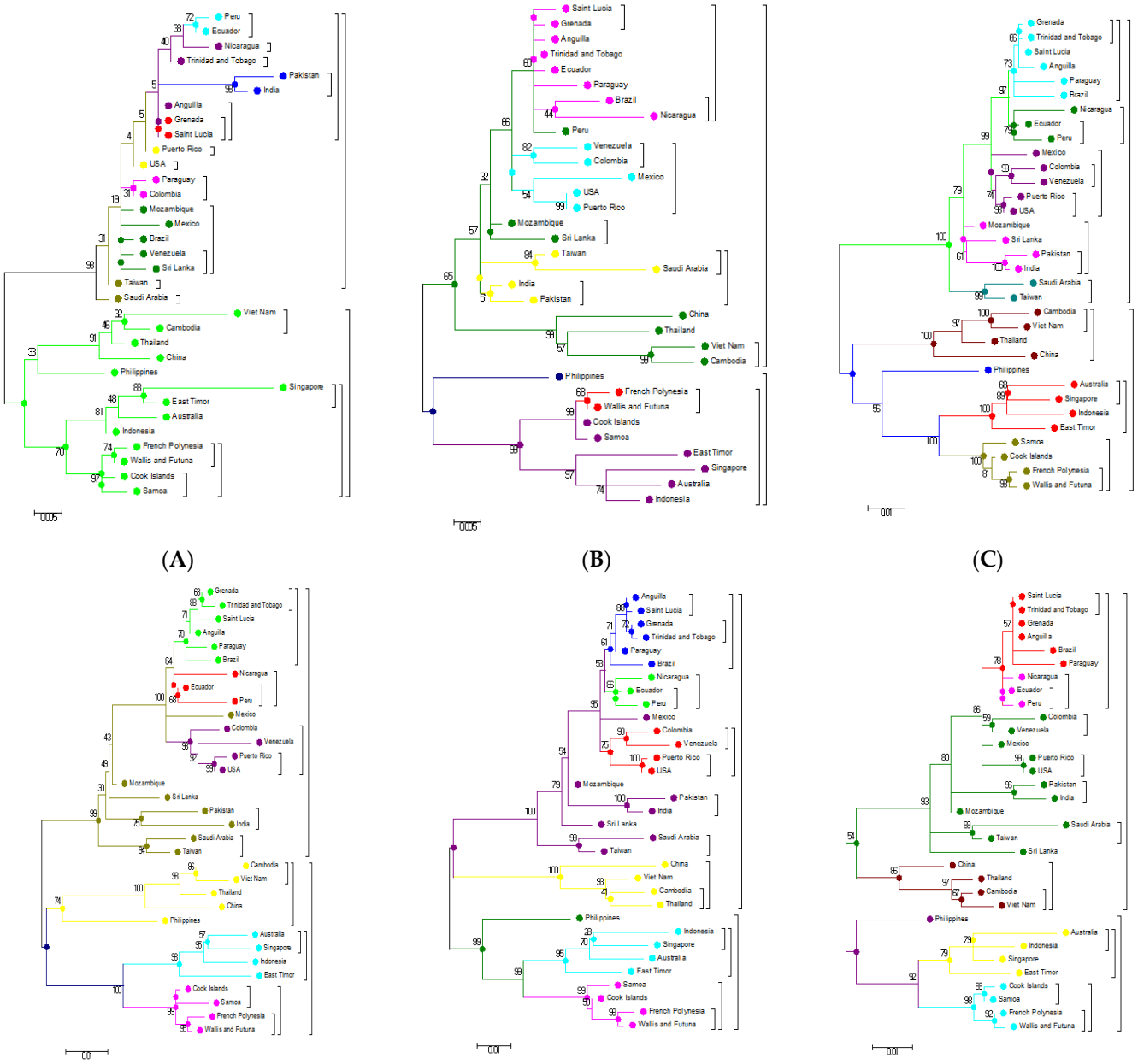

Figure 4. *Cont.*

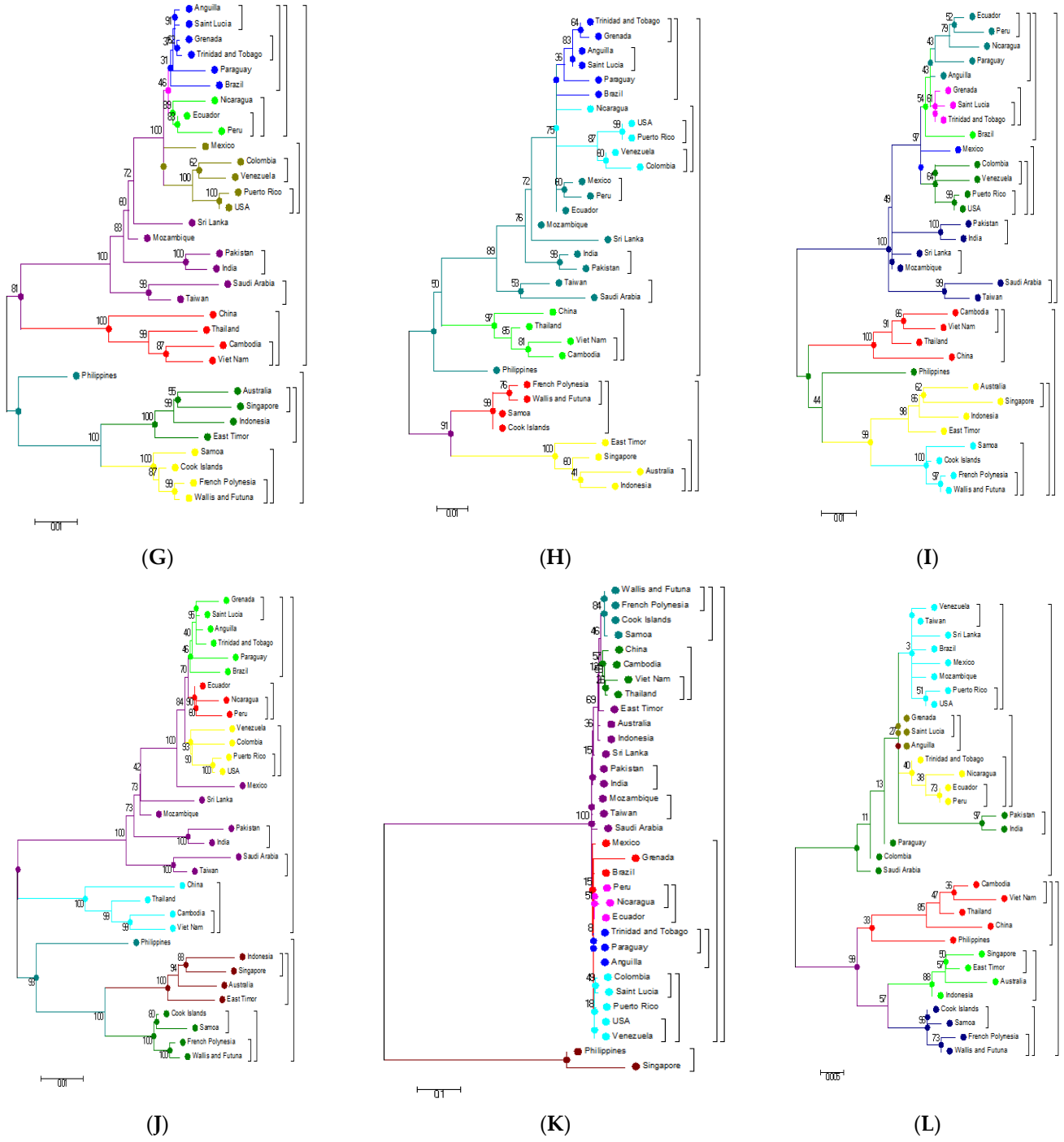

Figure 4. Molecular phylogenetic analysis using the maximum likelihood method based on the general time-reversible model of DENV-3 individual gene nucleotide sequences. All sequences of the individual genes were separated from the complete genome sequences that were downloaded from the GenBank database for analysis: (**A**) C gene; (**B**) PrM/M; (**C**) E gene; (**D**) NS1 gene; (**E**) NS2A; (**F**) NS2B; (**G**) NS3; (**H**) NS4A; (**I**) NS4B; (**J**) NS5; (**K**) 3′ UTR; (**L**) 5′ UTR.

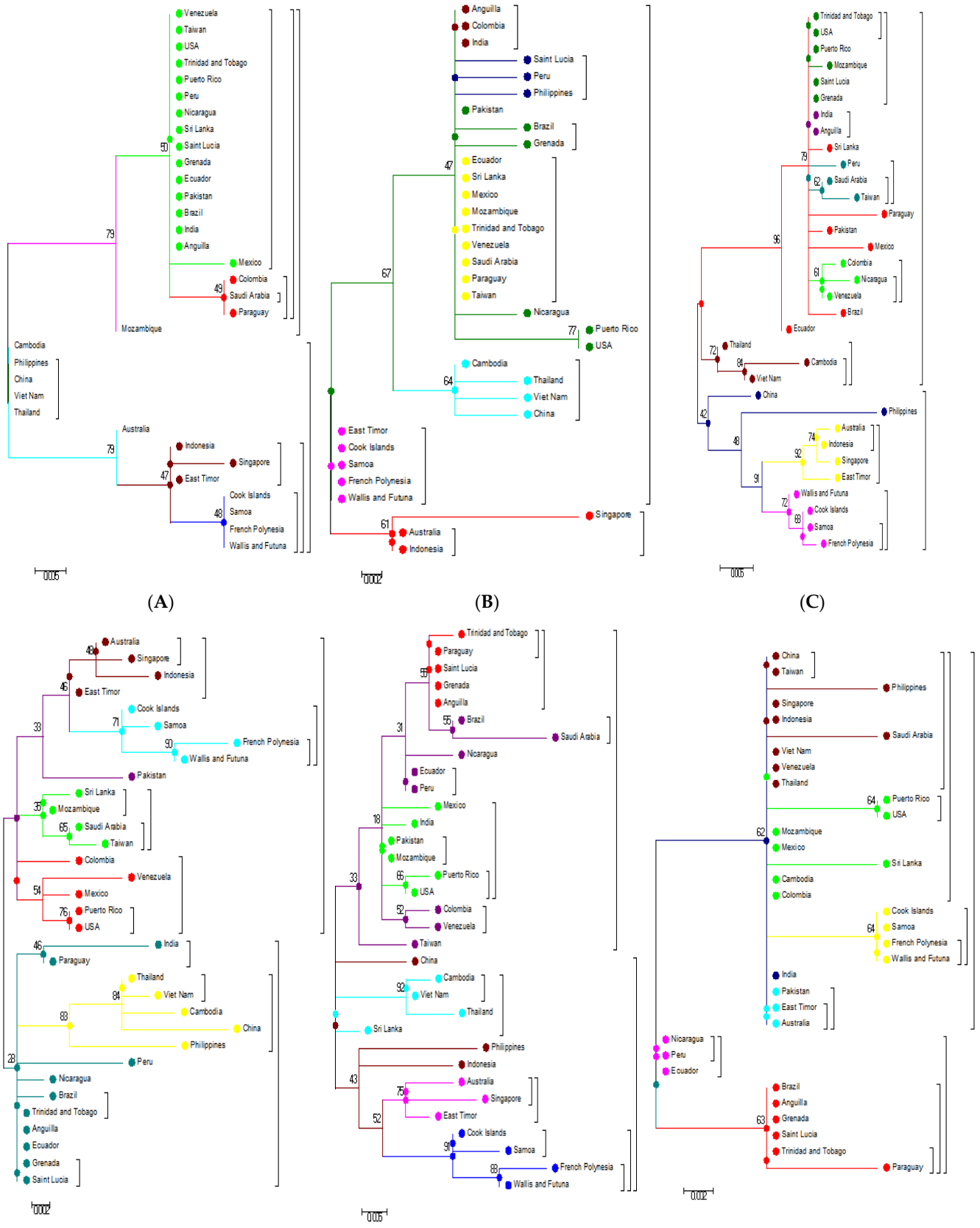

Figure 5. *Cont.*

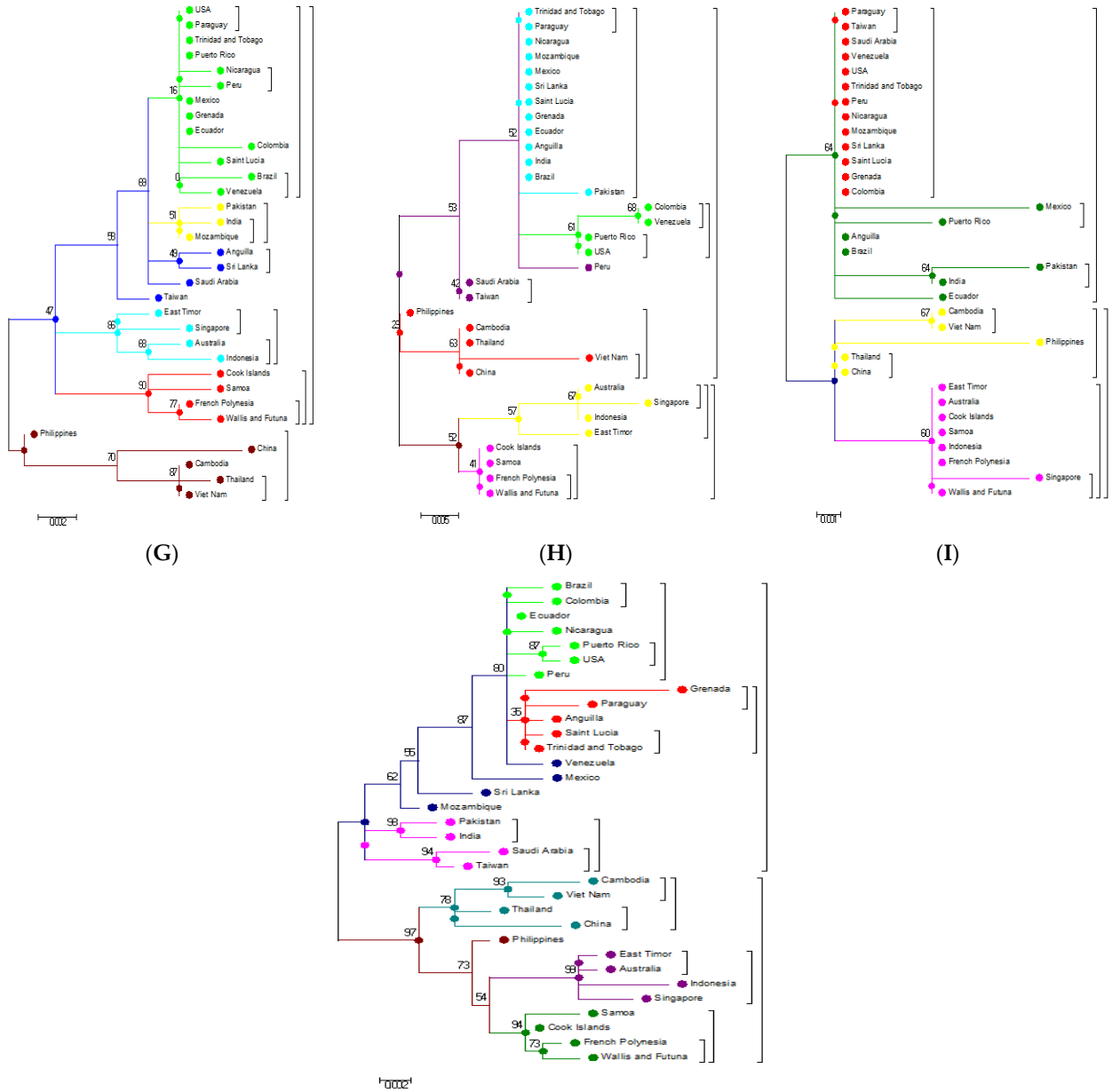

Figure 5. Molecular phylogenetic analysis using the maximum likelihood method based on the general time-reversible model of DENV-3 individual gene amino acid sequences. All sequences of the individual genes were separated from the complete genome sequences downloaded from the GenBank database for analysis. (**A**) C; (**B**) PrM/M; (**C**) E; (**D**) NS1; (**E**) NS2A; (**F**) NS2B; (**G**) NS3; (**H**) NS4A; (**I**) NS4B; (**J**) NS5.

3.5. Phylogenetic Analysis Based on Individual Proteins

Phylogenetic examination of the PrM/M gene shows a large deviation in the topologies of the first and second main clusters based on the analysis of individual genes (nucleotide) and their respective amino acid analysis (Figures 4B and 5B). The disagreement between the nucleotide and amino acid analysis of the NS1 gene resulted in the shifting of some strains in the subgroups of the first main cluster to the second cluster (Figures 4D and 5D). The NS2B analysis illustrates the significant deviation of the subgroups of the first and second main clusters (Figures 4F and 5F). Similarly, a comparison of the NS3 nucleotide and amino acid analysis shows topological deviations between the subgroups of the main

clusters, resulting in the Eastern Asian falling into the second main cluster (Figures 4G and 5G). Unlike C, E, PrM/M, NS1, NS2B, and NS3, the nucleotide and amino acid analysis of other genes such as NS4A and NS4B, as well as NS5, did not show significant deviations and movements between the two clusters (Figure 4H–J and Figure 5H–J).

4. Discussion

Previous studies have employed individual gene sequences of DENV-3 for phylogenetic investigations and classified them into various genotypes [18]. Most of such studies use E gene sequences for the genotyping of DENV-3 [19], although other genes have been used by some investigators [20]. For example, based on the phylogenetic analysis of E gene sequences, DENV-3 was split into four genotypes [21]: strains from South Pacific islands and Southeast Asia fall into genotype 1; genotype 2 comprises of strains from Thailand; genotype 3 consists of strains from East Africa, Samoa, and the Indian subcontinent; and strains from Puerto Rico Tahiti fell into genotype 4 [12]. Therefore, earlier studies were more focused on the sequence analysis of individual genes or a subset of those genes for genotyping of DENV-3 [22], especially the PrM/M, E, NS1, NS3, NS4A, and NS5 genes [23]. For example, during the 2015 epidemic of DENV in the province of Khyber Pakhtunkhwa, Pakistan, a phylogenetic analysis, made on the basis of the E and S1 genes, revealed that it belongs to DENV-3, showing maximum homology with strains previously reported from Pakistan and India [23]. This practice, although useful, is based on limited information, in most cases merely restricted to domestic sequences in one territory. However, more recently, investigators are using entire genome nucleotide or amino acid sequences for identifying genetic variation, constructing phylogenetic trees, and the classification of DENV genotypes. A recent study employing the entire genome of DENV-3 (10,672 bp) identified 388 mutations in the nucleotide sequences and 34 mutations in the amino acids [24].

Our investigation, by analyzing entire genomes of 33 DENV strains from around the world, confirmed the previous findings of Pakistani strains of DENV being placed in the DENV-3 genotype. Previous studies utilizing the complete genome sequences of DENV-3 have yielded similar results [25]. However, our investigation utilizing the entire genome yields additional important information. In particular, our analysis of amino acid sequences of the entire ORF produced some unexpected results, which is why we see several DENV strains swapping clusters after amino acid sequence analysis.

Previously, phylogenetic correlations using whole-genome sequences of Indian DENV-3 isolates described the presence of a discrete DENV-3 clade in India [26]. In the present study, our results confirmed the distinctiveness of the Indian DENV-3 isolates in the South-Central Asia subcluster through the analysis of complete genome nucleotide and amino acid sequences.

Phylogenetic trees generated from complete genomes using the ML technique presented two main clusters Oceania and the rest of the world (Figure 1). When the nucleotide sequences of the individual genes and their respective protein sequences were analyzed via p-blast, the topology network of the genomes changed considerably (Figures 4 and 5). When the analysis was carried out based on the C protein, DENV strains from countries such as Taiwan, the USA, Peru, Sri Lanka, Pakistan, Brazil, and India concentrated on a single node but were previously located on multiple nodes when the analysis was performed using nucleotide sequences of the C gene (Figures 4A and 5A). In the Oceania cluster, constructed based on the PrM/M gene, there are around nine countries, but due to changes in the PrM/M, protein only Singapore, Australia, and Indonesia are left in the cluster (Figures 4B and 5B). Similarly, for many other genes, strains from several countries changed clusters when phylogenetic trees were constructed based on amino acid sequences (Figures 4 and 5).

Before the start of this investigation, one of our assumptions was that phylogenetic analysis based on nucleotide and amino acid analysis yields the same results. However, our analysis with phylogenetic trees based on both nucleotide and amino acid sequences did not produce the same results. This might be due to the redundancy of the codons. Changes

at the nucleotide level are sometimes not reflected at the amino acid level. Our analysis based on amino acid sequences shows a different phylogenetic tree from the one which we constructed with nucleotide sequences (Figures 1 and 2). Some of the strains swapped clusters when the phylogenetic tree was constructed based on amino acid sequences.

Genomic regions, which displayed significant variability in our investigation and showed interesting, results include the PrM/M (Figures 4B and 5B), NS1 (Figures 4D and 5D), NS2B (Figures 4F and 5F), and NS3 genes (Figures 4G and 5G). These genes exhibited substantial variability both at the nucleotide and amino acid levels. In particular, phylogenetic analysis of the PrM/M gene showed large variability and, as a result, changed the topologies of certain strains between the first and second main clusters (Figures 4B and 5B). Some of the genes such as NS4A, NS4B, and NS5 did not show significant variation (Figure 4H–J and Figure 5H–J).

All the genes that improve viral fitness to survive and reproduce will keep changing to achieve even better functionality. Viral fitness depends more on certain genes than others. It has been reported previously that amino acid differences at position 390 of the E protein and in the 5′ and 3′ UTRs confer higher efficiency for viral replication in monocyte-derived dendritic cells and macrophages and thus improve the fitness of the virus [27]. On the other hand, the genes that do not confer survival advantage or are functionally constrained are less likely to change. Previous studies show that some of the nonstructural proteins such as NS3 and NS5 have limitations on how much they can change without compromising their function [28].

NS5 gene coding for an RNA-dependent RNA polymerase did not display significant variability in our analysis. Viral polymerases, like other important structural and nonstructural proteins, are under enormous selection pressure. However, unlike many structural proteins, enzymes cannot change too much so as not to compromise their functions. Viral polymerases are also constrained by the fact that functional redundancy for polymerases is much less than for other enzymes, further putting them under pressure. Therefore, due to functional limitations, polymerases are usually highly conserved with occasional changes that would further increase their fitness for viral replication.

Our extensive analysis of viral mutations and evolution from 33 complete viral genomes (DENV-3 isolates) shows substantial differences from previous results, which were based on the nucleotide and amino acid sequences of individual genes (E, PrM/M, NS1, NS2B, and NS3) [29]. The topologies of various genes become significantly different when whole-genome nucleotide as well as amino acid sequences are analyzed instead of individual genes. These results suggest that the evolution of the DENV-3 genome is guided by both structural as well as in nonstructural parts of the genome.

5. Conclusions

Our study shows that a phylogenetic analysis based on complete genome nucleotide sequences of the distinct DENV-3 genotypes from around the world produces phylogenetic trees, which are different from when the analysis is performed based on amino acid sequences. Our investigation also shows that both structural and nonstructural parts of the genome are likely to shape the evolution of the DENV-3 genome.

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