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Morning *vs.* evening administration of antiviral therapy in COVID-19 patients. A preliminary retrospective study in Ferrara, Italy

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Abstract. – OBJECTIVE: At the end of 2019, the Novel Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), spread rapidly from China to the whole world. Circadian rhythms can play crucial role in the complex interplay between viruses and organisms, and temporized schedules (chronotherapy) have been positively tested in several medical diseases. We aimed to compare the possible effects of a morning *vs.* evening antiviral administration in COVID patients.

PATIENTS AND METHODS: We retrospectively evaluated all patients admitted to COVID internal medicine units with confirmed SARS-CoV-2 infection, and treated with darunavir-ritonavir (single daily dose, for seven days). Age, sex, length of stay (LOS), pharmacological treatment, and timing of antiviral administration (morning or evening), were recorded. Outcome indicators were death or LOS, and laboratory parameters, e.g., variations in C-reactive protein (CRP) levels, ratio of arterial oxygen partial pressure (PaO_2, mmHg) to fractional inspired oxygen (FiO2) (PaO_2/FiO_2), and leucocyte count.

RESULTS: The total sample consisted of 151 patients, 33 (21.8%) of whom were selected for antiviral treatment. The mean age was 61.8±18.3 years, 17 (51.5%) were male, and the mean LOS was 13.4±8.6 days. Nine patients (27.3%) had their antiviral administration in the morning, and 24 (72.7%) had antiviral administration in the evening. No fatalities occurred. Despite the extremely limited sample size, morning group subjects showed a significant difference in CRP variation, compared to that in evening group subjects (-65.82±33.26 *vs.* 83.32±304.89, respectively, $p<0.032$). No significant differences were found for other parameters.

CONCLUSIONS: This report is the first study evaluating temporized morning *vs.* evening antiviral administration in SARS-CoV-2 patients. The morning regimen was associated with a significant reduction in CRP values. Further confirmations with larger and multicenter samples of patients could reveal novel potentially useful insights.

Key Words:
Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Chronobiology, Chronotherapy, Antiviral drugs, Darunavir, Ritonavir, C-reactive protein, Inflammation, Hospitalization, Outcome.

Introduction

At the end of 2019, another Novel Coronavirus, designated Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), was first reported in Wuhan, a city in the center of China, and then, rapidly spread to the whole world, since more than 800 thousand people in over 200 countries have become involved in the global pandemic[1]. A wide range of clinical pictures has been observed in affected patients, including from fatal, severe, mild, scarcely manifest, and even asymptomatic forms, and diverse immune responses may play

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a role. Our body defense is governed by the immune system, but a complex interplay between the host and infectious agent may depend on circadian rhythmicity of cellular processes and immune functions, thus affecting the patterns of host-virus interaction[2].

Biological rhythms exist at any level of living organisms and, according to their cycle length, may be divided into three main types: a) circadian rhythms (from the Latin circa-diem, period of approximately 24 hours, deriving from the duration of a cycle of earth rotation), b) ultradian rhythms (period <24 hours, e.g., hours, minutes or even seconds), and c) infradian rhythms (period > 24 hours, e.g., days, weeks, or months)[3]. Circadian clocks are autonomous cells, transcriptionally based, molecular mechanisms that confer the selective advantage of anticipation, enabling organisms to prepare for changes in their physical environments and respond to environmental factors in a temporally appropriate manner[4]. The anatomical center of the mammalian circadian clock lies within approximately 15,000 neurons of the suprachiasmatic nucleus, a region of the anterior hypothalamus, that directs the oscillatory nature of multiple physiological and metabolic functions, since the transcription of at least 10% of all cellular genes oscillates in a circadian manner[5].

Circadian rhythms can play a crucial role and determine the outcome of the interplay between hosts and microorganisms, including viruses. When a viral agent infects a cell, it immediately works to change the biological processes aimed at creating a more favorable environment for self-replication and spread. A recent review analyzed three different aspects of the interplay: (1) the circadian regulation of innate and adaptive immune systems, (2) the impact of the biological clock on viral infection, and (3) the clock deregulation operated by the viral perturbations[6]. Previous observations revealed that a circadian variation in the efficacy of vaccination exists. On the one hand, mice vaccinated at the time of high Toll-like receptor (TLR9) expression had an enhanced immune response[7]. On the other hand, in humans, a higher antibody response after morning vaccination than that after evening vaccination has been reported for both hepatitis A and influenza[8,9]. A growing body of evidence[10-15] shows that time-of-day-related treatment schedules and chronomodulated drug delivery may positively affect both the effectiveness and side effects of pharmacological therapy for several medical diseases, such as cardiovascular, rheumatologic, and oncologic diseases. Based on these premises, we aimed to evaluate the existence of a possible advantage of chronomodulated antiviral therapeutic strategies, comparing morning *vs.* evening administration.

Patients and Methods

Ferrara is a province located in the eastern part of the Emilia-Romagna region of Italy, with a total population of approximately 350,000 inhabitants. The General and University Hospital (Azienda Ospedaliero-Universitaria "S. Anna") has 626 beds and represents the hub and teaching hospital of the entire province. The "S. Anna" Hospital approached the Coronavirus outbreak with a series of organizational measures. A specific COVID-dedicated pathway was adopted, including an emergency department triage area, an infectious disease and a 'suspect' observational unit, three internal medicine units, one pulmonology unit, and one intensive care unit. We retrospectively evaluated all patients admitted to the internal medicine COVID units who had SARS-CoV-2 infection confirmed with polymerase chain reaction tested by nasopharyngeal swab. The internal medicine COVID units, accounting for 88 total beds, were opened on March 17, 2020 and received patients 24/24 hours and 7/7 days from the emergency department. On admission, each patient received a team evaluation by two specialists (internal medicine and infectious disease), who evaluated the time of infection, clinical signs and symptoms, and imaging and laboratory examinations, and prescribed the most appropriate therapeutic regimen, including or not antiviral drugs, that was immediately initiated. In our hospital, the association darunavir/ritonavir was the allowed available antiviral regimen, with a single daily dosage of 800 mg of darunavir-100 mg of ritonavir, for seven days. Thus, patients hospitalized in the morning received their first dose in the morning, while those hospitalized in the afternoon-evening hours received the drugs in the evening.

For the present study, we decided to stop enrolment on May 4, 2020, and we selected all patients treated with antiviral drugs. The study was conducted in agreement with the declaration of Helsinki of 1975, revised in 2013. Subject identifiers were deleted before data anal-

Table I. Characteristics of the investigated patients.

Total patients	n = 33
Age (years)	61.8 ± 18.3
Male [n (%)]	17 (51.5%)
Female [n (%)]	16 (48.5%)
Antiviral drugs evening administration [n (%)]	24 (72.7%)
Length of stay (days)	13.4 ± 8.6
Treatment other than antiviral drugs	
Hydroxychloroquine [n (%)]	29 (87.9%)
Steroids [n (%)]	3 (9.1%)
Azithromycin [n (%)]	20 (60.6%)
Antimicrobial therapy [n (%)]	22 (66.7%)
Low molecular weight heparin – prophylaxis dose [n (%)]	21 (63.6%)
Low molecular weight heparin – therapeutical dose [n (%)]	3 (9.1%)
Clinical data	
CRP variation (%)	42.64 ± 267.65
PaO_2/FiO_2 variation (%)	-0.374 ± 0.618
Lymphocytes count variation (%)	22.71 ± 49.65

ysis aimed to maintain data anonymity and confidentiality: therefore none of the patients could be identified, either in this paper or in the database. We recorded demographic data, such as age, sex, length of stay (LOS), general pharmacological treatment, and timing of antiviral administration (morning or evening). Our outcome indicators were: (1) death (or LOS), and (2) laboratory parameters, including variations of C-reactive protein (CRP) levels, ratio of arterial oxygen partial pressure (PaO_2, in mmHg) to fractional inspired oxygen (FiO_2) (PaO_2/FiO_2), and leucocyte count, from the first to the last day of the antiviral regimen. Descriptive analysis and comparison between subjects with morning *vs.* evening antiviral therapy administration was performed. Data are expressed as absolute numbers, percentages and means ± standard deviation. The chi-square test, Student's *t*-test, and Mann-Whitney-U test were used as appropriate. SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, 2004) was used for statistical analyses.

Results

Overall, 151 patients were hospitalized in the internal medicine COVID units during the study period, and antiviral treatment was prescribed in 33 (21.8%). The mean age was 61.8±18.3 years, 17 (51.5%) were male, and the mean LOS was 13.4±8.6 days. Table I shows the main characteristics of these 33 patients. Morning and evening antiviral administration was recorded in 9 (27.3%) and 24 (72.7%) patients, respectively. No fatalities occurred. No differences by sex were found. The results of the univariate analysis are reported in Table II. The only parameter with significant difference between the two groups was the CRP variation (-65.82±33.26% *vs.* 83.32±304.89%, $p<0.032$). Figure 1 graphically shows the CRP variation. A separate analysis (data not included) did not show significant differences for the subgroups of patients receiving or not receiving antimicrobial therapy, including azithromycin, likely due to the extremely limited number of cases.

Table II. Comparison between morning vs. evening administration of antiviral drugs.

	Morning administration (n = 9)	Evening administration (n = 24)	p
Male [n (%)]	4 (44.4%)	13 (54.2%)	NS
Female [n (%)]	5 (55.6%)	11 (45.8%)	
Age (years)	56.44 ± 12.37	63.79 ± 19.94	NS
Length of stay (days)	12.67 ± 8.63	13.65 ± 8.74	NS
CRP variation (%)	-65.82 ± 33.26	83.32 ±304.89	0.032
PaO_2/FiO_2 variation (%)	-0.372 ± 0.615	-0.375 ± 0.657	NS
Lymphocyte count variation (%)	39.97 ± 43	17.35 ± 51.73	NS

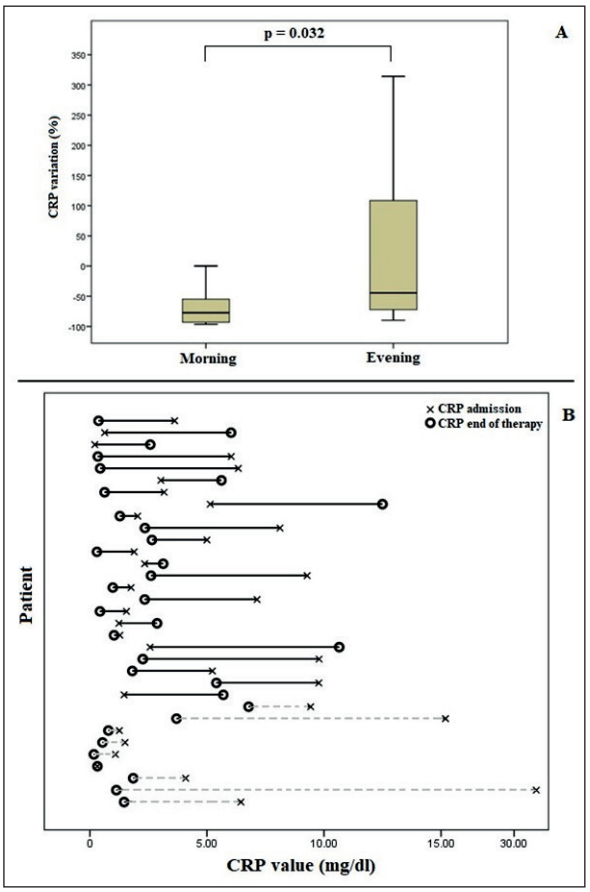

Figure 1. CRP variations after morning *vs.* evening antiviral therapy.

Discussion

This report is the first study evaluating the results of a temporized approach in patients hospitalized for SARS-CoV-2 infection. Morning *vs.* evening antiviral drug administration, evaluated at initiation and at the end of a 7-day regimen, was associated with a significant reduction in CRP values, with no differences for the other considered parameters.

The circadian regulation of the immune system response works to achieve time-dependent success against different pathogens in a rhythmic fashion[7,16,17]. In fact, immune cells of both the innate and adaptive components of the immune system possess molecular clock to manage their circadian rhythmic processes[18], such as lymphocyte migration through lymph nodes and lymph in mice[19]. For example, BMAL1 the heterodimeric transcription factor has been shown[20] to be a regulator of innate immunity, since BMAL1-deficient cells had increased susceptibility to infection by RNA viruses, e.g., respiratory syncytial virus and parainfluenza virus type 3. On the other hand, a circadian clock effect also exists for viral infection. Wild-type mice, kept in a controlled temperature and lighting environment (12/12-hour light/dark), and infected with murine herpesvirus at different times of the day, were evaluated for levels and spread of infection[21]. Interestingly, viral replication was increased tenfold in mice infected at the beginning of their resting phase (morning), compared to that found in mice infected during their activity phase (night). Again, the importance of BMAL1 was crucial, since BMAL1-deficient mice showed levels of virus replication independent of the time of day of viral infection[21]. This to confirm other studies[22] with BMAL1 knockout mice, showing that correct integrity of the molecular clockwork is important for the immune response towards viral infections. SARS-CoV-2, a single-stranded RNA-enveloped virus, targets cells through the viral structural spike (S) protein that binds to the angiotensin-converting enzyme 2 (ACE2) receptor. Following receptor binding, virus entry into the cell depends on subsequent S protein priming by a host type 2 transmembrane serine protease (TMPRSS2), favors internalization by the endocytic pathway[23,24]. Once inside the cell, viral polyproteins are translated and encode for the replicase-transcriptase complex, which is cleaved into the final products by viral proteases. The virus, then, synthesizes mRNA and genomic RNA *via* its RNA-dependent RNA polymerase, and structural proteins are synthesized leading to completion of assembly and release of viral particles[25,26]. The COVID-19 pandemic represents a novel task for scientists, and China has provided the largest amount of research data dealing with COVID-19 infection[27], but it has to be stressed that there is no evidence from randomized clinical trials (RCTs) that any potential therapy improves outcomes in patients with either suspected or confirmed COVID-19 thus far[28].

For the available antiviral drugs, lopinavir/ritonavir, a US Food and Drug Administration (FDA) approved oral combination agent for treating HIV, demonstrated *in vitro* activity against other novel coronaviruses *via* inhibition of 3-chymotrypsin-like protease[29]. An open-label randomized controlled trial, comparing the efficacy of lopinavir/ritonavir *vs.* standard care in patients hospitalized with severe COVID-19, did not find significant differences in either time to clinical improvement or 28-day mortality rates[30]. An-

other Chinese study[31] evaluated 47 patients with COVID-19 infection, in two subgroups according to whether they had been treated with adjuvant therapy plus lopinavir/ritonavir or not, during hospitalization. The changes in body temperature, routine blood tests and blood biochemistry between the two groups were observed and compared. Both groups achieved good therapeutic effects with body temperature, but the treated group showed reduced routine blood indexes, including abnormal proportions of white blood cells, lymphocytes and C-reactive protein, compared with those of the control group[31]. Darunavir has no human clinical data available, but *in vitro* cell models demonstrated activity against SARS-CoV-2, and a randomized controlled trial in association with of cobicistat is underway in China[28]. Oseltamivir, a neuraminidase inhibitor approved for the treatment of influenza, has no documented *in vitro* activity against SARS-CoV-2, and has no role in the management of COVID-19 once influenza has been excluded[28]. Umifenovir is a more promising antiviral agent with a unique mechanism of action targeting the S protein/ACE2 interaction and inhibiting membrane fusion of the viral envelope[32], it is currently approved in Russia and China for the treatment and prophylaxis of influenza, and limited clinical experience for COVID-19 has been described in China[28].

Limitations

We are aware of several limitations to this study. First, this was a retrospective study, based on nonrandomized consecutive patients. On the other hand, with the lack of any previous studies testing the hypothesis of a morning *vs.* evening administration of antiviral drugs, the time of therapy initiation depended on the time of admission. Second, there was a limited number of patients. However, other recent researches to evaluate the therapeutic effects and the possible advantages of the treatment with antiviral combinations were also conducted on limited samples. Third, under a strict chronobiologic point of view, hospitalization is per se a potential desynchronizing factor for circadian rhythms, secondary to forced time of light and meals. However, this limitation is common to investigation hospitalized patients.

Conclusions

A growing body of evidence[33-38] is accumulating on the potential advantages of a temporized approach to different diseases, so-called chronotherapy, aimed at obtaining better results or at least reduced side effects. Based on the theoretical premises of a circadian variation in either the immune response or viral activity, this retrospective study first provided the observation of a possible different morning *vs.* evening response to antiviral therapy in COVID-19 patients, at least regarding the inflammatory marker CRP. Interestingly, the finding of a statistically significant reduction in CRP values in the morning treatment group is even stronger, considering that this group accounted for only less than one-third of the total sample. Although, while no definite conclusions can be drawn from this small-sized study, in our opinion these preliminary findings could reveal the possibility of testing the hypothesis on larger and multicenter samples of patients, and obtaining potentially useful insights for the future.

Conflict of Interest

There are no financial or other conflicts of interest incurred by any of the authors due to the sources of funding, or utilized products, technology, or methods of our research and report of findings.

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Authors' Contribution

Conceptualization, A.D.G., F.F., E.C. and R.M.; methodology, A.D.G., and F.F.; software, A.D.G., F.F., S.G., and E.D.S.; validation, R.D.G., A.P., G.Z., and R.M.; formal analysis, A.D.G., F.F., S.G., and E.D.S.; investigation, A.D.G., S.G, and E.D.S.; resources, R.D.G., A.P., G.Z., and R.M.; data curation, A.D.G., S.G., E.D.S; writing-original draft preparation, A.D.G., F.F., E.C. and R.M.; writing-review and editing, R.D.G., A.P., G.Z., E.C. and R.M.; visualization, A.G.G., S.G., E.D.S., E.C. and A.P.; supervision, R.D.G., A.P., G.Z., and R.M.; project administration, R.D.G., G.Z., and R.M; funding acquisition, R.M.. All authors have read and agreed to the published version of the manuscript.

Appendix

List of Collaborators

OUTcome and COMorbidity Evaluation of INTernal MEDicine COVID19 (OUTCOME-INTMED-COV19) Study Collaborators. *Team Clinica Medica (Head: Roberto Manfredini) & Medicina Interna Universitaria (Head: Giovanni Zuliani)*: Benedetta Boari, Gloria Brombo, Eleonora Capatti, Andrea Cutini, Edoardo Dalla Nora, Andrea D'Amuri, Gloria Ferrocci, Francesca Di Vece, Laura Fornasari, Christian Molino, Elisa Misurati, Michele Polastri, Tommaso Romagnoli, Giovanni Battista Vigna, Alessandro Bella, Stefania Bonazzi, Beatrice Bonsi, Paola Chessa, Angela Colangiulo, Daniele Deplano, Valeria Fortunato, Enrico Giorgini, Patrizia Guasti, Gaetano Lo Coco, Mariarosaria Lopreiato, Francesco Luciani, Chiara Mancino, Lisa Marabini, Sara Morrone, Chiara Pazzaglini, Dario Pedrini, Chiara Pistolesi, Ugo Politti, Federica Ristè, Rossella Roversi, Alessandro Scopa, Chiara Marina Semprini, Daniela Tortola, Grazia Vestita, Alessandra Violi. *Team Medicina Interna Ospedaliera 2* (*Head: Roberto De Giorgio*): Tommaso Bachechi, Paolo Baldin, Antonella Cianci, Rossella Colonna, Roberto De Fazio, Lorenzo Di Candio, Ilaria Fiorica, Pierluigi Gaudenzi, Caterina Ghirardi, Lisa Giusto, Pierluigi Morandi, Claudia Parisi, Franco Ricci, Elena Satta, Francesco Strocchi, Federica Tordo Caprioli.

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