The Rationale for a Multi-Step Therapeutic Approach Based On Antivirals and Drugs with Immunomodulatory Activity in Patients With Coronavirus-Sars2-Induced Disease of Different Severity

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ABSTRACT

In December 2019 a novel human-infecting coronavirus, named SARS-CoV-2 has been recognized to cause a pneumonia epidemic outbreak with different degree of severity in Wuhan, Hubei Province in China. Since then this epidemic spread worldwide an in the last week Europe and Italy also have been involved. Effective preventive and therapeutic strategies are absolutely required to block this serious public health concern. Unfortunately, SARS-CoV-2 has been isolated only recently, therefore a few studies concerning its immunopathogenesis and tretament are available.

Therefore, on the basis of the assumption that the SARS-CoV-2 is genetically related to SARS-CoV (about 82% of genome homology) and that its characteristics, like the modality of transmission, the route of infection, the organ localization, the type of the immune response it may stimulate, the morbidity and the mortality rates are still poor-known, a literature search was performed to identify the reports assessing these elements in patients with SARS-CoV-induced infection. Therefore, we have analysed:

1) the structure of SARS CoV-2 and SARS CoV;

2) the clinical signs and symptoms and pathogenic mechanisms observed during the development of acute respiratory syndrome and the Cytokine Release Syndrome;

3) the modification of the cell microRNome and of the immune response in patients with SARS infection;

4) the possible role of some liposoluble compounds (such as vitamin A, D and E) in modulating directly or indirectly the replication ability of SARS-CoV-2 and host immune response.

Keywords: SARS; Covid-19; vitamins; therapy

Introduction

In December 2019 a novel human-infecting coronavirus, named SARS-CoV-2 has emerged as a very serious public health concern, causing a pneumonia epidemic outbreak in Wuhan, Hubei Province in China with different degree of severity [(1)]. This pathological condition has been defined as "coronavirus disease 2019" (abbreviated "COVID-19") and the most common clinical presentation in infected subjects is represented by flu-like symptoms in 80% of cases. About 10-15% of infected subjects develop a more serious respiratory form. It is characterized by an interstitial pneumonia with chest discomfort, severe dyspnoea, high fever and dry cough potentially evolving into acute respiratory failure with a severe respiratory distress syndrome in about 10% of infected subjects. The mortality rate is about 7% of affected patients [(2)]. However, patients may also present less common symptoms, like diarrhea, headache, myalgia or arthralgia, chills, nausea or vomiting, nasal congestion and conjunctival congestion (0.8%) [(3)]. The epidemic has been declared a "public health emergency of international concern" by the International Health Regulations Emergency Committee of the World Health Organization [(4)]. A dramatic situation is developing in Italy with a progressively increasing number of infected subjects, mainly rather old individuals. According to current data, about 15% of patients with CoV-2 infection develop severe forms of pneumonia, radiological signs of interstitial involvment at the computerized axial tomography. These subjects require intensive care and they are at high risk of death. The need for intensive care beds also is progressively increasing and this condition might lead to the collapse of the Italian Health System in a very short time (data from Ministero della Salute Italiano). Unfortunately, to date neither a vaccine nor specific proved effective treatments against this virus are available worldwide. Therefore, new therapeutic strategies are strongly required to efficaciously counteract CoV-2 as soon as possible and to establish effective antiviral approach. Unfortunately, it must be considered that this virus has been isolated only recently and a few articles describing its structure and genome organization have been published. Furthermore, no studies concerning immune response against SARS-CoV-2 and the alterations induced by this pathogen in cell structure and physiology have not been studied and are not well-known to date [(5)].

Immunopathogenesis of SARS-CoV-2 infection

In the last weeks bioinformatics analysis has been carried out on a virus genome from a patient with 2019-nCoV infection to compare it with other related coronavirus genomes [(6)]. According to the results, the genome of 2019-nCoV (now known as CoV-2) presents around 89% nucleotide identity with bat SARS-like-CoVZXC21 and about 82% with that of human SARS-CoV. A wide range of viruses and host factors mutually modulate their interaction, influence the antiviral immune response and contribute to determine the pathogenesis of SARS-CoV-2 [(7)]. Therefore, on the basis of the

assumption that the SARS-CoV-2 is genetically related to SARS-CoV, but that its characteristics, such as the modality of transmission, the route of infection, the organ localization, the type of the immune response it may stimulate, the morbidity and the mortality rates are still poor-known, we have performed a literature search to identify the reports assessing these elements in patients with SARS-CoV-induced infection, a better-defined pathologic condition since several years ago. This disease resembles CoV-2 mediated disease and it may help to understand COVID 19. According to these studies, it is conceivable that either a weak or an inappropriate host immune response against the virus may cause the either the severe distress respiratory syndrome or lead to an unfavourable outcome.

The aim of this paper is to examine the possible aspects of the complex loop which can develop between host and SARS-CoV-2 in brief as well as the factors and mechanisms involved in this intricate process as well as the possible immunoregulatory role of some compounds in this life-threatening condition. According to a schematic representation, some distinct phases may be recognized during the clinical course of SARS. In the first one a robust virus replication is detectable in these patients and it is often characterized by the appearance of fever, sore throat and non-productive cough. These symptoms generally subside in a few days with illness resolution. Nevertheless, in some individuals a second clinical phase develops. It is characterized by elevated fever, hypoxemia and progression to pneumonia. This step is associated with an exuberant host inflammatory response and with the sharp and vigorous decrease in virus titers [8]. Following this phase, about 20% of patients develop ARDS with a possible fatal outcome. Lung specimens obtained from patients who have died because of SARS show several histologic tissue modifications. In particular, the most frequent alterations are represented by: extensive cellular infiltrates in the interstitium and alveoli, diffuse alveolar damage (DAD) with alveolar hemorrage/edema, hyaline membrane formation, fibrin exudation, epithelial necrosis with thickening of alveolar septa in the earlier phases and the progression to fibrosis in septa and alveoli in later stages. In particular, DAD represent a critical and prominent histological feature detectable in the lungs from individuals, who have developed a fatal SARS-CoV-induced infection [9]. Furthermore, SARS-CoV genome and antigens have been observed in airway and alveolar epithelial cells, vascular endothelial cells, neutrophils, macrophages, monocytes and lymphocytes in samples from humans as well as from animal models [9; 10].

SARS-CoV2 genome organization and viral proteins

Viral particles consist of a genomic RNA 5’-capped and 3’-polyadenylated encoding four viral structural proteins: the spike (S) glycoprotein, the matrix (M) protein, the small envelope (E) protein

and the nucleocapsid (N) protein. It also includes multiple open reading frames (ORFs), codifying accessory proteins interposed among the structural genes (**Figure 1**) [11]. The viral envelope is composed of S, M, and E proteins, whereas N protein is detectable in the core of the viral particle, it interacts with the CoV RNA. It is involved in the transcription of viral RNA and, in association with E protein, it orchestrates the viral nucleocapsid assembly and in the generation of the mature viral envelopes. The S protein modulates the process of viral entry into host cells [12].

Defective and dysfunctional immune response in patients with Cov-2 related infection

A comprehensive theory of the pathogenesis for CoV-2 infectious disease is still lacking, but it has been proposed for SARS-CoV in the past [9] and some preliminary studies about CoV-2 have been published or are in progess [13].

Therefore, taking into account all available data in SARS-CoV infection and considering SARS-CoV-2 as a virus with similar characteristics and immunopathogenic effects to SARS-Co-V, it may be hypothesized that the deleterious events in patients with the most severe forms of the COVID-19 are the results of an inappropriate and inadequate immune response virus [9; 14; 15]. According to Gu's hypothesis, the SARS-CoV infects the human body through the respiratory tract, entering the epithelial cells of the trachea, bronchi, bronchioles, and lungs [9] (**Figure 4**). In this context, the virus colonizes also resident, infiltrating, and circulating immune cells. Then, the virus disseminates to all human organs, being carried by the infected circulating immune cells and spread to different types of cells in other organs. The immune cells of the spleen, peripheral and central lymph nodes, other lymphoid tissues are colonized and damaged by the virus. Furthermore, the mucosa of the intestine, the epithelium of the renal distal tubules, the neurons of the brain and macrophages in different organs are also involved. According to this hypothesis, it may be assumed that infected circulating immune cells spread to the mucosa-associated lymphoid tissue (MALT), Bronchus-associated lymphoid tissue (BALT) and Nasopharynxassociated lymphoid tissue (NALT). No data are available, concerning the possible virus-mediated alterations in the function of these lymphoid compartments in patients with CoV-2 infection. The immune defense is significantly impaired and infected patients may develop a pneumonia with different degrees of severity and experiment a rapid deterioration of clinical conditions. In particular, aged-subjects with chronic diseases have often a compromised immune function, generally develop more severe clinical pictures and present a more elevated mortality in comparison with healthy subjects [16]. According to Gu's study, the severity of the immune cell damage more than the extent of the lesions detectable in the lungs suggests the patient's immune status and his lymphocyte count probably represents the main predictor of his clinical evolution. Viral load also may exert a crucial impact on the strength and efficacy of the patient's immune response

[16]. During the course of SARS-CoV and CoV2 diseases, a activation of the immune response progressively develops, leading to a self-maintaining and self-increasing inflammatory state. High serum levels of pro-inflammatory cytokines (IFN-γ, IL-1, IL-6, IL-12, and TGFβ) [17; 18] and chemokines (CCL2, CXCL10, CXCL9, and IL-8) have been detected in SARS patients, who develop the most severe clinical forms of disease in comparison with subjects with a milder illness [19; 20; 21]. Furthermore, a strong pro-inflammatory Th1 and Th17 response has been observed in patients with MERS-CoV infection, with increased concentrations of IFN-γ, TNF-α, IL-15 and IL-17 [22]. In humans, Th17 cells can be induced by IL-6 and IL-1β [23]**.** Experimental research in *in vitro* models of cultured cells has examined the pattern of CoV proteins and has identified the potential pro-inflammatory role of some among them in the pathogenesis of SARS. In particular, nucleocapsid (N) and spike (S) CoV proteins possess direct binding sites on several specific DNA sequences, localized in the promoter region of a wide series of interleukins and cytokines [24; 25].

It may be hypothesized that CoV-2 induced disease with severe clinical courses and with a fatal outcome is characterized by a massive release of a wide spectrum of cytokines, leading to the "Cytokine Release Syndrome" [26]. A more detailed discussion of this topic is beyond the scope of this work and it will be the subject of a further paper. Therefore, on the basis of these concepts and observations, a proper modulation or control of the exuberant inflammatory response, developing in the course of SARS-CoV-2 infection might be a key strategy for the treatment of the patients with severe forms of CoV-2 infections and, probably, it might also prevent the evolution of the illness towards an unfavourable outcome.

Factors involved in the inflammatory immune response in patients with SARS-CoV-2

Multiple factors may contribute to explain the exuberant inflammatory response, detectable in this severe disease and should be considered in the strategy of treatment. Overall, these elements may contribute to determine the differences in clinical course and severity of illness in patients with COVID-19. The following points should be considered:

i) Rapididy of viral replication and load of viral proteins, mainly proteins causing the release of IL-1, IL-6, IL-8 and TNF-α;

ii) Anatomical human compartment or organ predominantly infected by the virus;

iii) Cytochine storm and antiviral impaired immune response.

Possible role of some drugs and liposoluble compounds (such as liposoluble vitamins) in modulating directly or indirectly the replication ability of SARS-CoV-2 and host immune response.

On the basis of all these immunopathogenic and clinical observations and considerations, a potential useful therapeutic rescue strategy for the treatment of patients affected by severe forms of SARS-CoV-2 infection could include:

i) antiviral therapy with the current available drugs, which have been demonstrated to be effective in reducing or in inhibiting replication of RNA-viruses (HCV, HIV and Ebola virus) in previous trials or of CoV-2 itself in very preliminary reports and anedoctical cases. This therapy should be administered as soon as possible to counteract CoV-2 replication with the main purpose to decrease the synthesis and the release of some crucial viral proteins (nucleocapsid and spike proteins) detectable in the cytoplasm and in the nucleus of the infected cells. The inhibition in the synthesis of these proteins should promote the decrease of their amounts and remove the persisting stimulus, which induce the transcription and the translation of the pro-inflammatory cytokines. This strategy may prevent the persistence of the self-maintaining and self-stimulating pro-inflammatory loop in the body tissues of infected individuals, mainly in the lung, associated with the release of the pro-inflammatory cytokines. The result of this therapy is the inhibition of the so called "cytokine storm" and the block of its related-deleterious effects **(Figures 2 and 3)**. To date, some drugs have demonstrated potential efficacy in the treatment of CoV-2 infected individuals, including: a) approved nucleoside analogues (favipiravir and ribavirin) and experimental nucleoside analogues (remdesivir and galidesivir) able to inhibit the RNA-dependent RNA polymerase and to block viral RNA synthesis in a broad spectrum of RNA viruses, including human coronaviruses [(27)]; b) Approved protease inhibitors including disulfiram, lopinavir, indinavir, saquinavir, ritonavir, atazanavir and darunavir have been shown to have activity against SARS-CoV-2 [(28)].

ii) Immunomodulatory therapy, including: a) monoclonal antibodies against IL-6 (as suggested in preliminary reports) and eventually against IL-1 and/or IL-8 as well as against cyclo-oxigenase inhibitors, like aspirin or FANS with the purpose to stop or to prevent the strong inflammatory response and the release of further cytokines and mediators of inflammation.

Very preliminary observation suggests that the block of IL-6 pathway cascade, may have a beneficial effect in patients with severe forms of SARS. Tocilizumab is a humanized anti-IL-6 receptor subunit alpha (anti-IL-6R) monoclonal antibody approved in numerous countries throughout the world, for the treatment of Rheumatoid Arthritis (RA), with moderate to severe active rheumatoid arthritis, refractory to methotrexate ory to methotrexate [(29)]. In patients with RA, the inhibition of IL-6 leads to Th1 and Th17 suppression and Th2 expansion via activation of T-regulatory cells [(30; 31)].

It is conceivable that the observed improvement in clinical conditions of patients suffering from severe forms of CoV-2 infections depends on the attenuation of the Cytokine Release Syndrome (CRS). Well-designed clinical trials are need in a very short time to test the efficacy and the safety of

this potentially very promising therapeutic approach (unpublished observations). No data are available on the possible efficacy and safety of Acetylsalicylic acid in the as well as the duration for an effective treatment. To date, the use of aspirin as an option for the treatment of acute respiratory distress syndrome, with the purpose to inhibit COX-2 activity has ben proposed [32]. Inhibition of COX-2 might attenuate the CRS, but only one experimental study in animals has tested a possible role of aspirin in acute lung injury. Aspirin has been reported to protect mice in a two-event model of transfusion-related acute lung injury [33]. The lack of studies on this topic makes it difficult to hypothesize a role of aspirin in the treatment of these patients and requires further studies.

iii) Other possible, but, to date, not tested anti-SARS-CoV-2 compounds with potential usefulness against virus or against its related complications may be represented by some liposoluble vitamins. Therapeutic regimens with fatsoluble vitamins administrations (such as A, D and E) are based on their immunoregulatory activity and on their ability to exert a protective role for the maintenance of a proper functioning of the immune response. The rationale for the use of these compounds with the purpose to treat CoV-2 infection deserves a conceptual explanation. Fatsoluble vitamins possess numerous cellular targets and can modulate a wide variety of cell activities at various levels [34]. In this paper we will consider in brief the regulatory activities of fatsoluble vitamins on the immune system functions and on the inflammatory response. These compounds possess pleiotropic effects and may exert a systemic direct antiviral- or immunomodulatory-effects.

The following points have to be considered:

i) A large series of clinical studies has shown that the serum concentrations of vitamin A, E and D are decreased in patients with some chronic viral infections, like HBV and HCV, in comparison to uninfected individuals as well as in aged patients [35].

ii) Vitamin D, E and A deficiency is associated with higher levels of viral replication as well as with higher titres of inflammatory cytokines, like IL-6 and TNF-α [36; 37; 38].

Vitamin E has been shown in several trials to enhance the immune response and resistance to infections [39]. All-trans retinoic acid (ATRA) is an active metabolite of vitamin A and it has been shown to modulate immunity. It induces the differentiation of $CD4^+$ T cells into Treg cells but inhibits the differentiation of Th17 cells, thereby it contribute to the maintainance of the Th17/Treg cell balance [40].

Some vitamins, like vitamin E, D and A have been used in clinical trials for the treatment of patients with persistent viral infections [41; 42; 43; 44; 45; 46]. Possible antiviral role of vitamin E has been already suggested several years ago in clinical trials, involving a small samples of children [47; 48] and adult patients [49], suffering from HBeAg-positive and HBeAg-positive/negative chronic hepatitis with very interesting and promising results. The possible rationale of vitamin E use in these patients and the

potential targets of direct or indirect- antiviral- effects mediated by vitamin E have been widely discussed in a previous systematic review [43].

iii) Fatsoluble vitamins possess well-known multiple nuclear and cytoplasmic targets in all the different types of mammalian cells and they may modulate and regulate an elevated number of intra- and extracellular- pathways via a direct binding to regulatory regions in a large series of genes critical for the maintenance of cell homeostasis, via modulation of a wide series of cell functions [43; 50].

On the basis of these brief revision of cell targets for vitamin A, D and E and the availoable clinical trials about the potential beneficial role of these compounds in the modulation of immune response leading to the improvement of antiviral response derives the conceptual rationale for the inclusion of vitamin A, D and E in a possible multitherapeutic protocol for the treatment of patients with CoV-2 related infection.

These vitamins may contribute to improve normal immune response, by restoring the normal immune system activity, mainly by counteracting Th1/Th2/Th17 umbalance and modulating the amounts and the ratio among the pro-inflammatory and anti-inflammatory cytokines. As reported in the studies vitamin D also is able both alone and in association with Tocilizumab to block IL-6 and to promote the generation of $Foxp3^+$ T cells and to counteract IL-17 production. These cells modulate the immune response and contribute to turn off the production of proinflammarory cytokines. Furthermore, vitamin E also is able to prevent IL-6 release. A very recent report has shown that SARS-CoV-2 viral load (RNAemia) in serum is closely associated with drastically elevated IL-6 level in patients with severe disease (data not published). The combined use of fatsoluble vitamins might exert an even more beneficial effect in elderly patients, who are chatacterized by an impairment of immune system function. These individuals are characterized by a very high mortality in Italy during this epidemic outbreak (unpublished data) [51; 52; 53].

Furthermore, these compounds present an additional anti-inflammatory activity mediated by the production of microRNA-122. These elements are short cell RNAs which exert a wide series of regulatory cell activities and modulate also antiviral immune response.

According Gu's hypothesis, the immune system dysfunction is the most important cause of clinical deterioration and possible unfavourable outcome in the individuals with CoV-disease. Therefore, the possible usefulness of immune system restoration mediated using these fatsoluble vitamins might represent a crucial strategy with the purpose to prevent or in the progressively inhibit the cytokine release syndrome. However, in their use with this indication fatsoluble vitamins A, D, E should be considered not only as physiological substances but also as real drugs with potential useful or dangerous effects. Unfortunately, to date no studies have assessed the blood concentration of these

liposoluble vitamins in patients with SARS-CoV-2 as well as it is unknown whether deficiency in that vitamin may be associated with a more severe course and outcome of this disease. Therefore, trials evaluating blood concentration of these compounds should be performed as soon as possible and the possible inclusion of fatsoluble vitamins in the treatment schedules of COVID 19 patients should be considered. However, the possible side effects of these compounds should be considered and the dosage of blood liposoluble vitamins should be provided. Based on all these pathogenic considerations, a possible protocol proposal for the treatment of patients with CoV-2 should consist of the following schedule already in the early phase of the disease:

i) Antiviral drugs to block viral replication and, mainly, the release of high amounts of viral proteins able to trigger a robust proinflammatory response;

ii) immunomodulatory compounds with the purpose of restoring the umbalanced and dysregulated immune system function, including fatsoluble vitamins in association with Tocilizumab.

The early administration of this drugs could prevent the development od CRS with the subsequent clinical deterioration and deaths as well as it could be decreased the need of intensive care beds.

In conclusion, in this paper we have provided a rapid excursus on available data about a very life-threatening disease worldwide, known as SARS-CoV 2, then we have examined the crucial mechanisms potentially involved in the development this severe illness. Since our research we have identified the possible viral and host cell targets and suggested a rationale for an early poly-therapeutic approach. Unfortunately, several problems are also evident, including: the dosage of antiviral drugs, of fatsoluble vitamins and Tociluzimab as well as the potential side effects of these treatment. Well-designed and well-sized protocols are needed to improve our knowledge in the immunopathogenesis of this complex disease, with the purpose to contribute to the control of thispublic health emergency.

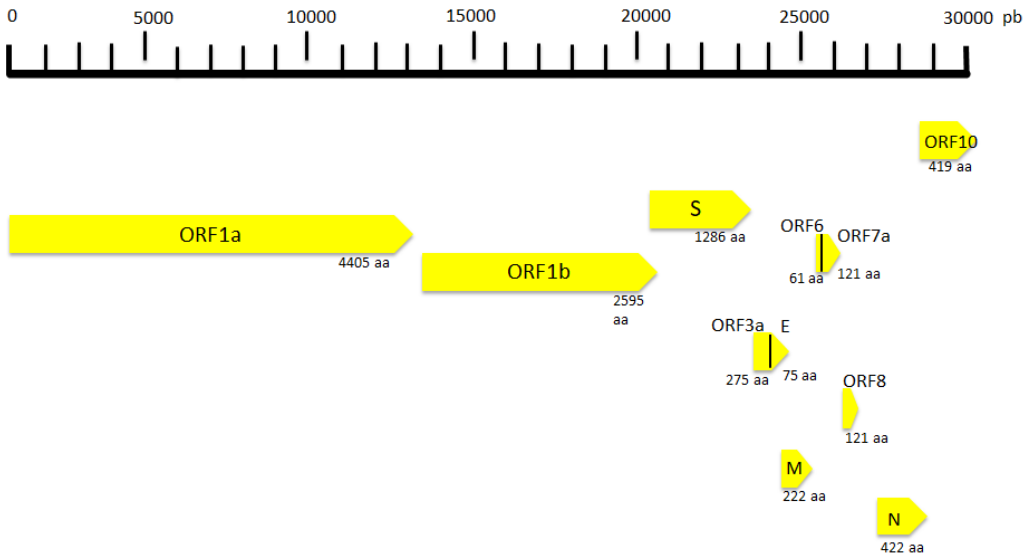

FIGURE LEGENDS

Figure 1. Coronavirus genoma and its structural and nonstructural proteins. ORF: Open Reading Frame; N: Nucleocapside Protein; S: Spike protein; M: Matrix Protein.

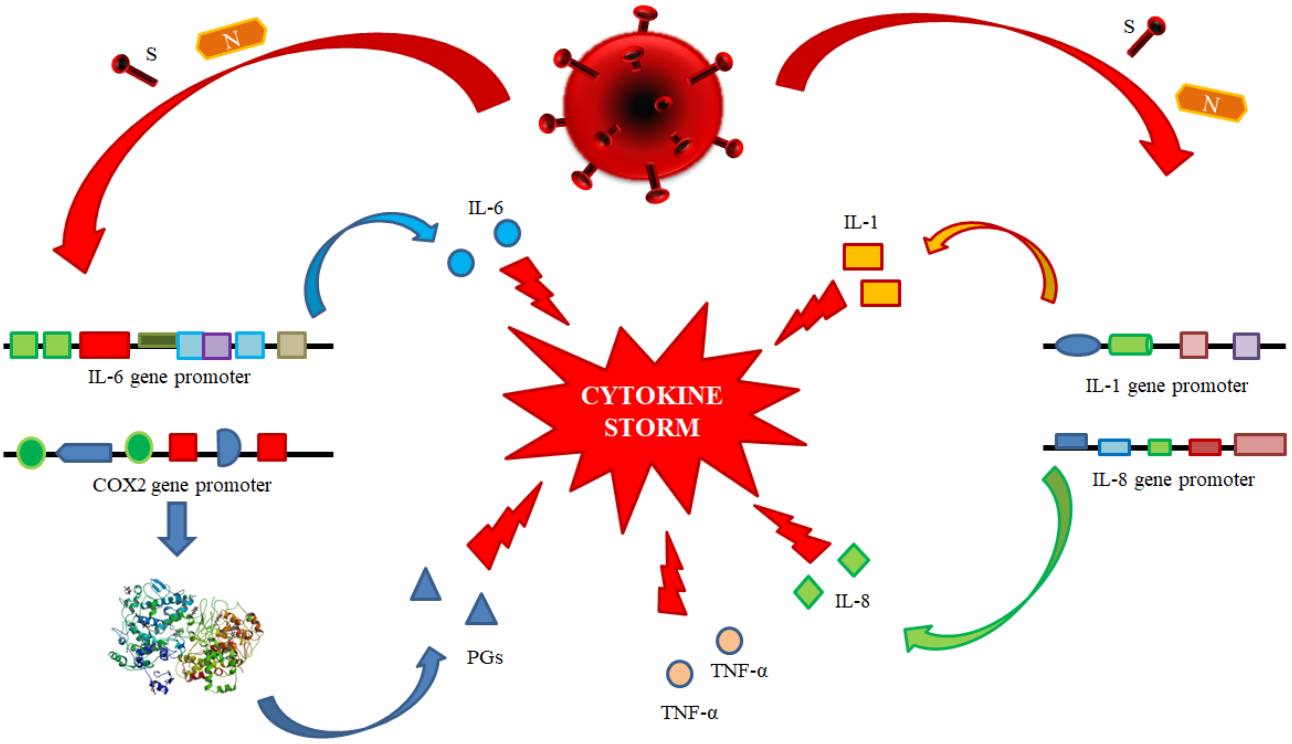

Figure 2. Pathogenetic mechanisms involved in the Cytokine Storm Syndrome. N and S viral proteins possess some target sequences on the DNA in the nucleus of human cells. In particular, some binding motifs are detectable in the promoter of some cell genes, encoding key cytokines or enzymes involved in inflammatory process, like IL-1, IL 6, IL 8, TNFα and COX-2. A high viral replication in infected cells may be associated with the production of elevated N and S protein amounts. A high viral replication in infected cells may be associated with the release of elevated N and S protein amounts. The binding to the promoters of the proinflammatory cytokines and enzymes may induce a hyper activation in the transduction and translation of these genes. As consequence, elevated amounts of proinflammatory cytokines are synthesized and secreted. The massive release of these mediators is associated with the development of the Cytokine Release Syndrome. Subjects with an immune system dysregulation (Aged individuals with chronic diseases and impaired immune system function are particularly at risk to develop this life-threatening condition).

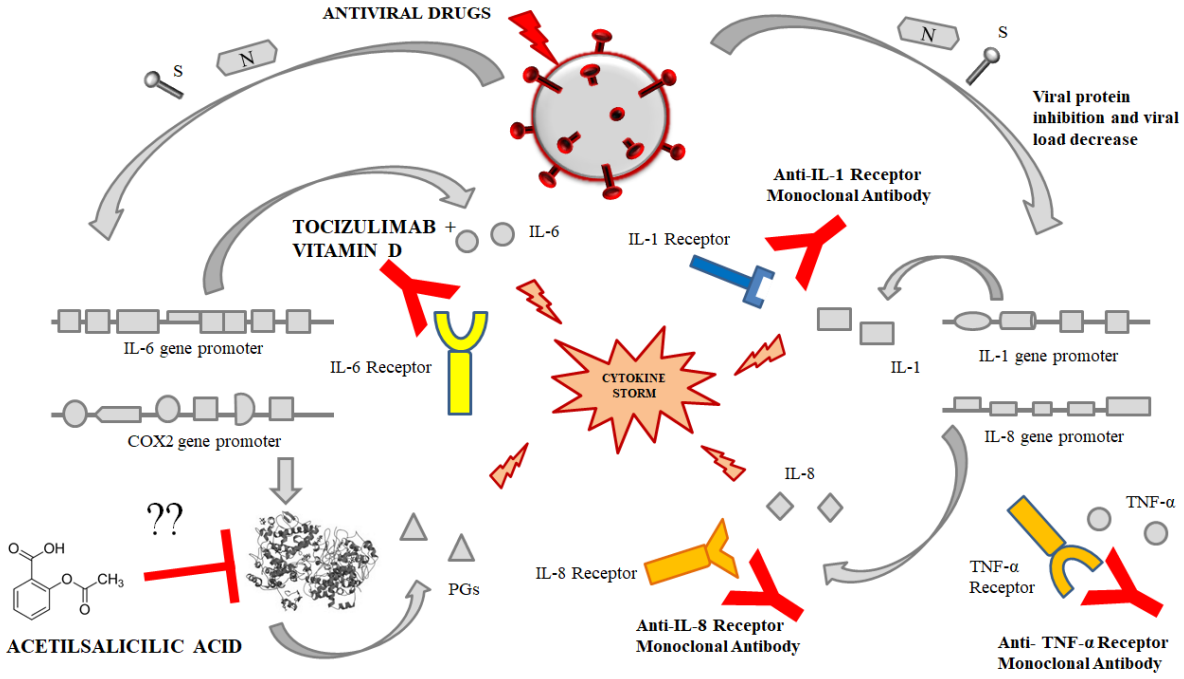

Figure 3. Possible or putative therapeutic targets. The figure shows the potentially useful for the prevention or treatment of CRS by means of aceetilsalicilic acid (although perplexity has been expressed about this treatment), monoclonal antibodies against the Receptors of some Interleukins

like IL-6, IL-1 alone or in cooperation with some liposoluble vitamins (mainly vitamin D). This figure provides the conceptual hypothesis that multiple therapeutic targets may be considered. to date there are no certainties on the efficacy of any therapies, alone or in combination, which may have some efficacy in the treatment of cytokine release syndrome in patients with CoV-2 infection.

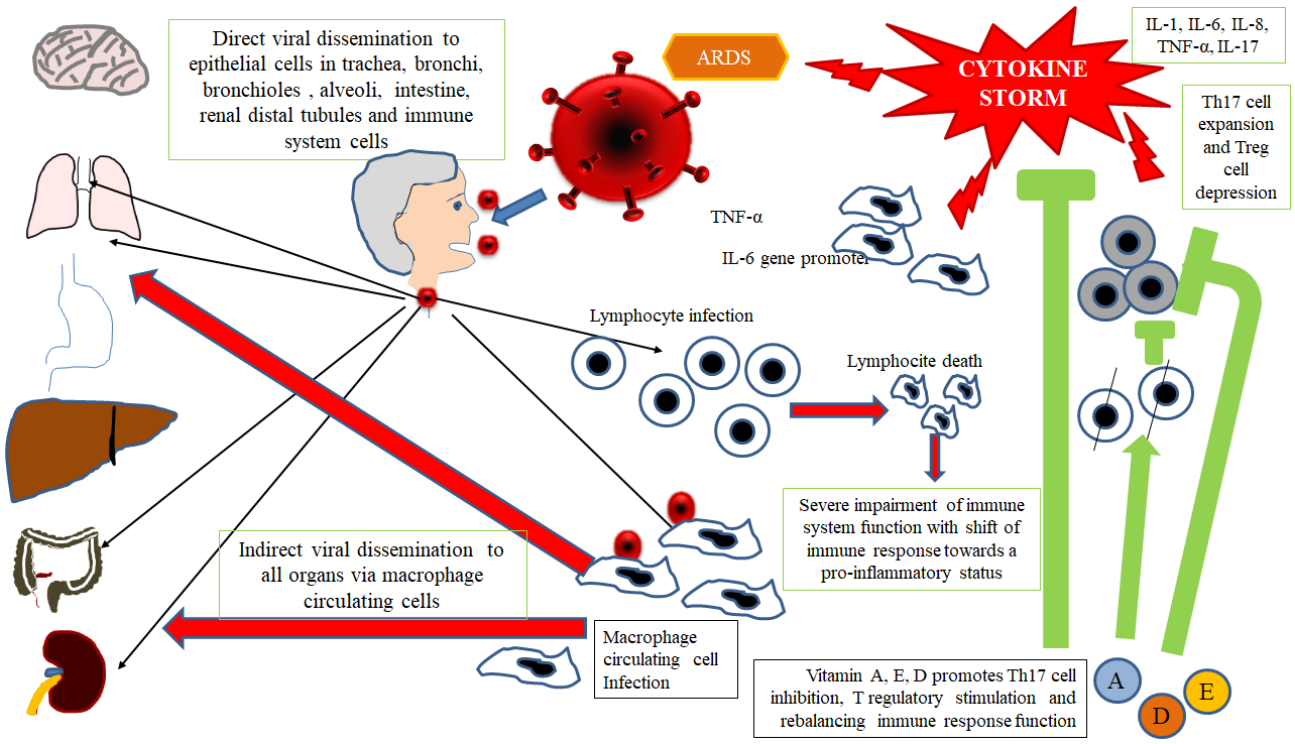

Figure 4. Gu's hypothesis, concerning SARS-CoV infection. A similar scheme may be considered with the purpose to explain the pathogenesis of SARS-CoV-2. The SARS-CoV infects the human body through the respiratory tract, entering the epithelial cells of the trachea, bronchi, bronchioles, and lungs. In this context, the virus colonizes also resident, infiltrating, and circulating immune cells. Then, the virus disseminates to all human organs, being carried by the infected circulating immune cells and spread to different types of cells in other organs. The immune cells of the spleen, peripheral and central lymph nodes, other lymphoid tissues are colonized and damaged by the virus. Furthermore, the mucosa of the intestine, the epithelium of the renal distal tubules, the neurons of the brain and macrophages in different organs are also involved. According to this hypothesis, it may be assumed that infected circulating immune cells spread to the mucosa-associated lymphoid tissue (MALT) and Bronchus-associated lymphoid tissue (BALT) The immune defense is significantly

impaired and infected patients may develop a pneumonia with different degrees of severity and experiment a rapid deterioration of clinical conditions. Aged subjects with chronic diseases have often a compromised immune function, generally develop more severe clinical pictures and present a more elevated mortality in comparison with healthy subjects. the severity of the immune cell damage more than the extent of the lesions detectable in the lungs suggests the patient's immune status and his lymphocyte count probably represents the main predictor of his clinical evolution. Viral load also may exert a crucial impact on the strength and efficacy of the patient’s immune response. The possible action of Fatsoluble vitamins in improving immune response activity is indicated.

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