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The Potential Impact of Zinc Supplementation on COVID-19 Pathogenesis

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During the current corona pandemic, new therapeutic options against this viral disease are urgently desired. Due to the rapid spread and immense number of affected individuals worldwide, cost-effective, globally available, and safe options with minimal side effects and simple application are extremely warranted. This review will therefore discuss the potential of zinc as preventive and therapeutic agent alone or in combination with other strategies, as zinc meets all the above described criteria. While a variety of data on the association of the individual zinc status with viral and respiratory tract infections are available, study evidence regarding COVID-19 is so far missing but can be assumed as was indicated by others and is detailed in this perspective, focusing on re-balancing of the immune response by zinc supplementation. Especially, the role of zinc in viral-induced vascular complications has barely been discussed, so far. Interestingly, most of the risk groups described for COVID-19 are at the same time groups that were associated with zinc deficiency. As zinc is essential to preserve natural tissue barriers such as the respiratory epithelium, preventing pathogen entry, for a balanced function of the immune system and the redox system, zinc deficiency can probably be added to the factors predisposing individuals to infection and detrimental progression of COVID-19. Finally, due to its direct antiviral properties, it can be assumed that zinc administration is beneficial for most of the population, especially those with suboptimal zinc status.

Keywords: zinc, COVID-19, SARS-CoV2, 2019-nCoV, coronaviridae, zinc deficiency, impaired immune system

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INTRODUCTION

The importance of the trace element zinc for the development and function of the immune system across all kinds of species has been proven in numerous studies (1–3). As zinc deficiency results in altered numbers and dysfunction of all immune cells, subjects with suboptimal zinc state have an increased risk for infectious diseases, autoimmune disorders, and cancer (3–6). In addition to malnutrition, risk groups for zinc deficiency include the elderly and patients with various inflammatory and autoimmune diseases, which will be discussed in detail later in the article (7, 8). As mild zinc deficiency is largely sub-clinical, it is unnoticed in most people. However, the World Health Organization (WHO) assumes that at least one third of the world population is affected by zinc deficiency (9). The fact that zinc deficiency is responsible for 16% of all deep respiratory infections world-wide (9) provides a first strong hint on a link of zinc deficiency with the risk of infection and severe progression of COVID-19 and suggests potential benefits of zinc supplementation.

The most common symptoms of COVID-19 are impaired smell and taste, fever, cough, sore throat, general weakness, pain as aching limbs, runny nose, and in some cases diarrhea (10). In the subsequent chapters, we will associate most of those symptoms with altered zinc homeostasis and explain how zinc might prevent or attenuate those symptoms, as summarized in **Figure 1**, and thus should be regarded as promising cost-effective, globally available therapeutic approach for COVID-19 patients, for which minimal to no side effects are known.

ZINC PROTECTS THE HUMAN BODY FROM ENTERING OF THE VIRUS

The entry of infectious agents into the human body is prevented by tissue barriers equipped with cilia and mucus, anti-microbial peptides like lysozymes and interferons. Regarding SARS-CoV2, the angiotensin-converting enzyme 2 (ACE2) and the cellular protease TMPRSS2 are the major mechanism for entering the cells (11).

a) Mucociliar clearance of viruses is affected by zinc
   Infections with coronaviruses go along with damage of the ciliated epithelium and ciliary dyskinesia consecutively impairing the mucociliar clearance (12). It was shown that physiological concentrations of zinc increase ciliary beat frequency (13). Moreover, zinc supplementation in zinc deficient rats had a positive effect on the number and the length of bronchial cilia (14) (**Figure 1.4**). Improved ciliary clearance does not only improve the removal of virus particle, it also reduces the risk of secondary bacterial infections, as discussed later. Alterations of the extracellular matrix, as monitored by increased epidermal growth factor and proliferating cell nuclear antigen (PCNA) immunostaining of rat lungs during zinc deficiency have also been described (15).
b) Zinc is essential for preserving tissue barriers
   Disturbances in the integrity of the respiratory epithelia facilitate the entry of the virus as well as co-infecting pathogens and can lead to pathogens entering the blood stream. An *ex-vivo* model of the chronic obstructive pulmonary disease (COPD) showed that decreasing zinc levels raised the leakage of the epithelium of the respiratory tract (16), while zinc supplementation improved lung integrity in a murine model of acute lung injury *in vivo* (17). Increased apoptosis and E-cadherin/beta-catenin proteolysis were amongst the underlying mechanisms (17–19). The expression of tight junction proteins like Claudin-1 and ZO-1 was found to be zinc-dependent, offering another explanation for zinc's positive effects on lung integrity (16). In addition, an inhibitory effect of zinc on LFA-1/ICAM-1 interaction weakened inflammation in the respiratory tract via reduction of leukocyte recruitment (20). Furthermore, high zinc levels improved the tolerance of the lung towards damage induced by mechanical ventilation (21) (**Figure 1.4**).
c) Zinc-dependent alterations in gene expression by pneumocytes could affect viral entering
   ACE-2, mainly expressed on pneumocytes type 2, is a zinc-metalloenzyme. Zinc binds to its active center and is thus essential for its enzymatic activity. If zinc binding also affects the molecular structure of ACE-2 and thereby its binding affinity to the virus, remains to be tested (22, 23). However, this is likely as zinc is important for stabilizing protein structures and altering substrate affinity of various metalloproteins (24, 25). Finally, zinc homeostasis might affect ACE-2 expression, as zinc-dependent expression was reported for other zinc-metalloenzyme such as metallothionein and matrix metalloproteinases (26). This suggestion is strengthened by the finding that ACE-2 expression is regulated by Sirt-1 (27, 28). As zinc decreases Sirt-1 activity (27), it might decrease ACE-2 expression and thus viral entry into the cell (**Figure 1.2**).

A lack of adequate secretion of type I and type II interferons was reported for COVID-19 patients (29). For human interferon alpha (IFN-α) it was shown that zinc supplementation can reconstitute its expression by leukocytes and potentiates its anti-viral effect via JAK/STAT1 signaling as observed for rhinovirus-infected cells (30, 31). However, as it was suggested that SARS-CoV2 might take advantage of the interferon-dependent expression of ACE2, which was recently addressed by Ziegler et al. (32), the overall effects of zinc need to be carefully evaluated in future studies.

ZINC DIRECTLY INHIBITS VIRAL REPLICATION

As a virus, SARS-CoV2 is highly dependent on the metabolism of the host cell. Direct antiviral effects of zinc have been demonstrated in various cases, which was reviewed in great detail (33–37). Examples include coronaviridae, picornavirus, papilloma virus, metapneumovirus, rhinovirus, herpes simplex virus, varicella-zoster virus, respiratory syncytial virus, human immunodeficiency virus (HIV), and the hepatitis C virus (34, 35, 37–39). It was suggested that zinc can prevent fusion with the host membrane, decreases the viral polymerase function, impairs protein translation and processing, blocks viral particle release, and destabilizes the viral envelope (35, 37, 40). Low-dose zinc supplementation together with small concentrations of the zinc ionophores pyrithione or hinokitol decreased RNA synthesis in influenza, poliovirus, picornavirus, the equine arteritis virus, and SARS-CoV by directly inhibiting the RNA-dependent RNA polymerase of the virus (34, 41). There is evidence that zinc can enhance the effect of chloroquine, another known zinc ionophore, while zinc ionophores like epigallocatechin-gallate or quercetin remain to be tested (42–45). There are close similarities of SARS-CoV2 and other coronaviridae like SARS-CoV and Middle East respiratory syndrome-related coronavirus (MERS-CoV) (46). Also, the alcohol-aversive drug disulfiram can bind the papain-like proteases of SARS-CoV and MERS-CoV resulting in release of cysteine-bound zinc that results in protein destabilization (47). Detailed studies on zinc's effect specifically on SARS-CoV2 are highly required (**Figure 1.3**).

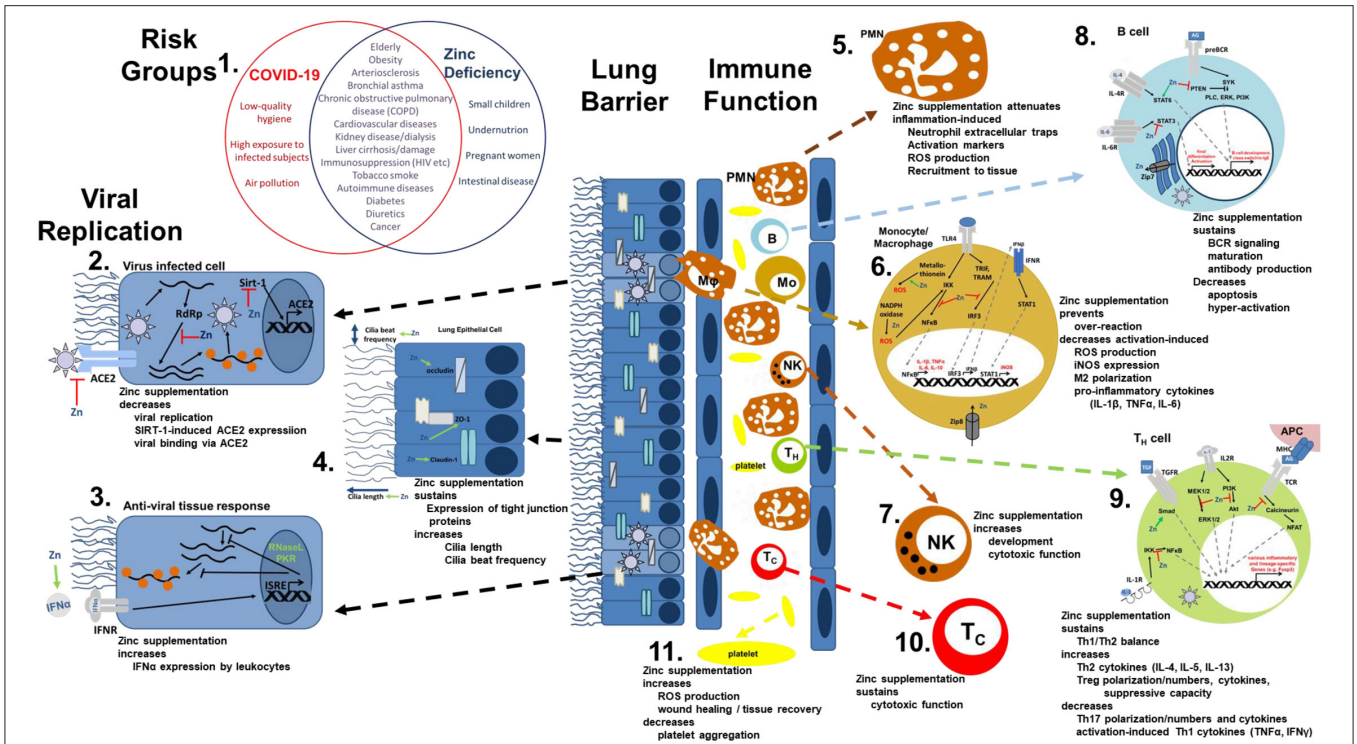

FIGURE 1 | Viral mechanism of COVID-19 and how they might be opposed by zinc data. **(1)** There is an impressive intersection of known risk factors for zinc deficiency (blue circle) and the predisposition for a severe COVID-19 infection (red circle). **(2,3)** Zinc (Zn) supplementation might already prevent the viral entry and also suppresses its replication, while it supports the anti-viral response of the host cells. **(4)** As zinc is known to increase ciliary length and movements and also sustains tissue integrity, entrance of the virus is impeded. **(5–10)** The importance of zinc on the development and function of the immune cells is manifold. It should be underlined, that zinc's effects should not generally be described as activating or inhibiting, as zinc in various cases normalizes overshooting immune reactions and balances the ratios of the various immune cell types. Zinc thus prevents for example that high levels of inflammatory mediators including reactive oxygen and nitrogen species destroy the host tissue. **(11)** On first view it appears contradicting, that zinc increases activation induced production of reactive oxygen species in platelets, while it is generally considered as anti-oxidative. However, in case of platelets, up to a certain threshold, ROS production is essential, as it can prevent the formation of platelet aggregates. In summary, zinc therefore might be able to prevent vascular complications observed in COVID-19 patients. Details for each point can be found in the text. ACE2, angiotensin converting enzyme 2; AG, antigen; IFN, interferon; IFNR, interferon receptor; ISRE, interferon-sensitive response element; APC, antigen presenting cell; IKK, IκB kinase; IL, interleukin; iNOS, inducible nitric oxide synthase; IRF3, IFN regulatory factor 3; MHC, major histocompatibility complex; MEK1/2, mitogen-activated protein kinase kinase 1/2; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor kappa B; PKR, protein kinase R; Akt, protein kinase B; PI3K, phosphatidylinositol-3 kinases; ROS, reactive oxygen species; RdRP, RNA-dependent RNA polymerase; RNase L, ribonuclease L; Sirt-, Sirtuin 1; STAT, signal transducer and activators of transcription; TCR, T cell receptor; Tc, cytotoxic T cell; TH, helper T cell; TGF, transforming growth factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β; TLR, toll-like receptor; TNF, tumor necrosis factor; Zip, Zrt- and Irt-like protein; ZO-1, zona occludens.

ZINC BALANCES THE IMMUNE RESPONSE DURING INFECTIOUS DISEASES

One of the hallmarks of COVID-19 is an imbalanced immune response (48). Due to hyper-inflammation, immune products including pro-inflammatory cytokines like interleukin (IL)-6, C-reactive protein (CRP), tumor necrosis factor (TNF)α and IL-1β (summarized as cytokine storm or cytokine release syndrome), reactive oxygen, and nitrogen species in connection with the recruitment of high numbers of strongly activated immune cells to the lungs, the destruction of the tissue, permanent lung damage and death due to systemic inflammation, and organ failure are expected, while the anti-inflammatory response is insufficient (48–52). A high number of patients develop an acute respiratory distress syndrome (ARDS) accompanied by high alveolar leakage leading to alveolar and interstitial edema with severely limited oxygen exchange (53). Advanced SARS-CoV2 infections are characterized by a systemic involvement with organ complications and accompanying cytokine storm (52, 54).

There is no doubt on the anti-inflammatory and anti-oxidative properties of zinc and underlying mechanisms have been the focus of numerous studies (1–3, 6, 55–60). A detailed description of zinc metabolism in airway epithelium and during inflammation of the airways has been published by Zalewski et al. (61). On the other hand, zinc deficiency was associated with elevated levels of pro-inflammatory mediators, increased reactive oxygen species (ROS) levels and pre-disposing for severe progression of inflammatory diseases, especially those affecting the lung, often reversible by zinc supplementation (6, 17, 56, 62–66) (**Figures 1.5,1.6**). As one example, exposure to organic dust increased lung damage, inflammation and

macrophage hyper-activation in animals with zinc deficiency, predisposing these animals to pulmonary fibrosis, while zinc supplementation 24 h before induction of acute lung injury significantly attenuated the inflammatory reaction and tissue damage (17, 67). Regarding systemic inflammatory diseases the number of studies showing benefits of especially preventive zinc supplementation is constantly increasing (17, 18, 58, 65, 68). Amongst the underlying mechanism, zinc's role as second messenger and importance in regulating intracellular signaling as detailed in **Figure 1** were described as well as zinc's effects on the epigenome (56, 57, 69–74).

Furthermore, leukocytosis with neutrophilia and lymphopenia, especially affecting $CD8^+$ T cells, were associated with poor prognosis of COVID-19 and the recovery of lymphocyte counts lead to clinical recovery (75, 76). Similar changes in lymphopoiesis and myelopoiesis have been described in zinc deficient rodents, which were normalized when zinc was supplemented (17, 19). Circulating and lung-resident T cells from COVID-19 patients showed increased expression of markers for T cell exhaustion like Tim-3 and PD-1 (77). The extent of these changes had an impact on the patient's prognosis (50). During the past decades, an immense literature was generated on the need of zinc for lymphocyte development and function and that zinc supplementation (6, 19, 63, 64, 78, 79) can reverse lymphopenia. Enumerating all findings and underlying mechanisms is beyond the scope of this article, and a lot of aspects have been discussed in related publications (36) but as one of the many key roles of zinc in the context of T cell function, zinc is indispensable in the signal cascade of the T cell receptor and IL-2 as a second messenger (78, 80) (**Figure 1.9**). The B cell compartment also strongly benefits from a balanced zinc homeostasis, as zinc is required for B cell maturation and function (72, 81) (**Figure 1.8**). Also important to mention, but neglected by previous related articles, is that there is evidence (82, 83) that SARS-CoV2 can directly infect T cells as well as B cells and impair their cell specific function. This could explain the impact of SARS-CoV2 infection on lymphoid tissues like the human spleen and lymph nodes (84). However, as data are limited to *in vitro* experiments, this needs to be verified *in vivo* as well as if zinc affects the virus-induced changes in T and B cells.

Additionally, granulocytes play a vital role during the inflammation-induced destruction of the lung (85). Recent evidence suggests that lipopolysaccharide-induced hyper-activation, recruitment and formation of neutrophil extracellular traps are attenuated by zinc supplementation *in vivo* and that cytokine expression, phagocytosis and burst, chemotaxis and degranulation, and intracellular signaling are zinc regulated (17, 86, 87) (**Figure 1.5**). Important defense mechanisms of the innate immunity include the toll like receptors. For instance, *in silico* data suggest that toll-like receptor (TLR)-4 can potentially recognize outer components of SARS-CoV2's like the viral spikes (88), while intracellular receptors including TLR3, TLR7/8, and TLR9 can recognize viral dsRNA, ssRNA, and unmethylated CpG DNA respectively (89–92). Intranasal pretreatment with a TLR3 agonist and, to a lesser extent, with TLR9, TLR7/8, or TLR4 agonists, provided a high level of protection against infections by SARS coronavirus and influence virus in mice, suggesting that TLR signaling can induce protective antiviral immunity (93). This might be a completely novel approach to consider regarding COVID-19 as well. Zinc is an essential regulator in TLR-4- and TLR-3-induced signaling in innate immune cells (94). Thus, zinc deficiency potentially disturbs the innate immune response toward SARS-CoV2, enabling the virus to easily spread throughout the host without an adequate immune response (**Figure 1.6**).

Clinical improvement of COVID-19 patients was correlated to an increase of $CD14^+$ monocytes and NK cells in the recovery phase (48). For a physiological inflammatory response and phagocytic activity macrophages need sufficient intracellular zinc levels (1). In addition, for NK cells and cytotoxic T cells it was shown that zinc supplementation increased their cytotoxicity toward target cells (1, 2, 95) (**Figures 1.7,1.10**).

In summary zinc's (re-)balancing power regarding immune cell numbers and functions might be highly beneficial in regard to therapy of COVID-19.

ZINC SUPPLEMENTATION IN RESPIRATORY INFECTIONS

Our suggested benefits of zinc supplementation to prevent and treat COVID-19 are supported by a row of successful supplementation studies focusing on respiratory tract infection, of which we listed some selected publications in **Table 1**. In most cases, prophylactic zinc supplementation was more effective than therapeutic proceedings (106–108, 111). Up to 30% of the everyday respiratory infections, briefly named "common cold," are due to infections with coronaviruses (112). Studies showed reduced symptom severity, reduced frequency, and duration of the common cold after zinc administration (99, 100, 113, 114) depending on dosage, zinc compound and the start time after initial symptoms (115). Most importantly, zinc supplementation of children revealed significant benefits in various studies (96, 106) and reduced 15% pneumonia-specific morality and 19% of pneumonia morbidity in developing countries (116).

RISK GROUPS AND SYMPTOMS OF COVID-19 AND ZINC DEFICIENCY REVEAL A LARGE OVERLAP

As illustrated in **Figure 1.1**, the intersection between risk groups of COVID-19 and zinc deficiency is impressive. In patients with chronic obstructive pulmonary disease (COPD), bronchial asthma, cardiovascular diseases, autoimmune diseases, kidney diseases, dialysis, obesity, diabetes, cancer, atherosclerosis, liver cirrhosis, immunosuppression, and known liver damage low serum zinc levels are regularly observed (4, 117). At the same time, these groups are particular at risk for COVID-19 (10, 51, 118, 119). For example 57.5% elderly and nursing home residents in the U.S., for which high incidence of respiratory tract infections is described, showed significantly decreased zinc intake levels and are considered subjects with high risk regarding COVID-19 (120). Moreover, other studies showed that serum zinc levels were inversely correlated with pneumonia and cystic

TABLE 1 | Selected zinc supplementation studies in respiratory infections.

Compound	Conc. [mg/d]	Duration	Disease	Effect	References
Treatment					
Zinc bis-glycinate	30 (elemental)	Max 7 days/dis-charge from the hospital	Lower RTI (Children)	Reduction of days of ALRI and shorter hospital stay	(96)
Zinc acetate	20	5d	Lower RTI (children)	Increased recovery rates (boys)	(97)
Zinc gluconate	10	6 mo	Lower RTI (children)	Decreased episodes of infection, more infection free days	(98)
Zinc gluconate Zinc actetate Gluconate nasal gel SULFITE nasal spray	60–313 76.8–102.4 2.1 0.044	Until symptoms are gone	Common cold	Variable results but generally reduced duration if supplementation started within first 24 h	Meta-study of 16 studies (99)
Zinc acetate vs. zinc gluconate	80–92 192–207	Until symptoms are gone	Common cold	<75 mg/day: reduced duration; zinc acetate better than gluconate	Meta-study of 7 studies (100)
Zinc gluconate	30 (elemental)	12 mo	Cystic fibrosis (children)	Reduced duration of antibiotics	(101)
Prophylactic					
Zinc sulfate	20 (elemental)	2 wk/6 mo follow-up	Lower RTI (children)	Reduced morbidity	(102)
Zinc sulfate	20 to ZD children	14d, 6 mo follow-up	Upper and lower RTI (children)	Decreased incidence and duration of upper and lower RTI	(103)
Zinc oxide	5	12 mo	Upper RTI (children)	Decreased incidence	(104)
Zinc gluconate	10	6 mo	Lower RTI	Decreased incidence	(105)
Zinc acetate, gluconate, methionine, sulfate	Min 70 mg/wk	>3 mo	Lower RTI	Decreased incidence (depending on criteria)	Meta-study of 10 studies (106)
Zinc in mineral mix	6 (f)−7.5 (m)	12 mo	Naturally occurring pneumonia	Decreased incidence and duration, decreased duration of antimicrobial therapy	(107)
Zinc sulfate	60–90	12 mo	Ventilation associated pneumonia	Decreased incidence	(108)
Zinc gluconate	Up to 12x 23 mg/d	Until symptoms are gone	Common cold	Decreased clinical score	(109)
Zinc sulfate	15	7 mo	Common cold	Decreased incidence	(110)
Murine models					
Zinc-enriched rodent diet	ZD: 50 ppm–ZS: 100 ppm	18d ZD followed by 3d ZS	Sepsis-induced ALI	Decrease in inflammation, lung damage, and mortality (vs. ZD mice)	(68)
Zinc aspartate	30 μg/ mouse	24 h	Acute lung injury (LPS inhalation), mice	Decreased hyper-inflammation, tissue damage	(17)

Conc, concentration; d, day; mo, months; ref, reference; RTI, respiratory tract infection; ZD, zinc deficiency; ZS, zinc supplementation; wk, week.
Single studies are not included in the meta-studies.

fibrosis (121, 122). On the other hand, zinc supplementation was able to reconstitute immune function in elderly and zinc deficient individuals (107, 123), which remains to be addressed for SARS-CoV2 infections (36). In this regard, the low response of older patients with low serum zinc to a 23-valent pneumococcal polysaccharide vaccination compared to those with higher zinc level (124), should be mentioned. However, zinc's role in the response to vaccination is generally discussed controversially and no data are available for vaccination against any corona virus.

Several studies indicate that there is an association between chemosensory dysfunctions and COVID-19 (125–133). Smell or taste is largely decreased, which might be a good disease biomarker (133). It was suggested that this might either be due to direct destruction of sensory cells by the virus, as ACE-2 is highly

expressed by the oral mucosa, or by viral entry into the brain and neuronal pathologies as was described for other SARS-CoV (133, 134). Zinc deficiency was related to significantly reduced taste sensitivity and impaired saliva secretion in humans and animals, which might involve zinc's importance for the action of carbonic anhydrase (135–140). Results from supplementation studies largely describe improvements in chemosensory functions (140, 141), but some studies did not find any effects (142) or even more severe disturbances when very high zinc concentrations were used (143). This is possibly due to investigating olfactory diseases of various origins, the lack of controlled trials and inclusion of observable studies. Thus, the benefits of zinc supplementation alone or in combination with common medical strategies should be tested for taste and smell diseases according to the available guidelines (144).

About 50% of patients that died of COVID-19 had bacterial or fungal co-infections (145), underlining the importance of sustaining the immune function by a sufficient zinc supply (1, 2, 36). In animal experiments it was shown that zinc restriction made mice highly susceptible to bacterial infection with *streptococcus pneumoniae* (146). As mentioned earlier, marginal zinc deficiency affects one third of the worldwide population and most subjects with COVID-19 are at risk of zinc deficiency (**Figure 1**). During physiological inflammatory responses, zinc is additionally redistributed to the tissues, resulting in serum hypozincemia (1, 65). In combination with the pre-existing suboptimal zinc supply, this will decrease serum zinc levels to critically low values and thereby significantly increase the susceptibility for co-infections with detrimental progression. In critically ill patients persistent low serum zinc was associated with recurrent sepsis and serum zinc levels were inversely correlated with mortality from sepsis (62), underlining the potential benefits of monitoring the zinc status of the patients and implementing zinc supplementation into therapy of COVID-19.

Vascular complications resulting from atherosclerosis development, microangiopathic organ failure, and venous thromboembolism were found as a major cause of death in COVID-19 patients (147–149), suggesting an important role of disease-induced coagulopathy, which, however, needs further investigation. Zinc influences thrombocyte aggregation and coagulation (150). Recently, a functional association between zinc and ROS production in platelets was described, indicating that zinc could decrease thrombus formation in a clinical context (151). Complications of SARS-CoV2 infections also include tissue damage affecting the gastrointestinal system (152), the liver (153), heart (154), nervous system (155), kidneys (156), blood vessels (149), and the skin (157). In this regard it should be mentioned that a balanced zinc homeostasis is essential for wound healing and tissue recovery after mechanical and inflammation-mediated damage (158, 159), adding more potential benefits of zinc supplementation of COVID-19 patients (**Figure 1.11**).

CONCLUSION

In this perspective, we reviewed the most important literature on the role of zinc homeostasis during viral infections, focusing on the potential benefits of zinc supplementation to prevent and treat SARS-CoV2 infections. Although data specifically on SARS-CoV2 are unfortunately still pending and randomized controlled studies have not been conducted, the enumerated evidence from the literature strongly suggests great benefits of zinc supplementation. Zinc supplementation improves the mucociliary clearance, strengthens the integrity of the epithelium, decreases viral replication, preserves antiviral immunity, attenuates the risk of hyper-inflammation, supports anti-oxidative effects and thus reduces lung damage and minimized secondary infections. Especially older subjects, patients with chronic diseases and most of the remaining COVID-19 risk groups would most likely benefit. Although studies are needed testing the effect of zinc as therapeutic option for established disease, preventive supplementation of subjects from risk groups should begin now, as zinc is a cost-efficient, globally available and simple to use option with little to no side effects. The first clinical trials on zinc supplementation alone and in combination with other drugs such as chloroquine have been registered (124, 160–162). Thus, first results and treatment regimens regarding zinc supplementation for COVID-19 risk groups and patients can be anticipated soon.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR'S NOTE

LR is a member of ZINC Net.

AUTHOR CONTRIBUTIONS

IW, BR, and LR drafted and corrected the text, table, and figure. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Wessels I, Maywald M, Rink L. Zinc as a gatekeeper of immune function. *Nutrients.* (2017) 9:1286. doi: 10.3390/nu9121286
2. Gammoh NZ, Rink L. Zinc in infection and inflammation. *Nutrients.* (2017) 9:624. doi: 10.20944/preprints201705.0176.v1
3. Roohani N, Hurrell R, Kelishadi R, Schulin R. Zinc and its importance for human health: an integrative review. *J Res Med Sci.* (2013) 18:144–57.
4. Wessels I, Rink L. Micronutrients in autoimmune diseases: possible therapeutic benefits of zinc and vitamin D. *J Nutr Biochem.* (2020) 77:108240. doi: 10.1016/j.jnutbio.2019.108240

5. Haase H, Schomburg L. You'd better zinc-trace element homeostasis in infection and inflammation. *Nutrients*. (2019) 11:2078. doi: 10.3390/nu11092078
6. Prasad AS. Effects of zinc deficiency on Th1 and Th2 cytokine shifts. *J Infect Dis.* (2000) 182(Suppl. 1):S62–8. doi: 10.1086/315916
7. Suita S, Ikeda K, Nagasaki A, Hayashida Y. Zinc deficiency during total parenteral nutrition in childhood. *J Pediatric Surg.* (1978) 13:5–9. doi: 10.1016/S0022-3468(78)80202-4
8. Hotz C, Brown KH. Identifying populations at risk of zinc deficiency: the use of supplementation trials. *Nutr Rev.* (2001) 59:80–4. doi: 10.1111/j.1753-4887.2001.tb06992.x
9. World Health Organization. The World Health report 2002. *Midwifery*. (2003) 19:72–3. doi: 10.1054/midw.2002.0343
10. Eurosurveillance ET. Updated rapid risk assessment from ECDC on coronavirus disease 2019 (COVID-19) pandemic: increased transmission in the EU/EEA and the UK. *Euro Surveill.* (2020) 25:2003121. doi: 10.2807/1560-7917.ES.2020.25.10.2003121
11. Hoffmann M, Kleine-Weber H, Krüger N, Müller M, Drosten C, Pöhlmann S. The novel coronavirus 2019 (2019-nCoV) uses the SARS-coronavirus receptor ACE2 and the cellular protease TMPRSS2 for entry into target cells. *bioRxiv.* (2020). doi: 10.1101/2020.01.31.929042. [Epub ahead of print].
12. Chilvers MA, McKean M, Rutman A, Myint BS, Silverman M, O'Callaghan C. The effects of coronavirus on human nasal ciliated respiratory epithelium. *Eur Respir J.* (2001) 18:965–70. doi: 10.1183/09031936.01.00093001
13. Woodworth BA, Zhang S, Tamashiro E, Bhargave G, Palmer JN, Cohen NA. Zinc increases ciliary beat frequency in a calcium-dependent manner. *Am J Rhinol Allergy.* (2010) 24:6–10. doi: 10.2500/ajra.2010.24.3379
14. Darma A, Athiyyah AF, Ranuh RG, Merbawani W, Setyoningrum RA, Hidajat B, et al. Zinc supplementation effect on the bronchial cilia length, the number of cilia, and the number of intact bronchial cell in zinc deficiency rats. *Indones Biomed J.* (2020) 12:78–84. doi: 10.18585/inabj.v12i1.998
15. Biaggio VS, Salvetti NR, Pérez Chaca MV, Valdez SR, Ortega HH, Gimenez MS, et al. Alterations of the extracellular matrix of lung during zinc deficiency. *Br J Nutr.* (2012) 108:62–70. doi: 10.1017/S00071145110 05290
16. Roscioli E, Jersmann HP, Lester S, Badiei A, Fon A, Zalewski P, et al. Zinc deficiency as a codeterminant for airway epithelial barrier dysfunction in an *ex vivo* model of COPD. *Int J Chron Obstruct Pulmon Dis.* (2017) 12:3503–10. doi: 10.2147/COPD.S149589
17. Wessels I, Pupke JT, Trotha K-T von, Gombert A, Himmelsbach A, Fischer HJ, et al. Zinc supplementation ameliorates lung injury by reducing neutrophil recruitment and activity. *Thorax.* (2020) 75:253–61. doi: 10.1136/thoraxjnl-2019-213357
18. Bao S, Knoell DL. Zinc modulates cytokine-induced lung epithelial cell barrier permeability. *Am J Physiol Lung Cell Mol Physiol.* (2006) 291:L1132–41. doi: 10.1152/ajplung.00207.2006
19. Fraker PJ, King LE. Reprogramming of the immune system during zinc deficiency. *Annu Rev Nutr.* (2004) 24:277–98. doi: 10.1146/annurev.nutr.24.012003.132454
20. Novick SG, Godfrey JC, Pollack RL, Wilder HR. Zinc-induced suppression of inflammation in the respiratory tract, caused by infection with human rhinovirus and other irritants. *Med Hypotheses.* (1997) 49:347–57. doi: 10.1016/S0306-9877(97)90201-2
21. Boudreault F, Pinilla-Vera M, Englert JA, Kho AT, Isabelle C, Arciniegas AJ, et al. Zinc deficiency primes the lung for ventilator-induced injury. *JCI Insight.* (2017) 2:e86507. doi: 10.1172/jci.insight.86507
22. Speth R, Carrera E, Jean-Baptiste M, Joachim A, Linares A. Concentration-dependent effects of zinc on angiotensin-converting enzyme-2 activity (1067.4). *FASEB J.* (2014) 28(1_supplement):1067.4.
23. Reeves PG, O'Dell BL. Effects of dietary zinc deprivation on the activity of angiotensin-converting enzyme in serum of rats and guinea pigs. *J Nutr.* (1986) 116:128–34. doi: 10.1093/jn/116.1.128
24. Christianson DW, Alexander RS. Carboxylate-histidine-zinc interactions in protein structure and function. *J. Am. Chem. Soc.* (1989) 111:6412–9. doi: 10.1021/ja00198a065
25. Cox E. Zinc-dependent protein folding. *Curr Opin Chem Biol.* (2000) 4:162–5. doi: 10.1016/S1367-5931(99)00070-8
26. Cao J-W, Duan S-Y, Zhang H-X, Chen Y, Guo M. Zinc deficiency promoted fibrosis via ROS and TIMP/MMPs in the myocardium of mice. *Biol Trace Elem Res.* (2019) 196:145–52. doi: 10.1007/s12011-019-01902-4
27. Rosenkranz E, Metz CH, Maywald M, Hilgers R-D, Weßels I, Senff T, et al. Zinc supplementation induces regulatory T cells by inhibition of Sirt-1 deacetylase in mixed lymphocyte cultures. *Mol Nutr Food Res.* (2016) 60:661–71. doi: 10.1002/mnfr.201500524
28. Patel VB, Zhong J-C, Grant MB, Oudit GY. Role of the ACE2/Angiotensin 1-7 axis of the renin-angiotensin system in heart failure. *Circ Res.* (2016) 118:1313–26. doi: 10.1161/CIRCRESAHA.116.307708
29. Blanco-Melo D, Nilsson-Payant BE, Liu W-C, Møller R, Panis M, Sachs D, et al. SARS-CoV-2 launches a unique transcriptional signature from *in vitro*, *ex vivo*, and *in vivo* systems. *bioRxiv.* (2020). doi: 10.1101/2020.03.24.004655. [Epub ahead of print].
30. Berg K, Bolt G, Andersen H, Owen TC. Zinc potentiates the antiviral action of human IFN-alpha tenfold. *J Interferon Cytokine Res.* (2001) 21:471–4. doi: 10.1089/10799900152434330
31. Cakman I, Kirchner H, Rink L. Zinc supplementation reconstitutes the production of interferon-alpha by leukocytes from elderly persons. *J Interferon Cytokine Res.* (1997) 17:469–72. doi: 10.1089/jir.1997.17.469
32. Ziegler CGK, Allon SJ, Nyquist SK, Mbano IM, Miao VN, Tzouanas CN, et al. SARS-CoV-2 receptor ACE2 is an interferon-stimulated gene in human airway epithelial cells and is detected in specific cell subsets across tissues. *Cell.* (2020) 181:1016–35.e19. doi: 10.1016/j.cell.2020.04.035
33. Ishida T. Review on the role of Zn2+ ions in viral pathogenesis and the effect of Zn2+ Ions for host cell-virus growth inhibition. *AJBSR.* (2019) 2:28–37. doi: 10.34297/AJBSR.2019.02.000566
34. Krenn BM, Gaudernak E, Holzer B, Lanke K, van Kuppeveld FJ, Seipelt J. Antiviral activity of the zinc ionophores pyrithione and hinokitiol against picornavirus infections. *J Virol.* (2009) 83:58–64. doi: 10.1128/JVI.01543-08
35. Read SA, Obeid S, Ahlenstiel C, Ahlenstiel G. The role of zinc in antiviral immunity. *Adv Nutr.* (2019) 10:696–710. doi: 10.1093/advances/nmz013
36. Skalny AV, Rink L, Ajsuvakova OP, Aschner M, Gritsenko VA, Alekseenko SI, et al. Zinc and respiratory tract infections: perspectives for COVID19 (Review). *Int J Mol Med.* (2020) 46:17–26. doi: 10.3892/ijmm.2020.4575
37. Suara RO, Crowe JE. Effect of zinc salts on respiratory syncytial virus replication. *Antimicrob Agents Chemother.* (2004) 48:783–90. doi: 10.1128/AAC.48.3.783-790.2004
38. Cai H, Zhang Y, Ma Y, Sun J, Liang X, Li J. Zinc binding activity of human metapneumovirus M2-1 protein is indispensable for viral replication and pathogenesis *in vivo*. *J Virol.* (2015) 89:6391–405. doi: 10.1128/JVI.03488-14
39. Lazarczyk M, Favre M. Role of Zn2+ ions in host-virus interactions. *J Virol.* (2008) 82:11486–94. doi: 10.1128/JVI.01314-08
40. Kümel G, Schrader S, Zentgraf H, Daus H, Brendel M. The mechanism of the antiherpetic activity of zinc sulphate. *J Gen Virol.* (1990) 71:2989–97. doi: 10.1099/0022-1317-71-12-2989
41. te Velthuis AJ, van den Worm SH, Sims AC, Baric RS, Snijder EJ, van Hemert MJ. Zn(2+) inhibits coronavirus and arterivirus RNA polymerase activity *in vitro* and zinc ionophores block the replication of these viruses in cell culture. *PLoS Pathog.* (2010) 6:e1001176. doi: 10.1371/journal.ppat.10 01176
42. Shittu MO, Afolami OI. Improving the efficacy of chloroquine and hydroxychloroquine against SARS-CoV-2 may require zinc additives - a better synergy for future COVID-19 clinical trials. *Infez Med.* (2020) 28:192–7.
43. Dabbagh-Bazarbachi H, Clergeaud G, Quesada IM, Ortiz M, O'Sullivan CK, Fernández-Larrea JB. Zinc ionophore activity of quercetin and epigallocatechin-gallate: from Hepa 1-6 cells to a liposome model. *J Agric Food Chem.* (2014) 62:8085–93. doi: 10.1021/jf5014633
44. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) *in vitro*. *Cell Res.* (2020) 30:269–71. doi: 10.1038/s41422-020-0282-0
45. Xue J, Moyer A, Peng B, Wu J, Hannafon BN, Ding W-Q. Chloroquine is a zinc ionophore. *PLoS ONE.* (2014) 9:e109180. doi: 10.1371/journal.pone.0109180

46. Liu J, Zheng X, Tong Q, Li W, Wang B, Sutter K, et al. Overlapping and discrete aspects of the pathology and pathogenesis of the emerging human pathogenic coronaviruses SARS-CoV, MERS-CoV, and 2019-nCoV. *J Med Virol.* (2020) 92:491–4. doi: 10.1002/jmv.25709
47. Lin M-H, Moses DC, Hsieh C-H, Cheng S-C, Chen Y-H, Sun C-Y, et al. Disulfiram can inhibit MERS and SARS coronavirus papain-like proteases via different modes. *Antiviral Res.* (2018) 150:155–63. doi: 10.1016/j.antiviral.2017.12.015
48. Wen W, Su W, Tang H, Le W, Zhang X, Zheng Y, et al. Immune cell profiling of COVID-19 patients in the recovery stage by single-cell sequencing. *Cell Discov.* (2020) 6:31. doi: 10.1038/s41421-020-00187-5
49. Cheng ZJ, Shan J. 2019 Novel coronavirus: where we are and what we know. *Infection.* (2020) 48:155–63. doi: 10.1007/s15010-020-01401-y
50. Chen G, Di Wu, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest.* (2020) 130:2620–9. doi: 10.1172/JCI137244
51. Guan W-J, Ni Z-Y, Hu Y, Liang W-H, Ou C-Q, He J-X, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
52. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. COVID-19: consider cytokine storm syndromes and immunosuppression. *Lancet.* (2020) 395:1033–4. doi: 10.1016/S0140-6736(20)30628-0
53. Matthay MA, Zemans RL, Zimmerman GA, Arabi YM, Beitler JR, Mercat A, et al. Acute respiratory distress syndrome. *Nat Rev Dis Primers.* (2019) 5:18. doi: 10.1038/s41572-019-0069-0
54. Wichmann D, Sperhake J-P, Lütgehetmann M, Steurer S, Edler C, Heinemann A, et al. Autopsy findings and venous thromboembolism in patients with COVID-19: a prospective cohort study. *Ann Intern Med.* (2020). doi: 10.7326/M20-2003. [Epub ahead of print].
55. Beck FW, Prasad AS, Kaplan J, Fitzgerald JT, Brewer GJ. Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. *Am J Physiol.* (1997) 272:E1002–7. doi: 10.1152/ajpendo.1997.272.6.E1002
56. Wessels I, Haase H, Engelhardt G, Rink L, Uciechowski P. Zinc deficiency induces production of the proinflammatory cytokines IL-1β and TNFα in promyeloid cells via epigenetic and redox-dependent mechanisms. *J Nutr Biochem.* (2013) 24:289–97. doi: 10.1016/j.jnutbio.2012.06.007
57. Liu M-J, Bao S, Gálvez-Peralta M, Pyle CJ, Rudawsky AC, Pavlovicz RE, et al. ZIP8 regulates host defense through zinc-mediated inhibition of NF-κB. *Cell Rep.* (2013) 3:386–400. doi: 10.1016/j.celrep.2013.01.009
58. Ganatra HA, Varisco BM, Harmon K, Lahni P, Opoka A, Wong HR. Zinc supplementation leads to immune modulation and improved survival in a juvenile model of murine sepsis. *Innate Immun.* (2017) 23:67–76. doi: 10.1177/1753425916677073
59. Choi S, Liu X, Pan Z. Zinc deficiency and cellular oxidative stress: prognostic implications in cardiovascular diseases. *Acta Pharmacol Sin.* (2018) 39:1120–32. doi: 10.1038/aps.2018.25
60. Prasad AS, Beck FW, Bao B, Fitzgerald JT, Snell DC, Steinberg JD, et al. Zinc supplementation decreases incidence of infections in the elderly: effect of zinc on generation of cytokines and oxidative stress. *Am J Clin Nutr.* (2007) 85:837–44. doi: 10.1093/ajcn/85.3.837
61. Zalewski PD, Truong-Tran AQ, Grosser D, Jayaram L, Murgia C, Ruffin RE. Zinc metabolism in airway epithelium and airway inflammation: basic mechanisms and clinical targets. a review. *Pharmacol Ther.* (2005) 105:127–49. doi: 10.1016/j.pharmthera.2004.09.004
62. Hoeger J, Simon T-P, Beeker T, Marx G, Haase H, Schuerholz T. Persistent low serum zinc is associated with recurrent sepsis in critically ill patients - a pilot study. *PLoS ONE.* (2017) 12:e0176069. doi: 10.1371/journal.pone.0176069
63. Ibs K-H, Rink L. Zinc-altered immune function. *J Nutr.* (2003) 133:1452S–6S. doi: *10.1093/jn/133.5.1452S*
64. Kahmann L, Uciechowski P, Warmuth S, Plümäkers B, Gressner AM, Malavolta M, et al. Zinc supplementation in the elderly reduces spontaneous inflammatory cytokine release and restores T cell functions. *Rejuvenation Res.* (2008) 11:227–37. doi: 10.1089/rej.2007.0613
65. Wessels I, Cousins RJ. Zinc dyshomeostasis during polymicrobial sepsis in mice involves zinc transporter Zip14 and can be overcome by zinc supplementation. *Am J Physiol Gastrointest Liver Physiol.* (2015) 309:G768–78. doi: 10.1152/ajpgi.00179.2015
66. Besecker BY, Exline MC, Hollyfield J, Phillips G, Disilvestro RA, Wewers MD, et al. A comparison of zinc metabolism, inflammation, and disease severity in critically ill infected and noninfected adults early after intensive care unit admission. *Am J Clin Nutr.* (2011) 93:1356–64. doi: 10.3945/ajcn.110.008417
67. Knoell DL, Smith DA, Sapkota M, Heires AJ, Hanson CK, Smith LM, et al. Insufficient zinc intake enhances lung inflammation in response to agricultural organic dust exposure. *J Nutr Biochem.* (2019) 70:56–64. doi: 10.1016/j.jnutbio.2019.04.007
68. Bao S, Liu M-J, Lee B, Besecker B, Lai J-P, Guttridge DC, et al. Zinc modulates the innate immune response *in vivo* to polymicrobial sepsis through regulation of NF-kappaB. *Am J Physiol Lung Cell Mol Physiol.* (2010) 298:L744–54. doi: 10.1152/ajplung.00368.2009
69. Gruber K, Maywald M, Rosenkranz E, Haase H, Plumakers B, Rink L. Zinc deficiency adversely influences interleukin-4 and interleukin-6 signaling. *J Biol Regul Homeost Agents.* (2013) 27:661–71.
70. Maywald M, Wessels I, Rink L. Zinc signals and immunity. *Int J Mol Sci.* (2017) 18:2222. doi: 10.3390/ijms18102222
71. Maywald M, Meurer SK, Weiskirchen R, Rink L. Zinc supplementation augments TGF-beta1-dependent regulatory T cell induction. *Mol Nutr Food Res.* (2017) 61:1–11. doi: 10.1002/mnfr.201600493
72. Ollig J, Kloubert V, Taylor KM, Rink L. B cell activation and proliferation increase intracellular zinc levels. *J Nutr Biochem.* (2019) 64:72–9. doi: 10.1016/j.jnutbio.2018.10.008
73. Reiber C, Brieger A, Engelhardt G, Hebel S, Rink L, Haase H. Zinc chelation decreases IFN-beta-induced STAT1 upregulation and iNOS expression in RAW 264.7 macrophages. *J Trace Elem Med Biol.* (2017) 44:76–82. doi: 10.1016/j.jtemb.2017.05.011
74. Kulik L, Maywald M, Kloubert V, Wessels I, Rink L. Zinc deficiency drives Th17 polarization and promotes loss of Treg cell function. *J Nutr Biochem.* (2019) 63:11–8. doi: 10.1016/j.jnutbio.2018.09.011
75. Chen X, Ling J, Mo P, Zhang Y, Jiang Q, Ma Z, et al. Restoration of leukomonocyte counts is associated with viral clearance in COVID-19 hospitalized patients. *medRxiv.* (2020). doi: 10.1101/2020.03.03.20030437. [Epub ahead of print].
76. Liu J, Li S, Liu J, Liang B, Wang X, Wang H, et al. Longitudinal characteristics of lymphocyte responses and cytokine profiles in the peripheral blood of SARS-CoV-2 infected patients. *EBioMedicine.* (2020) 55:102763. doi: 10.1016/j.ebiom.2020.102763
77. Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, et al. Reduction and functional exhaustion of t cells in patients with Coronavirus Disease 2019 (COVID-19). *Front Immunol.* (2020) **11**:827. doi: 10.3389/fimmu.2020.00827
78. Hönscheid A, Rink L, Haase H. T-lymphocytes: a target for stimulatory and inhibitory effects of zinc ions. *Endocr Metab Immune Disord Drug Targets.* (2009) 9:132–44. doi: 10.2174/187153009788452390
79. Prasad AS, Meftah S, Abdallah J, Kaplan J, Brewer GJ, Bach JF, et al. Serum thymulin in human zinc deficiency. *J Clin Invest.* (1988) 82:1202–10. doi: 10.1172/JCI113717
80. Kaltenberg J, Plum LM, Ober-Blöbaum JL, Hönscheid A, Rink L, Haase H. Zinc signals promote IL-2-dependent proliferation of T cells. *Eur J Immunol.* (2010) 40:1496–503. doi: 10.1002/eji.200939574
81. King LE, Fraker PJ. Variations in the cell cycle status of lymphopoietic and myelopoietic cells created by zinc deficiency. *J Infect Dis.* (2000) 182(Suppl 1):S16–22. doi: 10.1086/315923
82. Kam YW, Kien F, Roberts A, Cheung YC, Lamirande EW, Vogel L, et al. Antibodies against trimeric S glycoprotein protect hamsters against SARS-CoV challenge despite their capacity to mediate FcgammaRII-dependent entry into B cells *in vitro*. *Vaccine.* (2007) 25:729–40. doi: 10.1016/j.vaccine.2006.08.011
83. Jaume M, Yip MS, Cheung CY, Leung HL, Li PH, Kien F, et al. Anti-severe acute respiratory syndrome coronavirus spike antibodies trigger infection of human immune cells via a pH- and cysteine protease-independent FcγR pathway. *J Virol.* (2011) 85:10582–97. doi: 10.1128/JVI.00671-11

84. Chen Y, Feng Z, Diao B, Wang R, Wang G, Wang C, et al. The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) directly decimates human spleens and lymph nodes. *bioRxiv*. (2020). doi: 10.1101/2020.03.27.20045427. [Epub ahead of print].
85. Grommes J, Soehnlein O. Contribution of neutrophils to acute lung injury. *Mol Med*. (2011) 17:293–307. doi: 10.2119/molmed.2010.00138
86. Hasan R, Rink L, Haase H. Zinc signals in neutrophil granulocytes are required for the formation of neutrophil extracellular traps. *Innate Immun*. (2013) 19:253–64. doi: 10.1177/1753425912458815
87. Hasan R, Rink L, Haase H. Chelation of free Zn(2)(+) impairs chemotaxis, phagocytosis, oxidative burst, degranulation, and cytokine production by neutrophil granulocytes. *Biol Trace Elem Res*. (2016) 171:79–88. doi: 10.1007/s12011-015-0515-0
88. Choudhury A, Mukherjee S. *In silico* studies on the comparative characterization of the interactions of SARS-CoV-2 spike glycoprotein with ACE-2 receptor homologs and human TLRs. *J Med Virol*. (2020) 1–9. doi: 10.1002/jmv.25987. [Epub ahead of print].
89. Bieback K, Lien E, Klagge IM, Avota E, Schneider-Schaulies J, Duprex WP, et al. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. *J Virol*. (2002) 76:8729–36. doi: 10.1128/JVI.76.17.8729-8736.2002
90. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol*. (2000) 1:398–401. doi: 10.1038/80833
91. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*. (2001) 413:732–8. doi: 10.1038/35099560
92. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science*. (2004) 303:1526–9. doi: 10.1126/science.1093620
93. Zhao J, Wohlford-Lenane C, Zhao J, Fleming E, Lane TE, McCray PB, et al. Intranasal treatment with poly(I•C) protects aged mice from lethal respiratory virus infections. *J Virol*. (2012) 86:11416–24. doi: 10.1128/JVI.01410-12
94. Brieger A, Rink L, Haase H. Differential regulation of TLR-dependent MyD88 and TRIF signaling pathways by free zinc ions. *J Immunol*. (2013) 191:1808–17. doi: 10.4049/jimmunol.1301261
95. Rolles B, Maywald M, Rink L. Influence of zinc deficiency and supplementation on NK cell cytotoxicity. *J Funct Foods*. (2018) 48:322–8. doi: 10.1016/j.jff.2018.07.027
96. Rerksuppaphol S, Rerksuppaphol L. A randomized controlled trial of zinc supplementation in the treatment of acute respiratory tract infection in Thai children. *Pediatr Rep*. (2019) 11:7954. doi: 10.4081/pr.2019.7954
97. Mahalanabis D, Lahiri M, Paul D, Gupta S, Gupta A, Wahed MA, et al. Randomized, double-blind, placebo-controlled clinical trial of the efficacy of treatment with zinc or vitamin A in infants and young children with severe acute lower respiratory infection. *Am J Clin Nutr*. (2004) 79:430–6. doi: 10.1093/ajcn/79.3.430
98. Shah UH, Abu-Shaheen AK, Malik MA, Alam S, Riaz M, Al-Tannir MA. The efficacy of zinc supplementation in young children with acute lower respiratory infections: a randomized double-blind controlled trial. *Clin Nutr*. (2013) 32:193–9. doi: 10.1016/j.clnu.2012.08.018
99. Hulisz D. Efficacy of zinc against common cold viruses: an overview. *J Am Pharm Assoc*. (2003). (2004) 44:594–603. doi: 10.1331/1544-3191.44.5.594.Hulisz
100. Hemilä H. Zinc lozenges and the common cold: a meta-analysis comparing zinc acetate and zinc gluconate, and the role of zinc dosage. *JRSM Open*. (2017) 8:2054270417694291. doi: 10.1177/2054270417694291
101. Abdulhamid I, Beck FW, Millard S, Chen X, Prasad A. Effect of zinc supplementation on respiratory tract infections in children with cystic fibrosis. *Pediatr Pulmonol*. (2008) 43:281–7. doi: 10.1002/ppul.20771
102. Malik A, Taneja DK, Devasenapathy N, Rajeshwari K. Zinc supplementation for prevention of acute respiratory infections in infants: a randomized controlled trial. *Indian Pediatr*. (2014) 51:780–4. doi: 10.1007/s13312-014-0503-z
103. Khera D, Singh S, Purohit P, Sharma P, Singh K. Prevalence of zinc deficiency and effect of zinc supplementation on prevention of acute respiratory infections. *Turk Thorac J*. (2019) 1–14. doi: 10.2139/ssrn.3273670
104. Martinez-Estevez NS, Alvarez-Guevara AN, Rodriguez-Martinez CE. Effects of zinc supplementation in the prevention of respiratory tract infections and diarrheal disease in Colombian children: A 12-month randomised controlled trial. *Allergol Immunopathol*. (2016) 44:368–75. doi: 10.1016/j.aller.2015.12.006
105. Sazawal S, Black RE, Jalla S, Mazumdar S, Sinha A, Bhan MK. Zinc supplementation reduces the incidence of acute lower respiratory infections in infants and preschool children: a double-blind, controlled trial. *Pediatrics*. (1998) 102:1–5. doi: 10.1542/peds.102.1.1
106. Roth DE, Richard SA, Black RE. Zinc supplementation for the prevention of acute lower respiratory infection in children in developing countries: meta-analysis and meta-regression of randomized trials. *Int J Epidemiol*. (2010) 39:795–808. doi: 10.1093/ije/dyp391
107. Meydani SN, Barnett JB, Dallal GE, Fine BC, Jacques PF, Leka LS, et al. Serum zinc and pneumonia in nursing home elderly. *Am J Clin Nutr*. (2007) 86:1167–73. doi: 10.1093/ajcn/86.4.1167
108. Hasanzadeh Kiabi F, Alipour A, Darvishi-Khezri H, Aliasgharian A, Emami Zeydi A. Zinc supplementation in adult mechanically ventilated trauma patients is associated with decreased occurrence of ventilator-associated pneumonia: a secondary analysis of a prospective, observational study. *Indian J Crit Care Med*. (2017) 21:34–9. doi: 10.4103/0972-5229.198324
109. Al-Nakib W, Higgins PG, Barrow I, Batstone G, Tyrrell DA. Prophylaxis and treatment of rhinovirus colds with zinc gluconate lozenges. *J Antimicrob Chemother*. (1987) 20:893–901. doi: 10.1093/jac/20.6.893
110. Kurugöl Z, Akilli M, Bayram N, Koturoglu G. The prophylactic and therapeutic effectiveness of zinc sulphate on common cold in children. *Acta Paediatr*. (2006) 95:1175–81. doi: 10.1080/08035250600603024
111. Singh M, Das RR. Zinc for the common cold. *Cochrane Database Syst Rev*. (2013) 18:CD001364. doi: 10.1002/14651858.CD001364.pub4
112. Isaacs D, Flowers D, Clarke JR, Valman HB, MacNaughton MR. Epidemiology of coronavirus respiratory infections. *Arch Dis Child*. (1983) 58:500–3. doi: 10.1136/adc.58.7.500
113. Eby GA, Davis DR, Halcomb WW. Reduction in duration of common colds by zinc gluconate lozenges in a double-blind study. *Antimicrob Agents Chemother*. (1984) 25:20–4. doi: 10.1128/AAC.25.1.20
114. Godfrey JC, Conant Sloane B, Smith DS, Turco JH, Mercer N, Godfrey NJ. Zinc gluconate and the common cold: a controlled clinical study. *J Int Med Res*. (1992) 20:234–46. doi: 10.1177/030006059202000305
115. Wang MX, Win SS, Pang J. Zinc supplementation reduces common cold duration among healthy adults: a systematic review of randomized controlled trials with micronutrients supplementation. *Am J Trop Med Hyg*. (2020). doi: 10.4269/ajtmh.19-0718
116. Yakoob MY, Theodoratou E, Jabeen A, Imdad A, Eisele TP, Ferguson J, et al. Preventive zinc supplementation in developing countries: impact on mortality and morbidity due to diarrhea, pneumonia and malaria. *BMC Public Health*. (2011) 11(Suppl. 3):S23. doi: 10.1186/1471-2458-11-S3-S23
117. Ackland ML, Michalczyk A. Zinc deficiency and its inherited disorders -a review. *Genes Nutr*. (2006) 1:41–9. doi: 10.1007/BF02829935
118. Petrilli CM, Jones SA, Yang J, Rajagopalan H, O'Donnell LF, Chernyak Y, et al. Factors associated with hospitalization and critical illness among 4,103 patients with COVID-19 disease in New York city. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.04.08.20057794. [Epub ahead of print].
119. Vishnevetsky A, Levy M. Rethinking high-risk groups in COVID-19. *Mult Scler Relat Disord*. (2020) 42:102139. doi: 10.1016/j.msard.2020.102139
120. Briefel RR, Bialostosky K, Kennedy-Stephenson J, McDowell MA, Ervin RB, Wright JD. Zinc intake of the U.S. population: findings from the third National Health and Nutrition Examination Survey, 1988-1994. *J Nutr*. (2000) 130:1367S–73S. doi: 10.1093/jn/130.5.1367S

121. Bhat MH, Mudassir, Rather AB, Dhobi GN, Koul AN, Bhat FA, et al. Zinc levels in community acquired pneumonia in hospitalized patients; a case control study. *Egypt J Chest Dis Tubercul.* (2016) 65:485–9. doi: 10.1016/j.ejcdt.2015.12.020
122. Kamei S, Fujikawa H, Nohara H, Ueno-Shuto K, Maruta K, Nakashima R, et al. Zinc deficiency via a splice switch in zinc importer ZIP2/SLC39A2 causes cystic fibrosis-associated MUC5AC hypersecretion in airway epithelial cells. *EBioMedicine.* (2018) 27:304–16. doi: 10.1016/j.ebiom.2017.12.025
123. Haase H, Mocchegiani E, Rink L. Correlation between zinc status and immune function in the elderly. *Biogerontology.* (2006) 7:421–8. doi: 10.1007/s10522-006-9057-3
124. ClinicalTrials.gov. *A Study of Hydroxychloroquine and Zinc in the Prevention of COVID-19 Infection in Military Healthcare Workers*. (2020). Available online at: https://ClinicalTrials.gov/show/ (accessed May 15, 2020).
125. Yan CH, Faraji F, Prajapati DP, Boone CE, DeConde AS. Association of chemosensory dysfunction and Covid-19 in patients presenting with influenza-like symptoms. *Int Forum Allergy Rhinol.* (2020) **10**:806–13. doi: 10.1002/alr.22579
126. Yan CH, Faraji F, Prajapati DP, Ostrander BT, DeConde AS. Self-reported olfactory loss associates with outpatient clinical course in Covid-19. *Int Forum Allergy Rhinol.* (2020) **10**:821–31. doi: 10.1002/alr.22592
127. Soler ZM, Patel ZM, Turner JH, Holbrook EH. A primer on viral-associated olfactory loss in the era of COVID-19. *Int Forum Allergy Rhinol.* (2020) **10**:814–20. doi: 10.1002/alr.22578
128. Roland LT, Gurrola JG, Loftus PA, Cheung SW, Chang JL. Smell and taste symptom-based predictive model for COVID-19 diagnosis. *Int Forum Allergy Rhinol.* (2020) **10**:832–8. doi: 10.1002/alr.22602
129. Moein ST, Hashemian SM, Mansourafshar B, Khorram-Tousi A, Tabarsi P, Doty RL. Smell dysfunction: a biomarker for COVID-19. *Int Forum Allergy Rhinol.* (2020). doi: 10.1002/alr.22587. [Epub ahead of print].
130. Lechien JR, Hopkins C, Saussez S. Sniffing out the evidence; It's now time for public health bodies recognize the link between COVID-19 and smell and taste disturbance. *Rhinology.* (2020). doi: 10.4193/Rhin20.159. [Epub ahead of print].
131. Lechien JR, Chiesa-Estomba CM, Siati DR de, Horoi M, Le Bon SD, Rodriguez A, et al. Olfactory and gustatory dysfunctions as a clinical presentation of mild-to-moderate forms of the coronavirus disease (COVID-19): a multicenter European study. *Eur Arch Otorhinolaryngol.* (2020) 1–11. doi: 10.1007/s00405-020-05965-1
132. Russell B, Moss C, Rigg A, Hopkins C, Papa S, van Hemelrijck M. Anosmia and ageusia are emerging as symptoms in patients with COVID-19: What does the current evidence say? *Ecancermedicalscience.* (2020) 14:ed98. doi: 10.3332/ecancer.2020.ed98
133. Giacomelli A, Pezzati L, Conti F, Bernacchia D, Siano M, Oreni L, et al. Self-reported olfactory and taste disorders in SARS-CoV-2 patients: a cross-sectional study. *Clin Infect Dis.* (2020). doi: 10.1093/cid/ciaa330. [Epub ahead of print].
134. Netland J, Meyerholz DK, Moore S, Cassell M, Perlman S. Severe acute respiratory syndrome coronavirus infection causes neuronal death in the absence of encephalitis in mice transgenic for human ACE2. *J Virol.* (2008) 82:7264–75. doi: 10.1128/JVI.00737-08
135. Goto T, Komai M, Bryant BP, Furukawa Y. Reduction in carbonic anhydrase activity in the tongue epithelium and submandibular gland in zinc-deficient rats. *Int J Vitam Nutr Res.* (2000) 70:110–8. doi: 10.1024/0300-9831.70.3.110
136. Goto T, Komai M, Suzuki H, Furukawa Y. Long-term zinc deficiency decreases taste sensitivity in rats. *J Nutr.* (2001) 131:305–10. doi: 10.1093/jn/131.2.305
137. Goto T, Shirakawa H, Furukawa Y, Komai M. Decreased expression of carbonic anhydrase isozyme II, rather than of isozyme VI, in submandibular glands in long-term zinc-deficient rats. *Br J Nutr.* (2008) 99:248–53. doi: 10.1017/S0007114507801565
138. Tanaka M. Secretory function of the salivary gland in patients with taste disorders or xerostomia: correlation with zinc deficiency. *Acta Otolaryngol Suppl.* (2002) 122:134–41. doi: 10.1080/00016480260046526
139. Russell RM, Cox ME, Solomons N. Zinc and the special senses. *Ann Intern Med.* (1983) 99:227–39. doi: 10.7326/0003-4819-99-2-227
140. Sandstead HH, Henriksen LK, Greger JL, Prasad AS, Good RA. Zinc nutriture in the elderly in relation to taste acuity, immune response, and wound healing. *Am J Clin Nutr.* (1982) 36:1046–59. doi: 10.1093/ajcn/36.5.1046
141. Heckmann SM, Hujoel P, Habiger S, Friess W, Wichmann M, Heckmann JG, et al. Zinc gluconate in the treatment of dysgeusia–a randomized clinical trial. *J Dent Res.* (2005) 84:35–8. doi: 10.1177/154405910508400105
142. Sturniolo GC, D'Inca R, Parisi G, Giacomazzi F, Montino MC, D'Odorico A, et al. Taste alterations in liver cirrhosis: are they related to zinc deficiency? *J Trace Elem Electrolytes Health Dis.* (1992) 6:15–9.
143. Lyckholm L, Heddinger SP, Parker G, Coyne PJ, Ramakrishnan V, Smith TJ, et al. A randomized, placebo controlled trial of oral zinc for chemotherapy-related taste and smell disorders. *J Pain Palliat Care Pharmacother.* (2012) 26:111–4. doi: 10.3109/15360288.2012.676618
144. Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften. *Riech- und Schmeckstörungen (017/050)*. (2016). Available online at: https://www.awmf.org/leitlinien/detail/ll/017-050.html
145. Cox MJ, Loman N, Bogaert D, O'Grady J. Co-infections: potentially lethal and unexplored in COVID-19. *Lancet Microbe.* (2020) 1:e11. doi: 10.1016/S2666-5247(20)30009-4
146. Eijkelkamp BA, Morey JR, Neville SL, Tan A, Pederick VG, Cole N, et al. Dietary zinc and the control of Streptococcus pneumoniae infection. *PLoS Pathog.* (2019) 15:e1007957. doi: 10.1371/journal.ppat.1007957
147. Levi M, Thachil J, Iba T, Levy JH. Coagulation abnormalities and thrombosis in patients with COVID-19. *The Lancet Haematology.* (2020) 7:e438–40. doi: 10.1016/S2352-3026(20)30145-9
148. Gavriilaki E, Brodsky RA. Severe COVID-19 infection and thrombotic microangiopathy: success doesn't come easily. *Br J Haematol.* (2020) 189:e227–30. doi: 10.1111/bjh.16783
149. Varga Z, Flammer AJ, Steiger P, Haberecker M, Andermatt R, Zinkernagel AS, et al. Endothelial cell infection and endotheliitis in COVID-19. *Lancet.* (2020) 395:1417–8. doi: 10.1016/S0140-6736(20)30937-5
150. Taylor KA, Pugh N. The contribution of zinc to platelet behaviour during haemostasis and thrombosis. *Metallomics.* (2016) 8:144–55. doi: 10.1039/C5MT00251F
151. Lopes-Pires ME, Ahmed NS, Vara D, Gibbins JM, Pula G, Pugh N. Zinc regulates reactive oxygen species generation in platelets. *Platelets.* (2020). doi: 10.1080/09537104.2020.1742311. [Epub ahead of print].
152. Xiao F, Tang M, Zheng X, Liu Y, Li X, Shan H. Evidence for gastrointestinal infection of SARS-CoV-2. *Gastroenterology.* (2020) 158:1831–3.e3. doi: 10.1101/2020.02.17.20023721
153. Zhang C, Shi L, Wang F-S. Liver injury in COVID-19: management and challenges. *Lancet Gastroenterol Hepatol.* (2020) 5:428–30. doi: 10.1016/S2468-1253(20)30057-1
154. Inciardi RM, Lupi L, Zaccone G, Italia L, Raffo M, Tomasoni D, et al. Cardiac involvement in a patient with coronavirus disease 2019 (COVID-19). *JAMA Cardiol.* (2020). doi: 10.1001/jamacardio.2020.1096. [Epub ahead of print].
155. Baig AM. Updates on what ACS reported: emerging evidences of COVID-19 with nervous system involvement. *ACS Chem Neurosci.* (2020) **11**:1204–5. doi: 10.1021/acschemneuro.0c00181
156. Diao B, Wang C, Wang R, Feng Z, Tan Y, Wang H, et al. Human kidney is a target for novel severe acute respiratory syndrome coronavirus 2 (SARSCoV-2) infection. *medRxiv [Preprint]*. (2020). doi: 10.1101/2020.03.04.20031120
157. Galván Casas C, Català A, Carretero Hernández G, Rodríguez-Jiménez P, Fernández Nieto D, Rodríguez-Villa Lario A, et al. Classification of the cutaneous manifestations of COVID-19: a rapid prospective nationwide consensus study in Spain with 375 cases. *Br J Dermatol.* (2020) **183**:71–7. doi: 10.1111/bjd.19163
158. Lin P-H, Sermersheim M, Li H, Lee PH, Steinberg SM, Ma J. Zinc in wound healing modulation. *Nutrients.* (2017) 10:16. doi: 10.3390/nu10010016

159. Aydemir TB, Sitren HS, Cousins RJ. The zinc transporter Zip14 influences c-Met phosphorylation and hepatocyte proliferation during liver regeneration in mice. *Gastroenterology*. (2012) 142:1536–46.e5. doi: 10.1053/j.gastro.2012.02.046
160. ClinicalTrials.gov. *Coronavirus 2019 (COVID-19)- Using Ascorbic Acid and Zinc Supplementation*. (2020). Available online at: https://ClinicalTrials.gov/show/NCT04342728 (accessed May 15, 2020).
161. ClinicalTrials.gov. *Hydroxychloroquine and Zinc With Either Azithromycin or Doxycycline for Treatment of COVID-19 in Outpatient Setting*. (2020). Available from: https://ClinicalTrials.gov/show/NCT04370782 (accessed May 15, 2020).
162. ANZCTR.org.au. *High-Dose Intravenous Zinc (HDIVZn) As Adjunctive Therapy in COVID-19 Positive Critically Ill Patients: A Pilot Randomized Controlled Trial*. (2020). Available online at: https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=379579&isReview=true (accessed May 15, 2020).

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COVID-19: Poor outcomes in patients with zinc deficiency

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ABSTRACT

Background: Zinc is a trace element with potent immunoregulatory and antiviral properties, and is utilized in the treatment of coronavirus disease 2019 (COVID-19). However, we do not know the clinical significance of serum Zinc levels in COVID-19 patients. The aim of this study was to determine the clinical significance of serum zinc in COVID-19 patients and to establish a correlation with disease severity.
Methods: This was a prospective study of fasting zinc levels in COVID-19 patients at the time of hospitalization. An initial comparative analysis was conducted between COVID-19 patients and healthy controls. COVID-19 patients with zinc deficiency were compared to those with normal zinc levels.
Results: COVID-19 patients (n = 47) showed significantly lower zinc levels when compared to healthy controls (n = 45): median 74.5 (interquartile range 53.4–94.6) μg/dl vs 105.8 (interquartile range 95.65–120.90) μg/dl ($p < 0.001$). Amongst the COVID-19 patients, 27 (57.4%) were found to be zinc deficient. These patients were found to have higher rates of complications (p = 0.009), acute respiratory distress syndrome (18.5% vs 0%, p = 0.06), corticosteroid therapy (p = 0.02), prolonged hospital stay (p = 0.05), and increased mortality (18.5% vs 0%, p = 0.06). The odds ratio (OR) of developing complications was 5.54 for zinc deficient COVID-19 patients.
Conclusions: The study data clearly show that a significant number of COVID-19 patients were zinc deficient. These zinc deficient patients developed more complications, and the deficiency was associated with a prolonged hospital stay and increased mortality.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a major healthcare problem around the world, with significantly higher morbidity and mortality in patients with coexisting conditions such as diabetes mellitus and hypertension (Yang et al., 2020; Adhikari et al., 2020). The clinical presentation can be heterogeneous, ranging from asymptomatic to severe disease, which can be associated with a cytokine storm. The pathogenesis of COVID-19 is not fully understood, but is probably multifactorial, resulting in a systemic hyperinflammatory response and associated thromboembolic complications in severe cases (Yazdanpanah et al., 2020; Galimberti et al., 2020).

Zinc is a trace element with potent immunoregulatory and antiviral properties. Zinc is essential for growth, reproductive health, immunity, and neurobehavioral development (International Zinc Nutrition Consultative Group (IZiNCG) et al., 2004). The recommended daily intake of zinc ranges between 3 mg and 16 mg. Under physiological conditions, zinc is essential for cellular growth and the maturation of immune cells, particularly in the development and activation of T-lymphocytes (Wintergerst et al., 2006). Studies have shown that around 10% of our body proteins utilize zinc and that zinc is a cofactor in at least 200 immunomodulatory and antioxidant reactions (Iddir et al., 2020). Prolonged deficiency is associated with immune system dysfunction, sterility in males, neurosensory disorders, and decreased body mass (Prasad, 2008). Studies have also shown increased viral infection in patients with zinc deficiency (Read et al., 2019).

The antiviral property of zinc has been studied extensively in various viral infections, including coronavirus, hepatitis C virus, and HIV (Barocas et al., 2019). However, the exact role of zinc in

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SARS-CoV-2 is not well studied. The proposed mechanisms of the antiviral property of zinc include the inhibition of RNA synthesis, topoisomerase, and viral replication (Skalny et al., 2020).

To date, there is no definitive curative therapy for COVID-19. Therefore, the current treatment involves a multimodal approach with corticosteroids, antivirals, and anticoagulation therapy. Multivitamin supplements are not uncommon in 'flu' prescriptions. Supplementation with zinc is increasingly recommended in the management of COVID-19 patients (Alexander et al., 2020; Kumar et al., 2020). However, it is unclear whether these patients are actually deficient in zinc.

The aim of this study was to determine the clinical significance of serum zinc levels in COVID-19 patients and to establish a correlation with disease severity.

Methods

A prospective observational study was conducted from May 17 to May 27, 2020, in which serum zinc levels were tested in all consecutive SARS-CoV-2 RT-PCR-positive patients referred to Dr. Rela Institute and Medical Centre, Chennai, for the secondary and tertiary care management of COVID-19. This is a multi-speciality tertiary care institution, currently managing a significant volume of patients with COVID-19. After informed consent had been obtained, about 5 mL of blood was collected in a BD gel vacutainer following 6 h of fasting since the time of hospital admission. The biochemical analysis was performed on the serum sample after separation and the zinc level was measured with a fully automated Indiko Plus analyser (Thermo Scientific, USA) using a colorimetric method. The reference range used for the zinc concentration was 80–120 μg/dl. To verify the accuracy of the method, two levels of random controls were analysed (Randox chemistry control: Human Assay Control-2 LOT-1369 UN, Human Assay Control-3 LOT-1066 UE; Randox Immunoassay Control: level-1 LOT-1862, level-2 LOT-1877, and level-3 LOT 1867). Method performance was monitored by the analysis of the same control serum within the batches. The result obtained agreed with the certified values.

This study was performed following approval from the hospital ethics committee. Only SARS-CoV-2-positive adult patients admitted to the hospital during the study period were included. Patients already on zinc supplements, those who did not require hospital admission, and those who were unwilling to participate in the study were excluded from enrolment. Controls were hospital staff members from the outpatient department with no underlying comorbidities, who underwent a blood test to estimate zinc levels following informed consent.

A comparative analysis was conducted between COVID-19 patients and healthy volunteers. COVID-19 patients were further stratified according to their serum zinc concentration. A zinc level <80 μg/dl was defined as 'deficient'. COVID-19 patients with zinc deficiency were identified and compared to those with normal zinc levels. Corticosteroid therapy was initiated in patients with 'moderate' disease, defined as the presence of any of hypoxia (saturation <92%) measured by pulse oximetry, the requirement for oxygen therapy, tachycardia, or tachypnoea, and in patients with 'severe' disease, defined as any of oxygen saturation <90%, hypotension, acute respiratory distress syndrome (ARDS), or end organ damage. All patients received hydroxychloroquine, antibiotics, and multivitamins, including vitamin C 500 mg twice a day and zinc 150 mg once a day (after the test), Patients with moderate and severe disease received additional subcutaneous anticoagulation for the duration of their hospital stay as the standard of care. Patients were managed in the intensive care unit in case of clinical deterioration causing haemodynamic instability and invasive ventilation.

A descriptive statistical analysis was performed for all variables using IBM SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA), consisting of mean, standard deviation, percentage, median, and interquartile range (IQR; 25–75%). Proportions and associations between characteristics of the study groups were compared by Fisher's exact test. The Mann–Whitney *U*-test and *t*-test were used to compare continuous variables between the study groups. A univariate logistic regression analysis was conducted to determine the odds ratio (OR) and 95% confidence intervals (95% CI). Results were considered statistically significant when the *p*-value was <0.05.

Results

The comparative analysis of COVID-19 patients (n = 47) and healthy controls (n = 45) showed a median age of 34.0 years (range 18–77 years) versus 32.0 years (18–60 years) (p = 0.067) and a male to female sex ratio of 1.6:1 versus 2.1:1 (p = 0.09), respectively. COVID-19 patients had significantly lower zinc levels in comparison to the healthy controls: median 74.5 μg/dl (IQR 53.4–94.6 μg/dl) versus 105.8 μg/dl (IQR 95.65–120.90 μg/dl), $p<0.001$) (Figure 1). Five of the 45 healthy controls had low zinc levels (range 71.8–79.6 μg/dl).

COVID-19 patients: zinc deficiency versus normal levels

Amongst COVID-19 (n = 47) patients, 27 (57.4%) were found to be zinc deficient. A comparative analysis was conducted between COVID-19 patients with zinc deficiency and those with normal zinc levels. Majority of patients presented with fever and cough, and there was no statistically significant difference in these symptoms between the groups (p = 0.481 and p = 0.121). Other symptoms included sore throat, myalgia, and gastrointestinal symptoms, which were observed in both groups with no significant difference between them. Pre-existing comorbid conditions such as age >60 years (7.4% vs 10%, p = 1.0), diabetes mellitus (14.8% vs 15%, p = 1.0), hypertension (14.8% vs 25%, p = 0.40), coronary artery disease (3.7% vs 20%, p = 0.70), pregnancy (7.4% vs 0, p = 1.0), hypothyroidism (3.7% vs 0, p = 0.5), rheumatoid arthritis (3.7% vs 0, p = 1.0), obesity (0 vs 5%, p = 0.42), and bronchial asthma (0 vs 5%, p = 0.42) did not differ significantly between the zinc deficient COVID-19 patients and those with normal zinc levels. At the time of hospitalization, four (8.5%) patients required non-invasive oxygen therapy ranging from 2 to 8 L and four (8.5%) required mechanical ventilation. The disease severity of COVID-19 on admission was mild, moderate, and severe in 21 (77.8%) vs 18 (90%), 1 (3.7%) vs 2 (10%), and 5 (18.5%) vs 0 (p = 0.09) patients with zinc deficiency and those with normal zinc levels, respectively (Table 1).

In total, 14 (29.7%) patients received corticosteroids, commenced on day 5 (median 1–7 days) from the time of admission, of whom 12 (85.7%) had a zinc deficiency. Twelve patients received oxygen therapy during the hospital stay, including six patients on invasive mechanical ventilation.

Complications

Overall, zinc deficient patients developed more complications than those with normal levels: 19 (70.4%) vs 6 (30.0%), respectively (p = 0.009). A subgroup analysis showed that a higher number of patients in the zinc deficient group had ARDS (18.5% vs 0%, p = 0.063), hypotension (14.8% vs 0%, p = 0.126), and elevated interleukin-6 (IL-6) (33.3% vs 15%, p = 0.110) when compared to those with normal zinc levels (Table 2). Interestingly, the median peak IL-6 level was 67.8 pg/mL (IQR 23.8–158.1 pg/mL) vs 10.4 pg/mL (IQR 3.05–44.03 pg/mL) (p = 0.029) for zinc deficient and normal zinc level COVID-19 patients, respectively.

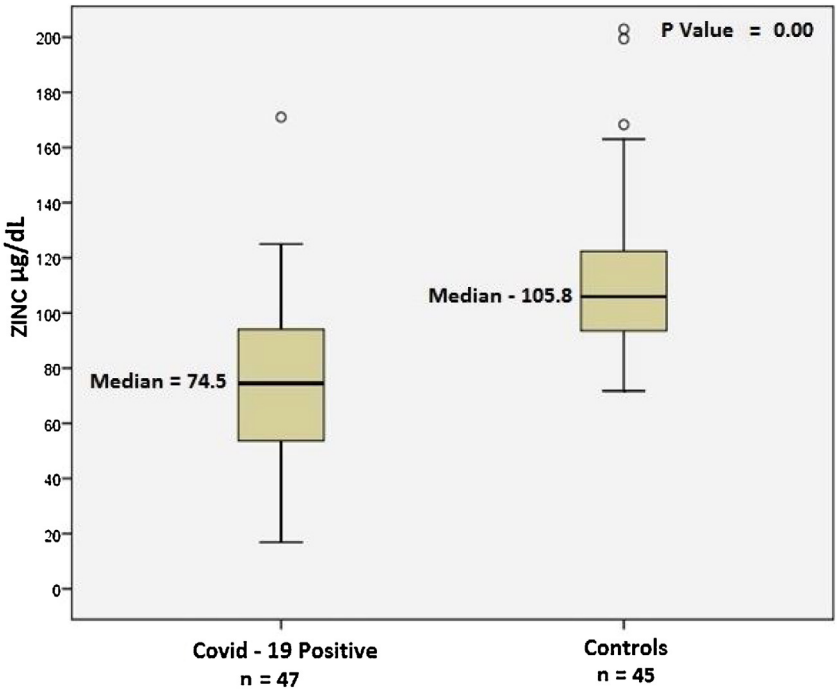

Figure 1. Serum zinc levels in patients with COVID-19 and healthy controls.

A higher number of zinc deficient COVID-19 patients had prolonged hospital stay (≥7 days) when compared to those with normal zinc levels (59.2% vs 30.0%, p = 0.047); the mean hospital stay was 7.9 days and 5.7 days, respectively (t = 2.036, df = 44.7, p = 0.048). Similarly, more proportion of patients in the zinc deficient group received corticosteroids (44.4% vs 10%, p = 0.022) and required intensive care unit (ICU) care (25.9% vs 10%, p = 0.266) when compared to patients with normal zinc levels, and the recorded deaths were higher in the zinc deficient group: 5 (18.5%) vs 0 (0%), p = 0.06. The clinical and treatment characteristics of the patients who died are shown in Table 3.

The OR of developing any complications in zinc deficient COVID-19 patients was 5.54 (95% CI 1.56–19.6, p = 0.008). On further analysis, the OR was 7.2 (95% CI 1.39–37.35, p = 0.02) for corticosteroid use, 3.39 (95% CI 0.99–11.57, p = 0.076) for hospital stay ≥7 days, 3.15 (95% CI 0.58–17.67, p = 0.266) for ICU admission, and 5.48 (95% CI 0.61–49.35, p = 0.129) for mortality.

Discussion

This appears to be the first clinical study correlating lower baseline zinc levels in patients with COVID-19 compared to healthy controls (74.5 µg/dl vs 105.8 µg/dl, p <0.001). Amongst COVID-19 patients, 57.4% (n = 27) were zinc deficient. However, we do not know whether zinc deficiency in these patients is a causation or an epiphenomenon. In vitro studies have shown that SARS-CoV-2 viral spike protein interacts with angiotensin-converting enzyme 2 (ACE2) and the serine protease transmembrane protease serine 2 (TMPRSS2) in the alveoli, permitting its entry into the cells. Interestingly, ACE2 is a zinc-dependent peptidyl dipeptide hydrolase composed of two subdomains (I and II), of which N-terminal containing subdomain I and C-terminus containing subdomain II are involved with zinc binding (Reeves and O'Dell, 1985). This process is facilitated and coordinated by amino acids His^{374}, His^{378}, Glu^{402} (HEXXH + E motif) and a molecule of water at subdomain I and by amino acids Arg^{169}, Trp^{477}, and Lys^{481} with a chloride ion at subdomain two (Towler et al., 2004). Earlier studies demonstrated that a decreased zinc level favours this interaction of ACE2 with SARS-CoV-2 spike protein and likewise that an increased zinc level inhibits ACE2 expression resulting in reduced viral interaction (Devaux et al., 2020; Li et al., 2020).

The zinc–viral particle interplay has been studied previously with other RNA viruses such as hepatitis C virus, coronavirus, HIV, and influenza virus (te Velthuis et al., 2010; Ferrari et al., 1999; Haraguchi et al., 1999; Ghaffari et al., 2019). Zinc supplements are traditionally prescribed for common cold ailments, usually caused by coronaviruses. Zinc supplements have been associated with a shortened duration of symptoms, reduced severity of illness, and more importantly with reduced morbidity and mortality in children (Wessels et al., 2020).

Zinc has been shown to exhibit antiviral properties by inhibition of RNA synthesis, viral replication, DNA polymerase, reverse transcriptase, and viral proteases (Read et al., 2019; Ko et al., 2018; Xue et al., 2014). However, the literature is unclear regarding SARS-CoV-2 and zinc. Interestingly, hydroxychloroquine, a drug used initially in the management of COVID-19, is an ionophore that transports zinc across the hydrophobic cell membrane (Xue et al., 2014; Rahman and Idid, 2020). Moreover, evidence specifically suggests that zinc supplements with antiviral drugs containing zinc ionophores precisely target and bind to SARS-CoV-2 preventing its replication within the infected host cells (te Velthuis et al., 2010). Intracellularly, zinc binds with RNA-dependent RNA polymerase causing elongation inhibition and decreased template binding of the viral mRNA (Rahman and Idid, 2020; te Velthuis et al., 2010).

Zinc plays a major role at various levels in the process of immune development and acts as an immunomodulator. Zinc deficiency has been associated with poor development of lymphoid tissue and reduced natural killer (NK) cell function leading to poor innate immunity (Shankar and Prasad, 1998). Zinc deficiency is associated with reduced macrophage activation and cytokine generation. Zinc is involved in T-cell and B-cell function. Thymulin, a zinc-dependent thymus hormone, binds to T-cell receptor and promotes T-cell maturation and cytotoxicity (Prasad, 2008). In addition, zinc deficiency is associated with down-regulation of interferon gamma, resulting in severe impairment of

Table 1
Comparison of variables in COVID-19 patients on admission: zinc deficient versus normal zinc level.

Variables	Zinc deficient COVID-19 patients (n = 27) (57.4%)		Normal zinc level COVID-19 patients (n = 20) (42.6%)		p-Value
	n	%	n	%	
Age (years), median (IQR)	33 (18–75)		35 (27–77)		0.546
Male to female sex ratio	1.7:1		3:1		0.529
Asymptomatic	1	3.7	2	10	0.567
Fever	20	74.1	17	85	0.481
Cough	12	44.4	4	20	0.121
Sore throat	5	18.5	1	5	0.221
Loose stools	4	14.8	4	20	0.707
Myalgia	6	22.2	4	25	0.5
Nausea	0	0	1	5	0.426
Anosmia	1	3.7	0	0	1.0
Dyspnoea	4	14.8	3	15	1.0
Comorbidities					
Diabetes mellitus	4	14.8	3	15	1.0
Systemic hypertension	4	14.8	5	25	0.405
Coronary artery disease	1	3.7	4	20	0.707
Pregnancy	2	7.4	0	0	1.0
Hypothyroidism	1	3.7	0	0	0.5
Rheumatoid arthritis	1	3.7	0	0	1.0
Obesity	0	0	1	5	0.426
Age >60 years	2	7.4	2	10	1.0
Bronchial asthma	0	0	1	5	0.426
Laboratory indices, median (IQR)					
Bilirubin (mg/dl) (Normal 0.2–1.2)	0.48 (0.35–0.48)		0.57 (0.38–0.90)		0.241
AST (U/l) (Normal 0–45)	28 (18–34)		25 (18–32)		0.639
ALT (U/l) (Normal 0–47)	18 (11–32)		22 (19–28)		0.517
Creatinine (mg/dl) (Normal 0.5–1.3)	0.80 (0.69–0.93)		0.96 (0.64–1.05)		0.166
LDH (U/l) (Normal 135–225)	264 (206.5–417.5)		200 (169–242)		0.006
Ferritin (ng/mL) (Normal 28–397)	216.0 (70.5–511.2)		202.3 (98.7–313.4)		0.622
CRP (mg/l) (Normal <5)	11.0 (3.5–48.5)		3.6 (1.3–35.8)		0.144
D-dimer (ng/mL) (Normal <250)	499.0 (237–603)		158.5 (106.75–487.5)		0.108
Fasting glucose (mg/dl) (Normal 70–100)	110 (93–128)		101.5 (92.7–142.5)		0.780
Triglyceride (mg/dl) (Normal <150)	103 (76–167)		124 (101.2–190.2)		0.165
Vitamin D (ng/mL) (Normal 0–30)	13.6 (11.3–25.7)		19.3 (12.9–22.2)		0.533
Disease severity on admission					0.09
Mild	21 (77.8%)		18 (90%)		
Moderate	1 (3.7%)		2 (10%)		
Severe	5 (18.5%)		0		

AST, aspartate aminotransferase; ALT, alanine aminotransferase; CRP, C-reactive protein; IQR, interquartile range; LDH, lactate dehydrogenase.

Table 2
Complications in COVID-19 patients during hospital stay: zinc deficient versus normal zinc level.

Complications	Zinc deficient COVID-19 patients (n = 27) (57.4%)		Normal zinc levelCOVID-19 patients (n = 20) (42.6%)		p-Value
	n	%	n	%	
Corticosteroids	12	44.48	2	10	0.022
ARDS	5	18.5	0	0	0.063
Hypotension	4	14.8	0	0	0.126
Sepsis	1	3.7	0	0	1.0
IL-6 > 7 pg/mL	9	33.3	3	15	0.110
Others[a]	2	7.4	1	5	1.0
ICU	7	25.9	2	10	0.266
Hospital stay ≥7 days	16	59.3	6	30	0.047
Death	5	18.5	0	0	0.06

ARDS, acute respiratory distress syndrome; ICU, intensive care unit; IL-6, interleukin 6.
[a] Others: melena, hyponatremia, and hypokalemia; one in each patient.

cell-mediated immunity. Also, it enhances the production of interleukins, particularly IL-2, via activation of nuclear factor kappa B (NF-κB) (Prasad et al., 2001). The above in vitro studies indicate that zinc deficiency is associated with immune dysfunction and the risk of infection. The role of zinc in the immunology of SARS-CoV-2 infection definitely warrants further clinical research.

ADAM enzymes (A disintegrin and metalloproteinase) are zinc-dependent cell surface proteins of the adamalysin protein family known to play a major role in inflammation. ADAM 17 catalyses the activation of the proinflammatory cytokine tumour necrosis factor alpha (TNF-α) and conversion of membrane-bound (m)IL-6 to soluble (s)-IL6. Targeting the inhibition of ADAM 17 at the zinc

Table 3
Clinical and treatment characteristics of patients with COVID-19 who died.

	Case 1	Case 2	Case 3	Case 4	Case 5
Age (years)/sex	40/M	51/F	50/F	72/F	75/F
Comorbidities	CAD	DM, HT	DM, HT, hypothyroid	DM, HT	Nil
Initial symptoms	Fever Myalgia Cough	Fever Anorexia	Fever Dyspnoea	Dyspnoea	Fever Dyspnoea
Duration of symptoms (days)	2	5	7	3	2
Complications	ARDS	Sepsis, ARDS	ARDS, MODS	ARDS	ARDS
Treatment	Methylprednisolone Supplements[a]Antibiotics Enoxaparin	Methylprednisolone Supplements[a]Piperacillin –tazobactam Enoxaparin	Methylprednisolone Supplements[a]Piperacillin –tazobactam Enoxaparin	Methylprednisolone Supplements[a]Piperacillin –tazobactam Enoxaparin	Methylprednisolone Supplements[a]M eropenem Enoxaparin
Hospital stay (days)	3	7	3	8	18
Admission zinc level (μg/dl)	36.4	47	57	59	81
WBC count ($\times 10^9$/l)	4.6	16.8	13.3	9.07	18.2
Lymphocyte count ($\times 10^9$/l)	0.73	0.67	1.33	1.18	1.27
CRP (ng/mL)	32.5	108.3	227	193.3	300.9
Ferritin (ng/dl)	979.8	203.6	636.5	514.5	1441

ARDS, acute respiratory distress syndrome; CAD, coronary artery disease; CRP, C-reactive protein; DM, type 2 diabetes mellitus; F, female; HT, systemic hypertension; M, male; MODS, multi-organ dysfunction syndrome; WBC, white blood cell.
[a] Supplements: vitamin C 500 mg twice a day and zinc 150 mg once a day.

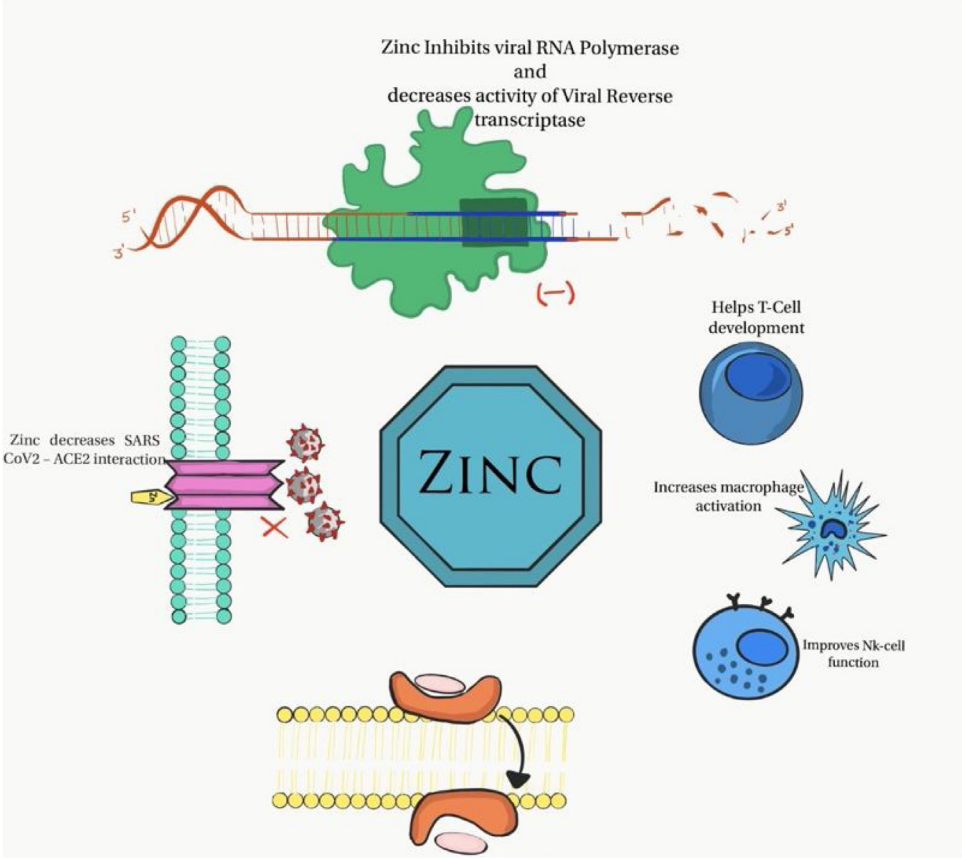

Figure 2. Illustration of antiviral and immunomodulatory properties of zinc in COVID-19.

cofactor site inhibits the enzyme, causing downregulation of inflammation by inhibiting these two pathways (Kato et al., 2006; Henry et al., 2020a).

These in vitro evidence suggests that zinc may have a pivotal role in COVID-19 (Figure 2). Therefore, zinc deficiency in COVID-19 patients may not be just a mere association. More studies are required to ascertain the relationship between COVID-19 and zinc.

The present study data clearly demonstrate a higher complication rate (70.4% vs 30.0%, p = 0.009) in zinc deficient COVID-19 patients, with an OR of 5.54. In addition, these patients showed an increased trend towards the development of ARDS (18.5% vs 0%, p = 0.06), longer hospital stays (mean 7.9 vs 5.7 days, p = 0.048), were more likely to have received corticosteroids (44.4% vs 10%, p = 0.02), and had increased mortality (5 (18.5%) vs 0 (0%), p = 0.06),

indicative of a severe disease spectrum in these patients. This study showed an association between the baseline zinc level and COVID-19 disease course, such that zinc deficient patients encounter more complications and mortality.

Lactate dehydrogenase (LDH) is an intracellular enzyme, present in most cells, that catalyses the interconversion of pyruvate and lactate. LDH is a marker of organ injury, particularly related to hypoxia (Shi et al., 2020). A pooled analysis of 1532 COVID-19 patients showing elevated LDH found that this was associated with a 6-fold increased risk of severe disease and 16-fold increased risk of death (Henry et al., 2020b). The elevated LDH in the present study was probably indicative of severe disease as a result of zinc deficiency.

This was a single-centre study with a limited number of patients requiring hospitalization for COVID-19. It would be interesting to study the zinc level and its role across the entire spectrum of the disease, including asymptomatic patients with no comorbid conditions who are otherwise managed with home isolation. Moreover, it is unclear whether low zinc is a simple association or a causative factor in COVID-19. The literature and our understanding of zinc in COVID-19 patients is currently limited. Clearly, a multi-centre study is required to throw more light on this specific issue.

In conclusion, this study clearly demonstrated that COVID-19 patients were zinc deficient when compared to healthy adults. It is convincing that low baseline zinc levels in these patients were associated with more complications, leading to prolonged hospitalization and increased mortality. It is not clear whether zinc supplementation after admission to hospital helps to reduce the severity of disease. It is worth exploring the exact role of zinc in COVID-19 patients and establishing the appropriate dosage to improve their survival. With more research, zinc could provide a cost-effective therapy for COVID-19, certainly the need of the hour in this pandemic.

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Ethical approval

Ethical approval was obtained.

Conflict of interest

None.

CRediT authorship contribution statement

Dinesh Jothimani: Conceptualisation, Methodology, Formal analysis, Writing - original draft. **Ezhilarasan Kailasam:** Methodology, Investigation. **Silas Danielraj:** Data curation, Formal analysis, Writing - review & editing. **Balaji Nallathambi:** Data curation, Writing - review & editing. **Hemalatha Ramachandran:** Formal analysis, Software. **Padmini Sekar:** Writing - original draft. **Shruthi Manoharan:** Visualization. **Vidyalakshmi Ramani:** Writing - review & editing. **Gomathy Narasimhan:** Writing - review & editing. **Ilankumaran Kaliamoorthy:** Writing - review & editing. **Mohamed Rela:** Supervision, Validation, Writing - review & editing.

References

Adhikari SP, Meng S, Wu YJ, et al. Epidemiology, causes, clinical manifestation and diagnosis, prevention and control of coronavirus disease (COVID-19) during the early outbreak period: a scoping review. Infect Dis Poverty 2020;9(1)29, doi:http://dx.doi.org/10.1186/s40249-020-00646-x Published 2020 March 17.

Alexander J, Tinkov A, Strand TA, Alehagen U, Skalny A, Aaseth J. Early nutritional interventions with zinc, selenium and vitamin D for raising anti-viral resistance against progressive COVID-19. Nutrients 2020;12(8)E2358, doi:http://dx.doi.org/10.3390/nu12082358 Published 2020 August 7.

Barocas JA, So-Armah K, Cheng DM, et al. Zinc deficiency and advanced liver fibrosis among HIV and hepatitis C co-infected anti-retroviral naïve persons with alcohol use in Russia. PLoS One 2019;14(6)e0218852, doi:http://dx.doi.org/10.1371/journal.pone.0218852 Published 2019 June 27.

Devaux CA, Rolain JM, Raoult D. ACE2 receptor polymorphism: susceptibility to SARS-CoV-2, hypertension, multi-organ failure, and COVID-19 disease outcome. J Microbiol Immunol Infect 2020;53(3):425–35, doi:http://dx.doi.org/10.1016/j.jmii.2020.04.015.

Ferrari E, Wright-Minogue J, Fang JW, Baroudy BM, Lau JY, Hong Z. Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in *Escherichia coli*. J Virol 1999;73(2):1649–54, doi:http://dx.doi.org/10.1128/JVI.73.2.1649-1654.1999.

Galimberti S, Baldini C, Baratè C, et al. The CoV-2 outbreak: how hematologists could help to fight Covid-19. Pharmacol Res 2020;157:104866, doi:http://dx.doi.org/10.1016/j.phrs.2020.104866.

Ghaffari H, Tavakoli A, Moradi A, et al. Inhibition of H1N1 influenza virus infection by zinc oxide nanoparticles: another emerging application of nanomedicine. J Biomed Sci 2019;26(1)70, doi:http://dx.doi.org/10.1186/s12929-019-0563-4 Published 2019 September 10.

Haraguchi Y, Sakurai H, Hussain S, Anner BM, Hoshino H. Inhibition of HIV-1 infection by zinc group metal compounds. Antiviral Res 1999;43(2):123–33, doi:http://dx.doi.org/10.1016/s0166-3542(99)00040-6.

Henry BM, Aggarwal G, Wong J, et al. Lactate dehydrogenase levels predict coronavirus disease 2019 (COVID-19) severity and mortality: a pooled analysis [published online ahead of print, 2020 May 27]. Am J Emerg Med 2020a;38:1722–6, doi:http://dx.doi.org/10.1016/j.ajem.2020.05.073.

Henry BM, Aggarwal G, Wong J, et al. Lactate dehydrogenase levels predict coronavirus disease 2019 (COVID-19) severity and mortality: a pooled analysis. Am J Emerg Med 2020b;, doi:http://dx.doi.org/10.1016/j.ajem.2020.05.073 Epub ahead of print. PMCID: PMC7251362.

Iddir M, Brito A, Dingeo G, et al. Strengthening the immune system and reducing inflammation and oxidative stress through diet and nutrition: considerations during the COVID-19 crisis. Nutrients 2020;12(6)E1562, doi:http://dx.doi.org/10.3390/nu12061562 Published 2020 May 27.

International Zinc Nutrition Consultative Group (IZiNCG), Brown KH, Rivera JA, et al. International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. Food Nutr Bull 2004;25(1 Suppl 2):S99–S203.

Kato GJ, McGowan V, Machado RF, et al. Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. Blood 2006;107(6):2279–85, doi:http://dx.doi.org/10.1182/blood-2005-06-2373.

Ko YL, Morihara D, Shibata K, et al. Factors attenuating zinc deficiency improvement in direct-acting antiviral agent-treated chronic hepatitis C virus infection. Nutrients 2018;10(11)1620, doi:http://dx.doi.org/10.3390/nu10111620 Published 2018 November 2.

Kumar A, Kubota Y, Chernov M, Kasuya H. Potential role of zinc supplementation in prophylaxis and treatment of COVID-19 [published online ahead of print, 2020 May 25]. Med Hypotheses 2020;144:109848, doi:http://dx.doi.org/10.1016/j.mehy.2020.109848.

Li MY, Li L, Zhang Y, Wang XS. Expression of the SARS-CoV-2 cell receptor gene ACE2 in a wide variety of human tissues. Infect Dis Poverty 2020;9(1)45, doi:http://dx.doi.org/10.1186/s40249-020-00662-x Published 2020 April 28.

Prasad AS. Zinc in human health: effect of zinc on immune cells. Mol Med 2008;14(5–6):353–7, doi:http://dx.doi.org/10.2119/2008-00033.Prasad.

Prasad AS, Bao B, Beck FW, Sarkar FH. Zinc activates NF-kappaB in HUT-78 cells. J Lab Clin Med 2001;138(4):250–6, doi:http://dx.doi.org/10.1067/mlc.2001.118108.

Rahman MT, Idid SZ. Can Zn Be a critical element in COVID-19 treatment? [published online ahead of print, 2020 May 26]. Biol Trace Elem Res 2020;1–9, doi:http://dx.doi.org/10.1007/s12011-020-02194-9.

Read SA, Obeid S, Ahlenstiel C, Ahlenstiel G. The role of zinc in antiviral immunity. Adv Nutr 2019;10(4):696–710, doi:http://dx.doi.org/10.1093/advances/nmz013.

Reeves PG, O'Dell BL. An experimental study of the effect of zinc on the activity of angiotensin converting enzyme in serum. Clin Chem 1985;31(4):581–4.

Shankar AH, Prasad AS. Zinc and immune function: the biological basis of altered resistance to infection. Am J Clin Nutr 1998;68(2 Suppl):447S–63S, doi:http://dx.doi.org/10.1093/ajcn/68.2.447S.

Shi J, Li Y, Zhou X, et al. Lactate dehydrogenase and susceptibility to deterioration of mild COVID-19 patients: a multicenter nested case-control study. BMC Med 2020;18(1)168, doi:http://dx.doi.org/10.1186/s12916-020-01633-7 Published 2020 June 3.

Skalny AV, Rink L, Ajsuvakova OP, et al. Zinc and respiratory tract infections: perspectives for COVID-19 (Review). Int J Mol Med 2020;46(1):17–26, doi:http://dx.doi.org/10.3892/ijmm.2020.4575.

te Velthuis AJ, van den Worm SH, Sims AC, Baric RS, Snijder EJ, van Hemert MJ. Zn(2+) inhibits coronavirus and arterivirus RNA polymerase activity in vitro and zinc ionophores block the replication of these viruses in cell culture. PLoS Pathog 2010;6(11)e1001176, doi:http://dx.doi.org/10.1371/journal.ppat.1001176 Published 2010 November 4.

Towler P, Staker B, Prasad SG, et al. ACE2 X-ray structures reveal a large hinge-bending motion important for inhibitor binding and catalysis. J Biol Chem 2004;279(17):17996–8007, doi:http://dx.doi.org/10.1074/jbc.M311191200.

Wessels I, Rolles B, Rink L. The potential impact of zinc supplementation on COVID-19 pathogenesis. Front Immunol 2020;11:1712, doi:http://dx.doi.org/10.3389/fimmu.2020.01712 Published 2020 July 10.

Wintergerst ES, Maggini S, Hornig DH. Immune-enhancing role of vitamin C and zinc and effect on clinical conditions. Ann Nutr Metab 2006;50(2):85–94, doi:http://dx.doi.org/10.1159/000090495.

Xue J, Moyer A, Peng B, Wu J, Hannafon BN, Ding WQ. Chloroquine is a zinc ionophore. PLoS One 2014;9(10)e109180, doi:http://dx.doi.org/10.1371/journal.pone.0109180 Published 2014 October 1.

Yang J, Zheng Y, Gou X, et al. Prevalence of comorbidities and its effects in patients infected with SARS-CoV-2: a systematic review and meta-analysis. Int J Infect Dis 2020;94:91–5, doi:http://dx.doi.org/10.1016/j.ijid.2020.03.017.

Yazdanpanah F, Hamblin MR, Rezaei N. The immune system and COVID-19: Friend or foe? [published online ahead of print, 2020 June 2]. Life Sci 2020;256:117900, doi:http://dx.doi.org/10.1016/j.lfs.2020.117900.

People with vitamin D deficiency at higher risk of severe COVID-19, says study

By Angela Betsaida B. Laguipo, BSNDec 4 2020

Caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the coronavirus disease 2019 (COVID-19) causes respiratory illness, vast inflammation, and in some cases, a cytokine storm – the latter of which can be fatal.

Some people are at a higher risk of severe coronavirus disease (COVID-19), typically due to underlying health conditions. As the pandemic evolves, however, many health experts have found that nutrient deficiency can also help contribute to severe illness.

In particular, recent evidence shows that vitamin deficiency can be tied to severe COVID-19.

A new study has revealed that vitamin D deficient patients are more likely to experience severe COVID-19.

Study: Effect of Vitamin D deficiency on COVID-19 status: A systematic review. Image Credit: Kavun Halyna / Shutterstock

The study, which appeared on the pre-print *medRxiv** server, tackled the findings of previous studies on vitamin D deficiency and COVID-19.

Vitamin D and COVID-19

One major micronutrient known to have a possible protective or mitigating effect against SARS-CoV-2 is vitamin D. Past studies have shown how vitamin D provides benefit for patients with

COVID-19. A naturally occurring vitamin in humans, vitamin D is produced when the skin is exposed to ultraviolet radiation from the sun. It can also be obtained from the diet.

Since COVID-19 is tied to immune hyperactivation, the protective effect of vitamin D has been attributed to its ability to suppress immune responses to the virus, thereby reducing the risk of severe acute respiratory distress syndrome (ARDS), a fatal complication of COVID-19 that is linked to an overreaction on the part of the immune system.

The dietary intake of vitamin D plays an imperative role in determining one's vitamin D status. Some foods, mainly coldwater fish like herring, sardines, mackerel, and salmon, contain vitamin D. Meanwhile, some doctors recommend vitamin D3 supplementation in the form of tablets.

The striking link between vitamin D deficiency and the development of common COVID-19 risk factors like obesity and older age has influenced scientists to theorize that vitamin D supplements could be used as a preventive and protective agent against SARS-CoV-2 infection.

Some researchers also noted that since COVID-19 is linked to immune hyperactivation and a cytokine storm, vitamin D can help prevent severe illness. Vitamin D regulates immunopathological inflammatory responses and supports innate antiviral effector mechanisms, hence boosting the immune system to work harder during an infection.

The study

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The study highlights the importance of having sufficient vitamin D levels in the body to combat the coronavirus pandemic.

To arrive at the study findings, the researchers conducted a meta-analysis by conducting a wide-reaching search for studies related to vitamin D and COVID-19 from three databases, including PubMed, ScienceDirect, and Google Scholar.

The search consisted of keywords such as vitamin D, 25-Hydroxyvitamin D, low vitamin D, COVID-19, SARS-CoV-2, coronavirus, or 2019-nCoV. They also searched for terms such as disease severity, ICU admission, and mortality.

Overall, the team included titles and abstracts of 2,774 articles. From there, they screened the full text of 17 articles.

The team found that in one particular study, vitamin D deficient patients were 5.84 times more likely to die from COVID-19 compared to people with sufficient vitamin D levels. Another study showed that vitamin D deficiency is tied to a higher risk of death.

Further readings of the selected articles showed that after controlling for confounders, patients with low 25-Hydroxyvitamin D have more risk of testing positive for COVID-19. The team also found that the SARS-CoV-2 positivity rate is lower in patients with adequate 25-Hydroxyvitamin D levels in the body.

"Findings from the study included suggest Vitamin D may serve as a mitigating effect for covid19 infection, severity, and mortality," the team concluded in the study.

"We recommend the need to encourage people to eat foods rich in vitamin D such as fish, red meat, liver, and egg yolks while at the same time providing vitamin D supplements for individuals with COVID-19 to boost their immune systems," the team added.

Finding ways to mitigate the ongoing pandemic is imperative. So far, 65.23 million people have been infected with SARS-CoV-2. Of these, at least 1.50 million have died.

Scientists and pharmaceutical companies are racing to develop effective antivirals and vaccines to mitigate or prevent SARS-CoV-2 infection. While the world waits for a safe and effective vaccine, it is crucial to observe basic infection control measures, such as physical distancing, regular hand hygiene, and masks. Also, sustaining the body with adequate amounts of nutrients can boost the immune system to fight off pathogens, as this meta-analysis's findings emphasize.

*Important Notice

medRxiv publishes preliminary scientific reports that are not peer-reviewed and, therefore, should not be regarded as conclusive, guide clinical practice/health-related behavior, or treated as established information.

Source:

- COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University (JHU) - https://gisanddata.maps.arcgis.com/apps/opsdashboard/index.html#/bda7594740fd40299423467b48e9ecf6

Journal reference:

- Das, P., Samad, N., Ahinkorah, B., Peprah, P. et al. (*2020*). Effect of Vitamin D deficiency on COVID-19 status: A systematic review. *medRxiv*. https://www.medrxiv.org/content/10.1101/2020.12.01.20242313v1

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https://www.forbes.com/sites/victoriaforster/2020/10/27/8-in-10-hospitalized-covid-19-coronavirus-patients-were-vitamin-d-deficient-new-study/?sh=77b8fe8a66ec

EDITORS' PICK|Oct 27, 2020, 02:30pm EDT| 78,913 views

8 In 10 Hospitalized Covid-19 Coronavirus Patients Were Vitamin D Deficient: New Study

Victoria Forster Contributor Healthcare
Cancer research scientist and childhood cancer survivor.

One study has found that over 8 in 10 hospitalized Covid-19 patients had a vitamin D deficiency. getty

Researchers in Spain have discovered that 82% of hospitalized patients with Covid-19 were vitamin D deficient in a new study published today. They studied levels of the vitamin in 216 patients admitted to the hospital for Covid-19 treatment in March this year, finding that 8 in 10 patients were considered clinically deficient.

The work published today in *The Journal of Clinical Endocrinology & Metabolism* also looked at a control group of 197 people who lived in the same geographical area and were similar to the Covid-19 patient group in age and sex. Among these people, 47% were vitamin D deficient.

However, it is important to point out that the conclusions of this study are correlative, not causative - meaning that it can't be concluded from this work that vitamin D deficiency was directly responsible for a higher chance of being hospitalized with Covid-19.

Vitamin D is essential for bone health, but is also thought to have beneficial effects on the immune system too with some evidence that having sufficient vitamin D may give some protection against respiratory tract infections. Despite its importance, vitamin D deficiency is common, with one study from 2011 estimating that 41.6% of U.S. adults are deficient. This number varies greatly depending on race with 82.1% of Black Americans being deficient and 69.2% of Hispanic Americans.

Vitamin D can be produced naturally by exposure to sunlight and found in some foods, including oily fish, eggs and fortified milk and plant-based milk substitutes.

Vitamin D is found in some foods, notably oily fish, fortified milks and eggs getty

The Spanish research is not alone in suggesting that vitamin D might be beneficial in protecting hospitalized individuals from adverse outcomes. Last month, a study from researchers in Boston found that patients over 40 were over 50% less likely to die from the infection if they had sufficient levels of vitamin D. However, the more recent study from Spain wasn't able to conclude any link between vitamin D deficiency and severity of disease. So the information regarding any benefit of vitamin D for Covid-19 is still a bit inconclusive.

In certain parts of the world, especially where sunlight hours are limited during winter, vitamin D supplementation is routinely recommended by physicians. So, should everyone start taking vitamin D now even if it isn't clear whether it might help with Covid-19? Well, not necessarily. It is important to note that too much vitamin D can be toxic and it can also interact with certain

medications. So, if in any doubt, check with your doctor about whether it is recommended and safe to take vitamin D supplements.

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Victoria Forster

I am a postdoctoral research scientist focusing on childhood cancers and new, targeted cancer therapies. As a survivor of childhood leukemia myself, I am a determined advocate for research into better, less-toxic cancer treatments and how to reduce the long-term side effects of current drugs. I am an award-winning science communicator and have written for The Times, The Guardian and various cancer-focused outlets. I am also a 2017 TED Fellow, having done my TED talk on cancer survivorship and I regularly do public talks on topics ranging from ‘Why haven’t we cured cancer yet?’ to ‘Cannabis and cancer; hype or hope?’. I am passionate about using social media to communicate science and frequently share pictures and stories from my own laboratory work in real-time on my Twitter account @vickyyyf, alongside commentary about important research breakthroughs. You can find out more about me and how to get in contact via my website drvickyforster.com. All of my articles reflect my personal views and not those of my employer

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Review

Clinical Trials for Use of Melatonin to Fight against COVID-19 Are Urgently Needed

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Abstract: The recent pandemic of COVID-19 has already infected millions of individuals and has resulted in the death of hundreds of thousands worldwide. Based on clinical features, pathology, and the pathogenesis of respiratory disorders induced by this and other highly homogenous coronaviruses, the evidence suggests that excessive inflammation, oxidation, and an exaggerated immune response contribute to COVID-19 pathology; these are caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This leads to a cytokine storm and subsequent progression triggering acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), and often death. We and others have reported melatonin to be an anti-inflammatory and anti-oxidative molecule with a high safety profile. It is effective in critical care patients by reducing their vascular permeability and anxiety, inducing sedation, and improving their quality of sleep. As melatonin shows no harmful adverse effects in humans, it is imperative to introduce this indoleamine into clinical trials where it might be beneficial for better clinical outcomes as an adjuvant treatment of COVID-19-infected patients. Herein, we strongly encourage health care professionals to test the potential of melatonin for targeting the COVID-19 pandemic. This is urgent, since there is no reliable treatment for this devastating disease.

Keywords: melatonin; COVID-19; inflammation; immune response; clinical trials

1. Introduction

As of today (22 July 2020) there have been more than 616,317 deaths worldwide from coronavirus (COVID-19), a newly emerged respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Regarding the most affected countries to date, the USA has reported 142,066 deaths, Brazil 81,487, the United Kingdom 45,422, Mexico 40,400, Italy 35,073, and France 30,165 [1]. These large numbers warrant urgent research to accelerate clinical trials with therapies that may reduce the alarmingly high death rate. The combined use of anti-viral and anti-inflammatory drugs may be more efficient than using either modality alone. One of the overlooked and promising candidates is melatonin, which may substantially enhance the actions of adjuvant treatments for COVID-19 by reducing symptoms such as pneumonia, acute lung injury (ALI), and acute respiratory distress syndrome (ARDS). At present, the low efficacy of anti-viral drugs on COVID-19 is not surprising. Due to increased drug resistance and continuously occurring mutations of the virus, we still lack ideal medicines to target this disease and new vaccines have to be repeatedly adapted to the continuously changing viral subtypes. In fact, the drugs in the market can only mitigate the mild to moderate

symptoms if used in the early stage of viral infection, and have reduced effects in patients with severe symptoms or those who are predisposed to complications. Thus, the clinical significance is limited, as well as because viral infectious diseases are self-limiting and the mild to moderately severe patients develop self-recovery without treatment. In viral infectious diseases, the key is to ameliorate the severe symptoms, including the massive tissue and organ injury and, finally, to control the mortality. It has been speculated that the most severe symptoms are beyond viral cytotoxicity *per se* and result from the overreaction of the innate immune response that causes destructive inflammation, as observed in the severe disease progression of coronavirus infections [2]. This may be one of the reasons why antiviral drugs have failed to be effective in severely infected patients. To compensate for the shortcomings of the anti-viral drugs, a more generalized and less virus-specific therapy which instead targets severe symptoms of the viral infection should be considered. Melatonin is a suitable candidate. Melatonin possesses an excellent anti-oxidative and anti-inflammatory capacity and it balances the overshooting innate immune response while promoting adaptive immunity [3–6]. Currently, an increasing number of publications has suggested or strongly recommended the use of melatonin to combat COVID-19.

2. Pathogenesis of COVID-19

To date, the effect of SARS-CoV-2 on humans have been clearly age-related. Thus, the excessive mortality rate occurs in the elderly with very few deaths from COVID-19 being recorded for individuals under the age of 20. Currently reported COVID-19-affected patients present varying symptoms including fever, dry cough, myalgia, fatigue, or diarrhea. In other cases, the acute progression of the disease results in ALI/ARDS, respiratory failure, heart failure, sepsis, and sudden cardiac arrest within a few days [7–9]. The pathogenic examination of lungs from mild COVID-19 patients revealed edema, proteinaceous exudate with globules, patchy inflammatory cellular infiltration, and moderate formation of hyaline membranes [10]. In a postmortem assessment of a COVID-19 patient with severe ARDS, specimens of the infected lungs demonstrated bilateral diffuse alveolar damage with edema, pneumocyte desquamation, and hyaline membrane formation [11]. Although these reports were performed for only a small number of cases, they do resemble the pathological features identified in SARS- or MERS-induced respiratory disorders [12].

As recently reviewed [13], SARS-CoV-2 shares 79.0% nucleotide identity with SARS-CoV and 51.8% identity with MERS-CoV, indicating their high genetic homology. In SARS-CoV and MERS-CoV, infected animal models revealed an inflammatory response which causes a "cytokine storm", subsequently triggering vascular leakage and abnormal innate and adaptive immune responses, including lymphopenia and an increase in neutrophils, thereby inducing ALI/ARDS or even death [14]. In the early stages of coronavirus infections, dendritic and epithelial cells are activated and cause a reported "deluge" of pro-inflammatory cytokines, i.e., elevated levels of interleukin-1β (IL-1β), IL-6, interferon-γ (IFN-γ), interferon-inducible protein 10 (IP-10) or IL-4, IL-10, and IL-17 [5,9]. It was documented that repressed immune functions in COVID-19 patients are accompanied by lymphopenia and neutropenia, as well as a decreased number of CD8+ T cells [7–9]. Furthermore, recent reports suggest that some COVID-19 patients, although negative for the viral nucleic acid assay, still sometimes present with a high level of inflammation. Altogether, the most recent findings indicate that inflammation is a major issue for COVID-19 patients in whom the immune system is severely attenuated due to the high cytokine production that contributes to the COVID-19 pathogenesis. It should be added that the amplification of the inflammatory response would promote programmed cell death (apoptosis) or necrosis of the affected cells, which would further trigger inflammation, followed by the increasing permeability of blood vessels and the aberrant accumulation of inflammatory cells, including monocytes, macrophages, and neutrophils. The resultant vicious circle intensifies the situation as the regulation of immune response is lost and the "cytokine storm" is further activated, leading to serious consequences. Similarly, this putative "cytokine burst" pathology associated with coronaviruses is also supported by experimental SARS-CoV models, one of which showed that the severity of ALI was accompanied by an elevated expression of inflammation-related genes rather

than increased viral titers. In another case, the ablation of IFN-α/β receptors or the depletion of inflammatory monocytes/macrophages caused a marked rise in the survival rate of coronavirus hosts without a change in viral load [15,16]. Both situations suggest a potential amplifying mechanism involved in CoV-induced ALI/ARDS regardless of the viral load. If a similar pathology also exists in COVID-19, the attenuation of the "cytokine storm" by targeting several key steps in the process could bring about improved outcomes. Herein, melatonin is not taken as a typical viricidal agent but it indirectly exerts anti-viral actions based on its well-reported anti-oxidative, anti-inflammatory, and immune system-enhancing properties [17–22] and, therefore, it may be useful to examine its potential effects in suppressing COVID-19 infections (see Figure 1).

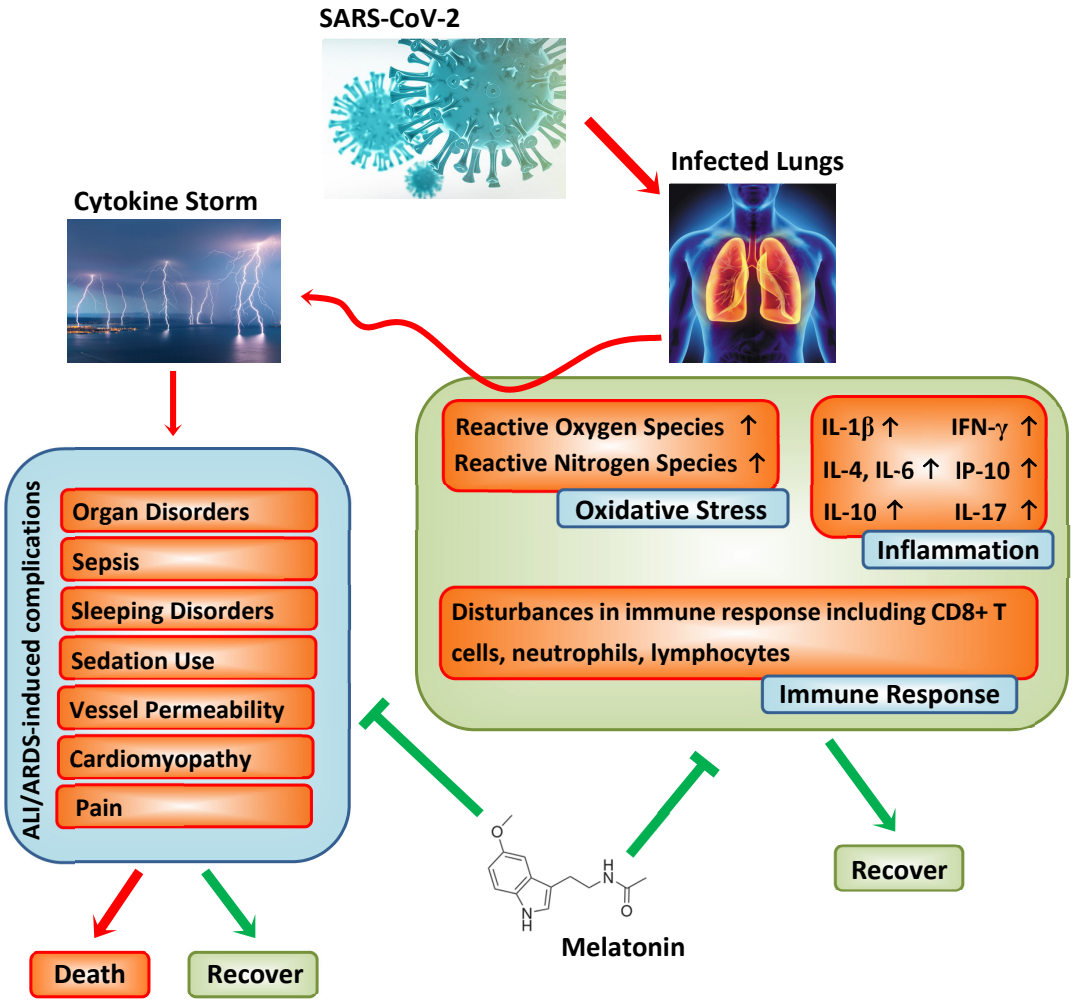

Figure 1. Pathogenesis of SARS-CoV-2 and adjuvant actions of melatonin. We postulate that melatonin significantly suppresses the immune response, the enhanced inflammation, and the excessive oxidative stress, triggering a "cytokine storm". The "cytokine storm" induces acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) accompanied by severe complications.

3. Melatonin and Its Anti-Inflammatory and Anti-Oxidative Properties

Melatonin (*N*-acetyl-5-methoxytryptamine) is a multifunctional molecule with the structure of methoxyindole. It is present in almost all biological systems, in both plants and in animals. With regard to its bioactivity, it regulates circadian and seasonal biorhythms in vertebrates. Synthesis of melatonin is continuous; however, the peak of its production and release from the pineal gland takes place only at night. In adults, approximately 30 μg of melatonin are estimated to be synthesized per day, and the maximal concentration in the blood is reached in the mid-dark period. Melatonin, released from the

pineal gland, is discharged into the cerebrospinal fluid and into the blood and is rapidly degraded in the liver. Melatonin has been successfully used to treat sleep disorders, atherosclerosis, respiratory diseases, and viral infections [17]. Melatonin is well known to possess potent anti-inflammatory capacities and acts via various pathways in terms of inflammatory diseases, including Sirtuin 1 (SIRT1), for the attenuation of lung injury and inflammation [23]. Similarly, melatonin suppresses nuclear factor kappa B (NF-κB) activation in ARDS, and down-regulates NF-κB activation in T cells and lung tissue [24–26]. NF-κB is a major transcription factor involved in the production of cytokines. Moreover, melatonin induces the nuclear translocation of NF-E2-related factor 2 (Nrf2), mediating activation of anti-oxidative phase II enzymes [19] crucial in protecting the lungs from injury. There is no clear evidence for the role of Nrf2 itself in CoV-induced ALI but the close interactions of SIRT1, NF-κB, and Nrf2 indicate their involvement in CoV-induced ALI/ARDS.

Many reports have confirmed the anti-inflammatory action of melatonin. Inflammation is known to be associated with an elevated production of cytokines and chemokines, while melatonin induces a significant reduction in pro-inflammatory cytokines [4,27]. Some of these actions are certainly mediated by melatonin membrane receptors, such as MT1 and MT2. Considering the receptor affinities, only low doses of this substance would be required; however, highly elevated doses reaching several hundred milligrams per day promote melatonin's receptor-independent antioxidant properties. Melatonin effectively scavenges a wide range of reactive oxygen/nitrogen species (ROS/RNS), including hydroxyl radicals and the commonly overlooked carbonate radical [21,28–30]. Among several possibilities of formation, its mitochondrial generation may be the most important one. Under conditions of reduced gas exchange, the organism tries to enhance the arterial blood supply by producing the relaxant nitric oxide (NO) at higher rates. At the same time, the hypoxic condition can impair the mitochondrial electron flux and cause electron dissipation, which results in superoxide formation. In the presence of high NO concentrations, superoxide combines with NO to form peroxynitrite ($OONO^-$). Interestingly, melatonin not only scavenges this oxidant, but also reduces its formation, by improving the mitochondrial electron flux and, thereby, decreasing superoxide generation. Generally, the protection of mitochondria by melatonin includes the prevention of the electron transport chain that causes enhanced free radical formation, control over the duration of permeability transition pore opening, and the maintenance of mitochondrial equilibrium redox balance to support mitochondrial integrity, which all have primary relevance to the return to a healthy state [31–36], in particular with regard to respiratory diseases, including the severe forms of COVID-19.

These anti-inflammatory and antioxidant properties of melatonin are also of substantial interest in pulmonary functioning under intensive care conditions. The artificial ventilation of patients bears the problem of causing undue mechanical stress to the lungs. Namely, ventilator-induced lung injury has been shown to initiate oxidative stress and inflammation. For instance, in a murine model, melatonin increased the level of the anti-inflammatory IL-10, along with improved oxygenation and reduced histological damage to the lungs [37]. Furthermore, a recent study using ramelteon, the melatoninergic agonist, in lung-injured rats revealed strong reductions in oxidative markers, reduced edema, neutrophil infiltration, the induction of apoptosis, decreased NF-κB activation and iNOS expression, and lower levels of TNFα, IL-1β, and IL-6 [38]. It should also be pointed out that the practical problem in patients with severe COVID-19 concerns the reduction of pulmonary gas exchange due to surfactant impairments by lipid peroxidation caused by infiltrating neutrophils. In vitro experiments have shown that melatonin can associate with surfactant lipids [39] and also reduce their peroxidation [40].

The anti-inflammatory actions of melatonin are also, in part, associated with mitochondrial functions, as recently outlined in the context of COVID-19 [41]. The protective mechanisms by which melatonin acts, especially under conditions of high-grade inflammation and in aging, have been reviewed [4,5]. Melatonin, in addition to its anti-inflammatory capacities, functions as an "anti-oxidative shield", activating anti-oxidative enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and phase-2 antioxidant enzymes while, on the other hand, down-regulating

pro-oxidative enzymes such as nitric oxide synthase [19,42]. Viral infections and their metabolism are major sources of oxidizing agents and the anti-oxidative actions of melatonin have been documented in ALI caused by sepsis or ischemia reperfusion [43,44]. Furthermore, in advanced ALI/ARDS patients who display severe inflammation, hypoxemia, and ventilation problems, Sarma and Ward [45] noticed elevated concentrations of oxygen leading to massive oxidant generation. Regarding the beneficial roles of melatonin in COVID-19 treatment, this indoleamine was successfully applied in infants with respiratory disease [46,47] and in advanced COVID-19 pneumonia patients [8], confirming its anti-oxidative and anti-inflammatory actions in the lung. An overview of the most important functions of melatonin is provided in Figure 2.

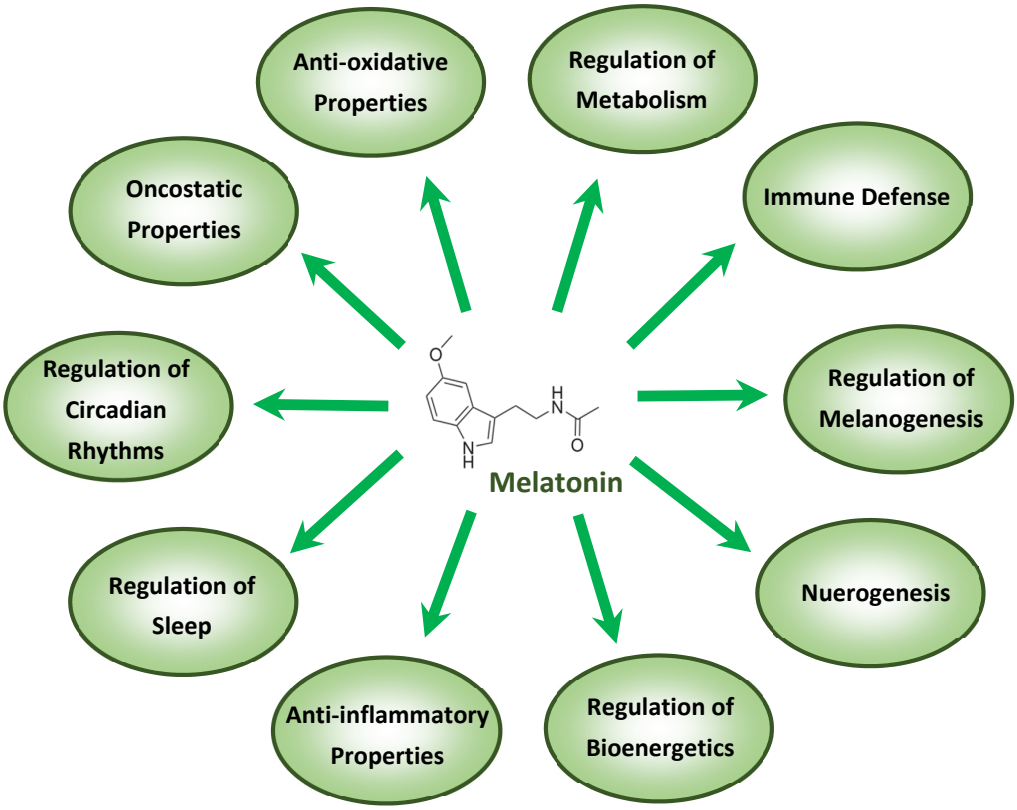

Figure 2. The most important functions of melatonin, some of which apply to the treatment of viral diseases including COVID-19.

4. Melatonin and Immunomodulation

From the moment the virus is inhaled and infects the epithelial cells of the respiratory tract, dendritic cells phagocytose the virus and present antigens to T cells. The resultant effector T cells function by killing the infected epithelial cells, and cytotoxic CD8+ T cells produce and release pro-inflammatory cytokines, which induce cell apoptosis [48]. Both the pathogen (CoV) and cell apoptosis trigger and amplify the immune response. Here, melatonin exerts many of its physiological actions by acting through membrane-bound MT1 and MT2 receptors, which belong to the superfamily of G-protein-coupled receptors containing the typical seven transmembrane domains and account for several of its immunological actions [49]. For instance, a resultant decrease in cyclic adenosine monophosphate (cAMP) concentration is observed upon the action of melatonin or the melatonin-mediated inhibition of cellular and humoral immune responses in mice [50]. This shows that, in animals and humans, melatonin affects both the cellular and humoral arms of the immune response [51,52]. The clinical characteristics of COVID-19 present serious disturbances in neutrophils, lymphocytes, and CD8+ T cells in peripheral blood [7,53] and melatonin exerts regulatory actions on

the immune system and directly enhances the immune response by improving the proliferation and maturation of natural killer cells, T and B lymphocytes, granulocytes, and monocytes in both bone marrow and other tissues [54]. The inflammasome NLRP3 is correlated with lung diseases caused by infection, including influenza A virus and bacteria [55,56]. Since it is part of the innate immune response during lung infection, COVID-19 triggers NLRP3 activation to amplify the inflammation. Knowing the anti-inflammatory capacity of melatonin, we urge the rational use of this substance for ALI/ARDS-mediated symptoms. Indeed, the melatonin-controlled regulation of NLRP3 was shown in radiation-induced lung injury or respiratory disturbances, where melatonin distinctly reduced the infiltration of macrophages and neutrophils into the lung by inhibition of the NLRP3 inflammasome [26,57–59].

5. Melatonin and Its Adjuvant Effects

As usual, drug interactions must be considered since they may limit the use of the drugs in practice. In consideration of the common beneficial action of melatonin and attenuated metabolic processes caused by viral infections, there are reasonable imperatives to propose that this indoleamine may limit symptoms associated with viral infections, including COVID-19. Currently, it is known that severe inflammation induces multiple perturbations, such as enhanced endothelial cell apoptosis or elevation of the production of vascular endothelial growth factor (VEGF), which contributes to edema and the massive release of immune cells, while melatonin is an effective suppressor of VEGF in vascular endothelial cells [60]. Moreover, melatonin was found to be an ameliorating agent against sepsis-induced cardiomyopathy [61,62]; this effect may be also beneficial for some COVID-19 patients in whom an increased risk of sepsis and cardiac arrest accompany severe ALI/ARDS development. In addition to its effects in the lungs, melatonin is also beneficial for patients with myocardial infarction, cardiomyopathy, hypertensive heart diseases, and pulmonary hypertension [63]. Moreover, melatonin exerts neurological protection by reducing the cerebral inflammatory response, cerebral edema, and blood–brain barrier permeability [64]. Furthermore, melatonin improves sleep quality in ICU patients [65] in whom deep sedation is associated with increased long-term mortality, and the application of melatonin reduces sedation use and the frequency of pain, agitation, and anxiety [66,67]. Thus, the advantages for the use of melatonin in COVID-19 patients not only focus on the attenuation of the viral-induced respiratory disorders, but also on the overall health improvement and prevention of patients' potential complications and their well-being.

Currently, accumulated evidence indicates that increased blood coagulation has a negative relationship with the symptoms of COVID-19 and anticoagulants are recommended to reduce the severity of COVID-19 symptoms in patients [68]. Melatonin exhibits anticoagulating activity and has been suggested to treat Ebola virus infection [69]. From this perspective, it should not be a problem to use melatonin with other anticoagulants in COVID-19 treatment. One of the main advantageous properties of melatonin is its short $T_{1/2}$ (52.8 ± 18.1 min) [70] If physicians identify a bleeding tendency in patients and if this bleeding tendency is related to melatonin, the withdrawal of melatonin will achieve rapid (short $T_{1/2}$) results without negative consequences. Therefore, the concomitant use of anticoagulants and melatonin is safe and it will not cause prolonged bleeding problems after its withdrawal.

Some drugs have already been suggested for the prevention and treatment of COVID-19, including chloroquine or hydroxychloroquine. However, some recent studies show that hydroxychloroquine is ineffective. Therefore, caution is suggested regarding its use. It is noteworthy that it was reported that the anti-malaria effectiveness of chloroquine was greatly increased by a melatonin antagonist, luzindole, and/or bright light at night, which reduced melatonin production. Simultaneously, even the research on the anti-malaria effect of melatonin antagonists reported that high doses of melatonin are beneficial for malaria treatment because they inhibit programmed cell death and oxidative stress [71]. Thus, applying melatonin as an adjuvant to chloroquine and hydroxychloroquine treatments of COVID-19 may reduce the necessary doses, and thus the toxicity, of these agents [72]. In addition to the drugs

mentioned above, methylprednisolone is used to relieve edema, which is justified in the case of SARS, where, as previously indicated, edema contributes significantly to lung dysfunction, leading to lung failure. The activity of melatonin as a protective drug compared to methylprednisolone was studied in mice with spinal cord injuries [73]. It was shown that the protective properties of melatonin were greater than those of the steroid. The combination of these drugs has led to even greater efficacy for relieving edema [74], so melatonin can be used in combination with prednisone to relieve edema with greater efficacy in patients suffering from pneumonia with SARS-CoV-2. Finally, ribavirin, remdesivir, and other nucleotide analogs targeting RNA-dependent RNA polymerase are a popular strategy. Indeed, neither humans nor animals have the polymerase enzyme, thus, in principle, substances of this group can be highly selective. Combining nucleotide analogs with melatonin may provide additional benefits. For example, melatonin increased ribavirin potency as an anti-influenza agent, probably due to the immunomodulatory functions of melatonin. In vitro studies have shown that ribavirin in combination with melatonin shows improved properties regarding the replication inhibition of respiratory syncytial virus [75].

6. Melatonin and Its Safety

Melatonin, in its original form, is a "human-friendly agent". Currently available products contain synthetic melatonin, which is structurally identical to that produced in the body. Melatonin is an endogenously synthesized molecule in the pineal gland and is present in almost all biological systems, including animals, plants, and microbes [33,76–79]. In addition to the documented anti-inflammatory benefits of melatonin, it has a very high safety profile even when used in high doses; there is no evidence that melatonin exaggerates inflammatory responses. In a randomized trial, oral intake of 25 mg/day melatonin for 6 months promoted a significant reduction in serum concentrations of IL-6 and IL-1β [80]. Similarly, in the acute phase of inflammation, including brain reperfusion [81], and coronary artery reperfusion [82], melatonin intake of 6 mg/day and 5 mg/day, respectively, for less than 5 days reduced levels of pro-inflammatory cytokines. Even doses of 1 g/day given for a month had no adverse effects in humans [83]. Additionally, Weishaupt et al. [84] treated severely affected ALS patients using 300 mg melatonin daily for 2 years, without any adverse effects. Furthermore, in acute cases after surgery, melatonin doses up to 50 mg/kg in patients showed no serious side effects [85]. The destructive inflammation and massive pathological alterations occurring in severe COVID-19 patients require adequate measures that are not satisfied by the so-called physiological levels of melatonin. For instance, the dose selected by Huang et al. [75] to treat the H1N1 virus-associated deadly influenza was inadequate. Melatonin, at a dose of 10 mg/kg/day (20 mg/kg/48 h), had a demonstrable but only slight effect, whereas a dose of 100 mg/kg/day (200 mg/kg/48 h) substantially reduced the mortality. If we convert this murine dose to the human dose according to standard dose translation, based on dividing the surface area by a factor 12.3 (120), the calculated equivalent human dose is 8.1 mg/kg/day (100/12.3 = 8.1). This dose is very similar to the dose used in two neonatal septic trials (8.1 and 8.2 mg/kg/day), as described previously [86,87]. Importantly, this dose would not cause obvious adverse effects, based on the outcomes of these clinical trials. Thus, the estimated dose to treat deadly viral infectious diseases, including COVID-19, is around 8 mg/kg/day. For a 75 kg individual, a daily dose of 600 mg may be warranted. All data indicate that large doses of melatonin, whether given chronically or for acute treatment do not cause intolerable or uncontrollable side effects and that the safety margin of melatonin for humans is as high as 3750 mg/day for a 75 kg individual [85]. Despite the high safety profile of melatonin, as summarized above, its actions in COVID-19 patients should be prudently screened for efficacy and safety.

7. Conclusions

Melatonin shows no harmful adverse effects in humans. Given its proven beneficial actions in multiple organs, it is imperative to introduce this indoleamine into clinical trials as an adjuvant treatment for COVID-19-infected patients. Its documented anti-inflammatory and anti-oxidative

properties, actions shared by its precursor *N*-acetylserotonin and down-stream metabolites [88], have been repeatedly confirmed in respiratory disorders in both animals and humans. Considering the wealth of scientific evidence related to its high efficacy coupled with its proven safety, we encourage healthcare professionals to seriously test the potential role of melatonin against COVID-19 infection. This is urgent, since there is no reliable treatment for this devastating disease.

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References

1. European Centre for Disease Prevention and Control. COVID-19 Situation Update Worldwide, as of 20 July 2020. Available online: https://www.ecdc.europa.eu/en/geographical-distribution-2019-ncov-cases (accessed on 22 July 2020).
2. Li, G.; Fan, Y.; Lai, Y.; Han, T.; Li, Z.; Zhou, P.; Pan, P.; Wang, W.; Hu, D.; Liu, X.; et al. Coronavirus infections and immune responses. *J. Med. Virol.* **2020**, *92*, 424–432. [CrossRef]
3. Kleszczyński, K.; Tukaj, S.; Kruse, N.; Zillikens, D.; Fischer, T.W. Melatonin prevents ultraviolet radiation-induced alterations in plasma membrane potential and intracellular pH in human keratinocytes. *J. Pineal Res.* **2013**, *54*, 89. [CrossRef]
4. Hardeland, R. Aging, melatonin, and the pro- and anti-inflammatory networks. *Int. J. Mol. Sci.* **2019**, *20*, 1223. [CrossRef]
5. Hardeland, R. Melatonin and inflammation-story of a double-edged blade. *J. Pineal Res.* **2018**, *65*, e12525. [CrossRef]
6. Luo, J.; Zhang, Z.; Sun, H.; Song, J.; Chen, X.; Huang, J.; Lin, X.; Zhou, R. Effect of melatonin on T/B cell activation and immune regulation in pinealectomy mice. *Life Sci.* **2020**, *242*, 117191. [CrossRef]
7. Chen, N.; Zhou, M.; Dong, X.; Qu, J.; Gong, F.; Han, Y.; Qiu, Y.; Wang, J.; Liu, Y.; Wei, Y.; et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: A descriptive study. *Lancet* **2020**, *395*, 507–513. [CrossRef]
8. Castillo, R.R.; Quizon, G.R.A.; Juco, M.J.M.; Roman, A.D.E.; de Leon, D.G.; Punzalan, F.E.R.; Guingon, R.B.L.; Morales, D.D.; Tan, D.X.; Reiter, R.J. Melatonin as adjuvant treatment for coronavirus disease 2019 pneumonia patients requiring hospitalization (MAC-19 PRO): A case series. *Melatonin Res.* **2020**, *3*, 297–310. [CrossRef]
9. Huang, C.; Wang, Y.; Li, X.; Ren, L.; Zhao, J.; Hu, Y.; Zhang, L.; Fan, G.; Xu, J.; Gu, X.; et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* **2020**, *395*, 497–506. [CrossRef]
10. Zeng, Z.; Xu, L.; Xie, X.Y.; Yan, H.L.; Xie, B.J.; Xu, W.Z.; Liu, X.A.; Kang, G.J.; Jiang, W.L.; Yuan, J.P. Pulmonary pathology of early phase COVID-19 pneumonia in a patient with a benign lung lesion. *Histopathology* **2020**. [CrossRef]
11. Xu, Z.; Shi, L.; Wang, Y.; Zhang, J.; Huang, L.; Zhang, C.; Liu, S.; Zhao, P.; Liu, H.; Zhu, L.; et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir. Med.* **2020**, *8*, 420–422. [CrossRef]
12. Liu, J.; Zheng, X.; Tong, Q.; Li, W.; Wang, B.; Sutter, K.; Trilling, M.; Lu, M.; Dittmer, U.; Yang, D. Overlapping and discrete aspects of the pathology and pathogenesis of the emerging human pathogenic coronaviruses SARS-CoV, MERS-CoV, and 2019-nCoV. *J. Med. Virol.* **2020**, *92*, 491–494. [CrossRef] [PubMed]
13. Zhang, R.; Wang, X.; Ni, L.; Di, X.; Ma, B.; Niu, S.; Liu, C.; Reiter, R.J. COVID-19: Melatonin as a potential adjuvant treatment. *Life Sci.* **2020**, *250*, 117583. [CrossRef] [PubMed]
14. Channappanavar, R.; Perlman, S. Pathogenic human coronavirus infections: Causes and consequences of cytokine storm and immunopathology. *Semin. Immunopathol.* **2017**, *39*, 529–539. [CrossRef] [PubMed]

15. Channappanavar, R.; Fehr, A.R.; Vijay, R.; Mack, M.; Zhao, J.; Meyerholz, D.K.; Perlman, S. Dysregulated type I interferon and inflammatory monocyte-macrophage responses cause lethal pneumonia in SARS-CoV-infected mice. *Cell Host Microbe* **2016**, *19*, 181–193. [CrossRef] [PubMed]
16. Smits, S.L.; de Lang, A.; van den Brand, J.M.A.; Leijten, L.M.; van IJcken, W.F.; Eijkemans, M.J.C.; van Amerongen, G.; Kuiken, T.; Andeweg, A.C.; Osterhaus, A.D.M.E.; et al. Exacerbated innate host response to SARS-CoV in aged non-human primates. *PLoS Pathog.* **2010**, *6*, e1000756. [CrossRef]
17. Anderson, G.; Reiter, R.J. Melatonin: Roles in influenza, Covid-19, and other viral infections. *Rev. Med. Virol.* **2020**, *30*, e2109. [CrossRef]
18. Boga, J.A.; Coto-Montes, A.; Rosales-Corral, S.A.; Tan, D.X.; Reiter, R.J. Beneficial actions of melatonin in the management of viral infections: A new use for this "molecular handyman"? *Rev. Med. Virol.* **2012**, *22*, 323–338. [CrossRef]
19. Kleszczyński, K.; Zillikens, D.; Fischer, T.W. Melatonin enhances mitochondrial ATP synthesis, reduces reactive oxygen species formation, and mediates translocation of the nuclear erythroid 2-related factor 2 resulting in activation of phase-2 antioxidant enzymes (γ-GCS, HO-1, NQO1) in ultraviolet radiation-treated normal human epidermal keratinocytes (NHEK). *J. Pineal Res.* **2016**, *61*, 187–197.
20. Kleszczyński, K.; Zwicker, S.; Tukaj, S.; Kasperkiewicz, M.; Zillikens, D.; Wolf, R.; Fischer, T.W. Melatonin compensates silencing of heat shock protein 70 and suppresses ultraviolet radiation-induced inflammation in human skin ex vivo and cultured keratinocytes. *J. Pineal Res.* **2015**, *58*, 117–126. [CrossRef]
21. Slominski, A.T.; Hardeland, R.; Zmijewski, M.A.; Slominski, R.M.; Reiter, R.J.; Paus, R. Melatonin: A cutaneous perspective on its production, metabolism, and functions. *J. Invest. Dermatol.* **2018**, *138*, 490–499. [CrossRef]
22. Tan, D.X.; Manchester, L.C.; Terron, M.P.; Flores, L.J.; Reiter, R.J. One molecule, many derivatives: A never-ending interaction of melatonin with reactive oxygen and nitrogen species? *J. Pineal Res.* **2007**, *42*, 28–42. [CrossRef]
23. Huang, C.C.; Chiou, C.H.; Liu, S.C.; Hu, S.L.; Su, C.M.; Tsai, C.H.; Tang, C.H. Melatonin attenuates TNF-α and IL-1β expression in synovial fibroblasts and diminishes cartilage degradation: Implications for the treatment of rheumatoid arthritis. *J. Pineal Res.* **2019**, *66*, e12560. [CrossRef]
24. Ling, Y.; Li, Z.Z.; Zhang, J.F.; Zheng, X.W.; Lei, Z.Q.; Chen, R.Y.; Feng, J.H. MicroRNA-494 inhibition alleviates acute lung injury through Nrf2 signaling pathway via NQO1 in sepsis-associated acute respiratory distress syndrome. *Life Sci.* **2018**, *210*, 1–8. [CrossRef]
25. Pedrosa, A.M.C.; Weinlich, R.; Mognol, G.P.; Robbs, B.K.; Viola, J.P.B.; Campa, A.; Amarante-Mendes, G.P. Melatonin protects CD4+ T cells from activation-induced cell death by blocking NFAT-mediated CD95 ligand upregulation. *J. Immunol.* **2010**, *184*, 3487–3494. [CrossRef]
26. Sun, C.K.; Lee, F.Y.; Kao, Y.H.; Chiang, H.J.; Sung, P.H.; Tsai, T.H.; Lin, Y.C.; Leu, S.; Wu, Y.C.; Lu, H.I.; et al. Systemic combined melatonin-mitochondria treatment improves acute respiratory distress syndrome in the rat. *J. Pineal Res.* **2015**, *58*, 137–150. [CrossRef]
27. Habtemariam, S.; Daglia, M.; Sureda, A.; Selamoglu, Z.; Gulhan, M.F.; Nabavi, S.M. Melatonin and respiratory diseases: A review. *Curr. Top. Med. Chem.* **2017**, *17*, 467–488. [CrossRef]
28. Tan, D.X.; Chen, L.D.; Poeggeler, B.; Manchester, L.C.; Reiter, R.J. Melatonin: A potent, endogenous hydroxyl radical scavenger. *Endocr. J.* **1993**, *1*, 57–60.
29. Reiter, R.J.; Poeggeler, B.; Tan, D.X.; Chen, L.D.; Manchester, L.C.; Guerrero, J.M. Antioxidant capacity of melatonin: A novel action not requiring a receptor. *Neuroendocrinol. Lett.* **1993**, *15*, 103–116.
30. Tan, D.X.; Reiter, R.J.; Manchester, L.C.; Yan, M.T.; El-Sawi, M.; Sainz, R.M.; Mayo, J.C.; Kohen, R.; Allegra, M.; Hardeland, R. Chemical and physical properties and potential mechanisms: Melatonin as a broad spectrum antioxidant and free radical scavenger. *Curr. Top. Med. Chem.* **2002**, *2*, 181–197. [CrossRef]
31. Acuña-Castroviejo, D.; Rahim, I.; Acuña-Fernández, C.; Fernández-Ortiz, M.; Solera-Marín, J.; Sayed, R.K.A.; Díaz-Casado, M.E.; Rusanova, I.; López, L.C.; Escames, G. Melatonin, clock genes and mitochondria in sepsis. *Cell. Mol. Life Sci.* **2017**, *74*, 3965–3987. [CrossRef]
32. Hardeland, R. Melatonin and the electron transport chain. *Cell. Mol. Life Sci.* **2017**, *74*, 3883–3896. [CrossRef] [PubMed]
33. Reiter, R.J.; Rosales-Corral, S.; Tan, D.X.; Jou, M.J.; Galano, A.; Xu, B. Melatonin as a mitochondria-targeted antioxidant: One of evolution's best ideas. *Cell. Mol. Life Sci.* **2017**, *74*, 3863–3881. [CrossRef] [PubMed]
34. Reiter, R.J.; Tan, D.X.; Rosales-Corral, S.; Galano, A.; Zhou, X.J.; Xu, B. Mitochondria: Central organelles for melatonin's antioxidant and anti-aging actions. *Molecules* **2018**, *23*, 509. [CrossRef]

35. Tan, D.X.; Manchester, L.C.; Qin, L.; Reiter, R.J. Melatonin: A mitochondrial targeting molecule involving mitochondrial protection and dynamics. *Int. J. Mol. Sci.* **2016**, *17*, 2124. [CrossRef]
36. Wongprayoon, P.; Govitrapong, P. Melatonin as a mitochondrial protector in neurodegenerative diseases. *Cell. Mol. Life Sci.* **2017**, *74*, 3999–4014. [CrossRef]
37. Pedreira, P.R.; García-Prieto, E.; Parra, D.; Astudillo, A.; Diaz, E.; Taboada, F.; Albaiceta, G.M. Effects of melatonin in an experimental model of ventilator-induced lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2008**, *295*, L820–L827. [CrossRef]
38. Wu, G.C.; Peng, C.K.; Liao, W.I.; Pao, H.P.; Huang, K.L.; Chu, S.J. Melatonin receptor agonist protects against acute lung injury induced by ventilator through up-regulation of IL-10 production. *Respir. Res.* **2020**, *21*, 65. [CrossRef]
39. Bouhafs, R.K.L.; Jarstrand, C. Effects of antioxidants on surfactant peroxidation by stimulated human polymorphonuclear leukocytes. *Free Radic. Res.* **2002**, *36*, 727–734. [CrossRef]
40. Ceraulo, L.; Ferrugia, M.; Tesoriere, L.; Segreto, S.; Livrea, M.A.; Liveri, V.T. Interactions of melatonin with membrane models: Portioning of melatonin in AOT and lecithin reversed micelles. *J. Pineal Res.* **1999**, *26*, 108–112. [CrossRef]
41. Tan, D.X.; Hardeland, R. Potential utility of melatonin in deadly infectious diseases related to the overreaction of innate immune response and destructive inflammation: Focus on COVID-19. *Melatonin Res.* **2020**, *3*, 120–143. [CrossRef]
42. Reiter, R.J.; Ma, Q.; Sharma, R. Treatment of Ebola and other infectious diseases: Melatonin "goes viral". *Melatonin Res.* **2020**, *3*, 43–57. [CrossRef]
43. Chen, H.H.; Chang, C.L.; Lin, K.C.; Sung, P.H.; Chai, H.T.; Zhen, Y.Y.; Chen, Y.C.; Wu, Y.C.; Leu, S.; Tsai, T.H.; et al. Melatonin augments apoptotic adipose-derived mesenchymal stem cell treatment against sepsis-induced acute lung injury. *Am. J. Transl. Res.* **2014**, *6*, 439–458. [PubMed]
44. Wang, M.L.; Wei, C.H.; Wang, W.D.; Wang, J.S.; Zhang, J.; Wang, J.J. Melatonin attenuates lung ischaemia-reperfusion injury via inhibition of oxidative stress and inflammation. *Interact. Cardiov. Thorac. Surg.* **2018**, *26*, 761–767. [CrossRef] [PubMed]
45. Sarma, J.V.; Ward, P.A. Oxidants and redox signaling in acute lung injury. *Compr. Physiol.* **2011**, *1*, 1365–1381. [PubMed]
46. Gitto, E.; Reiter, R.J.; Cordaro, S.P.; La, R.M.; Chiurazzi, P.; Trimarchi, G.; Gitto, P.; Calabrò, M.P.; Barberi, I. Oxidative and inflammatory parameters in respiratory distress syndrome of preterm newborns: Beneficial effects of melatonin. *Am. J. Perinatol.* **2004**, *21*, 209–216. [CrossRef] [PubMed]
47. Gitto, E.; Reiter, R.J.; Sabatino, G.; Buonocore, G.; Romeo, C.; Gitto, P.; Buggé, C.; Trimarchi, G.; Barberi, I. Correlation among cytokines, bronchopulmonary dysplasia and modality of ventilation in preterm newborns: Improvement with melatonin treatment. *J. Pineal Res.* **2005**, *39*, 287–293. [CrossRef]
48. Rogers, M.C.; Williams, J.V. Quis Custodiet Ipsos Custodes? Regulation of cell mediated immune responses following viral lung infections. *Annu. Rev. Virol.* **2018**, *5*, 363–383. [CrossRef]
49. Dubocovich, M.L.; Markowska, M. Functional MT1 and MT2 melatonin receptors in mammals. *Endocrine* **2005**, *27*, 101–110. [CrossRef]
50. Maestroni, G.J.; Conti, A.; Pierpaoli, W. Role of the pineal gland in immunity. Circadian synthesis and release of melatonin modulates the antibody response and antagonizes the immunosuppressive effect of corticosterone. *J. Neuroimmunol.* **1986**, *13*, 19–30. [CrossRef]
51. Guerrero, J.M.; Reiter, R.J. Melatonin-Immune system relationships. *Curr. Top. Med. Chem.* **2002**, *2*, 167–179. [CrossRef]
52. Maestroni, G.J. The immunotherapeutic potential of melatonin. *Expert Opin. Investig. Drugs* **2001**, *10*, 467–476. [CrossRef] [PubMed]
53. Liu, Y.; Yang, Y.; Zhang, C.; Huang, F.; Wang, F.; Yuan, J.; Wang, Z.; Li, J.; Feng, C.; Zhang, Z.; et al. Clinical and biochemical indexes from 2019-nCoV infected patients linked to viral loads and lung injury. *Sci. China Life Sci.* **2020**, *63*, 364–374. [CrossRef] [PubMed]
54. Miller, S.C.; Pandi-Perumal, S.R.; Esquifino, A.I.; Cardinali, D.P.; Maestroni, G.J.M. The role of melatonin in immuno-enhancement: Potential application in cancer. *Int. J. Exp. Pathol.* **2006**, *87*, 81–87. [CrossRef] [PubMed]

55. Shen, C.; Zhang, Z.; Xie, T.; Ji, J.; Xu, J.; Lin, L.; Yan, J.; Kang, A.; Dai, Q.; Dong, Y.; et al. Rhein suppresses lung inflammatory injury induced by human respiratory syncytial virus through inhibiting NLRP3 inflammasome activation via NF-κB pathway in mice. *Front. Pharmacol.* **2019**, *10*, 1600. [CrossRef] [PubMed]
56. Tate, M.D.; Ong, J.D.H.; Dowling, J.K.; McAuley, J.L.; Robertson, A.B.; Latz, E.; Drummond, G.R.; Cooper, M.A.; Hertzog, P.J.; Mansell, A. Reassessing the role of the NLRP3 inflammasome during pathogenic influenza a virus infection via temporal inhibition. *Sci. Rep.* **2016**, *6*, 27912. [CrossRef]
57. Wu, X.; Ji, H.; Wang, Y.; Gu, C.; Gu, W.; Hu, L.; Zhu, L. Melatonin alleviates radiation-induced lung injury via regulation of miR-30e/NLRP3 axis. *Oxidat. Med. Cell. Longev.* **2019**, *2019*, 4087298. [CrossRef]
58. Wu, H.M.; Xie, Q.M.; Zhao, C.C.; Xu, J.; Fan, X.Y.; Fei, G.H. Melatonin biosynthesis restored by CpG oligodeoxynucleotides attenuates allergic airway inflammation via regulating NLRP3 inflammasome. *Life Sci.* **2019**, *239*, 117067. [CrossRef]
59. Zhang, Y.; Li, X.X.; Grailer, J.J.; Wang, N.; Wang, M.; Yao, J.; Zhong, R.; Gao, G.F.; Ward, P.A.; Tan, D.X.; et al. Melatonin alleviates acute lung injury through inhibiting the NLRP3 inflammasome. *J. Pineal Res.* **2016**, *60*, 405–414. [CrossRef]
60. Cheng, J.; Yang, H.L.; Gu, C.J.; Liu, Y.K.; Shao, J.; Zhu, R.; He, Y.Y.; Zhu, X.Y.; Li, M.Q. Melatonin restricts the viability and angiogenesis of vascular endothelial cells by suppressing HIF-1alpha/ROS/VEGF. *Int. J. Mol. Med.* **2019**, *43*, 945–955.
61. Chen, J.; Xia, H.; Zhang, L.; Zhang, H.; Wang, D.; Tao, X. Protective effects of melatonin on sepsis-induced liver injury and dysregulation of gluconeogenesis in rats through activating SIRT1/STAT3 pathway. *Biomed. Pharmacother.* **2019**, *117*, 109150. [CrossRef]
62. Zhang, J.; Wang, L.; Xie, W.; Hu, S.; Zhou, H.; Zhu, P.; Zhu, H. Melatonin attenuates ER stress and mitochondrial damage in septic cardiomyopathy: A new mechanism involving BAP31 upregulation and MAPK-ERK pathway. *J. Cell. Physiol.* **2020**, *235*, 2847–2856. [CrossRef] [PubMed]
63. Nduhirabandi, F.; Lamont, K.; Albertyn, Z.; Opie, L.H.; Lecour, S. Role of toll-like receptor 4 in melatonin-induced cardioprotection. *J. Pineal Res.* **2016**, *60*, 39–47. [CrossRef] [PubMed]
64. Tordjman, S.; Chokron, S.; Delorme, R.; Charrier, A.; Bellissant, E.; Jaafari, N.; Fougerou, C. Melatonin: Pharmacology, functions and therapeutic benefits. *Curr. Neuropharmacol.* **2017**, *15*, 434–443. [CrossRef] [PubMed]
65. Lewis, S.R.; Pritchard, M.W.; Schofield-Robinson, O.J.; Alderson, P.; Smith, A.F. Melatonin for the promotion of sleep in adults in the intensive care unit. *Cochrane Database Syst. Rev.* **2018**, *5*, CD012455. [CrossRef] [PubMed]
66. Lewandowska, K.; Malkiewicz, M.A.; Sieminski, M.; Cubala, W.J.; Winklewski, P.J.; Medrzycka-Dabrowska, W.A. The role of melatonin and melatonin receptor agonist in the prevention of sleep disturbances and delirium in intensive care unit—A clinical review. *Sleep Med.* **2020**, *69*, 127–134. [CrossRef]
67. Mistraletti, G.; Umbrello, M.; Sabbatini, G.; Miori, S.; Taverna, M.; Cerri, B.; Mantovani, E.S.; Formenti, P.; Spanu, P.; D'Agostino, A.; et al. Melatonin reduces the need for sedation in ICU patients: A randomized controlled trial. *Minerva Anestesiol.* **2015**, *81*, 1298–1310.
68. Bi, X.; Su, Z.; Yan, H.; Du, J.; Wang, J.; Chen, L.; Peng, M.; Chen, S.; Shen, B.; Li, J. Prediction of severe illness due to COVID-19 based on an analysis of initial fibrinogen to albumin ratio and platelet count. *Platelets* **2020**, *31*, 674–679. [CrossRef]
69. Tan, D.X.; Korkmaz, A.; Reiter, R.J.; Manchester, L.C. Ebola virus disease: Potential use of melatonin as a treatment. *J. Pineal Res.* **2014**, *57*, 381–384. [CrossRef]
70. Galley, H.F.; Lowes, D.A.; Allen, L.; Cameron, G.; Aucott, L.S.; Webster, N.R. Melatonin as a potential therapy for sepsis: A phase I dose escalation study and an ex vivo whole blood model under conditions of sepsis. *J. Pineal Res.* **2014**, *56*, 427–438. [CrossRef]
71. Srinivasan, V.; Spence, D.W.; Moscovitch, A.; Pandi-Perumal, S.R.; Trakht, I.; Brown, G.M.; Cardinali, D.P. Malaria: Therapeutic implications of melatonin. *J. Pineal Res.* **2010**, *48*, 1–8. [CrossRef]
72. Michaelides, M.; Stover, N.B.; Francis, P.J.; Weleber, R.G.M. Retinal toxicity associated with hydroxychloroquine and chloroquine. *Arch. Ophthalmol.* **2011**, *129*, 30–39. [CrossRef]
73. Kaptanoglu, E.; Tuncel, M.; Palaoglu, S.; Konan, A.; Demirpençe, E.; Kilinç, K. Comparison of the effects of melatonin and methylprednisolone in experimental spinal cord injury. *J. Neurosur.* **2000**, *93*, 77–84. [CrossRef] [PubMed]

74. Cayli, S.R.; Kocak, A.; Yilmaz, U.; Tekiner, A.; Erbil, M.; Ozturk, C.; Batcioglu, K.; Yologlu, S. Effect of combined treatment with melatonin and methylprednisolone on neurological recovery after experimental spinal cord injury. *Eur. Spine J.* **2004**, *13*, 724–732. [CrossRef] [PubMed]
75. Huang, S.H.; Lia, C.L.; Chen, S.J.; Shia, L.G.; Lin, L.; Chen, Y.W.; Cheng, C.P.; Sytwu, H.K.; Shang, S.T.; Lin, G.J. Melatonin possesses an anti-influenza potential through its immune modulatory effect. *J. Funct. Foods* **2019**, *58*, 189–198. [CrossRef]
76. Hardeland, R.; Cardinali, D.P.; Srinivasan, V.; Spence, D.W.; Brown, G.M.; Pandi-Perumal, S.R. Melatonin—A pleiotropic, orchestrating regulator molecule. *Prog. Neurobiol.* **2011**, *93*, 350–384. [CrossRef]
77. Lanoix, D.; Lacasse, A.A.; Reiter, R.J.; Vaillancourt, C. Melatonin: The smart killer: The human trophoblast as a model. *Mol. Cell. Endocrinol.* **2012**, *348*, 1–11. [CrossRef]
78. Reiter, R.J.; Tan, D.X.; Zhou, Z.; Cruz, M.H.; Fuentes-Broto, L.; Galano, A. Phytomelatonin: Assisting plants to survive and thrive. *Molecules* **2015**, *20*, 7396–7437. [CrossRef]
79. Slominski, A.; Tobin, D.J.; Zmijewski, M.A.; Wortsman, J.; Paus, R. Melatonin in the skin: Synthesis, metabolism and functions. *Trends Endocrinol. Metab.* **2008**, *19*, 17–24. [CrossRef]
80. Sánchez-López, A.L.; Ortiz, G.G.; Pacheco-Moises, F.P.; Mireles-Ramírez, M.A.; Bitzer-Quintero, O.K.; Delgado-Lara, D.L.C.; Ramírez-Jirano, R.J.; Velázquez-Brizuela, I.E. Efficacy of melatonin on serum pro-inflammatory cytokines and oxidative stress markers in relapsing remitting multiple sclerosis. *Arch. Med. Res.* **2018**, *49*, 391–398. [CrossRef]
81. Zhao, Z.; Lu, C.; Li, T.; Wang, W.; Ye, W.; Zeng, R.; Ni, L.; Lai, Z.; Wang, X.; Liu, C. The protective effect of melatonin on brain ischemia and reperfusion in rats and humans: In Vivo assessment and a randomized controlled trial. *J. Pineal Res.* **2018**, *65*, e12521. [CrossRef]
82. Shafiei, E.; Bahtoei, M.; Raj, P.; Ostovar, A.; Iranpour, D.; Akbarzadeh, S.; Shahryari, H.; Anvaripour, A.; Tahmasebi, R.; Netticadan, T.; et al. Effects of N-acetyl cysteine and melatonin on early reperfusion injury in patients undergoing coronary artery bypass grafting: A randomized, open-labeled, placebo-controlled trial. *Medicine* **2018**, *97*, e11383. [CrossRef] [PubMed]
83. Nordlund, J.J.; Lerner, A.B. The effects of oral melatonin on skin color and on the release of pituitary hormones. *J. Clin. Endocrinol. Metab.* **1977**, *45*, 768–774. [CrossRef] [PubMed]
84. Weishaupt, J.H.; Bartels, C.; Pölking, E.; Dietrich, J.; Rohde, G.; Poeggeler, B.; Mertens, N.; Sperling, S.; Bohn, M.; Hüther, G.; et al. Reduced oxidative damage in ALS by high-dose enteral melatonin treatment. *J. Pineal Res.* **2006**, *41*, 313–323. [CrossRef] [PubMed]
85. Nickkholgh, A.; Schneider, H.; Sobirey, M.; Venetz, W.P.; Hinz, U.; Pelzl, L.H.; Gotthardt, D.N.; Cekauskas, A.; Manikas, M.; Mikalauskas, S.; et al. The use of high-dose melatonin in liver resection is safe: First clinical experience. *J. Pineal Res.* **2011**, *50*, 381–388. [CrossRef]
86. Henderson, R.; Kim, S.; Lee, E. Use of melatonin as adjunctive therapy in neonatal sepsis: A systematic review and meta-analysis. *Complement. Ther. Med.* **2018**, *39*, 131–136. [CrossRef]
87. El-Gendy, F.M.; El-Hawy, M.A.; Hassan, M.G. Beneficial effect of melatonin in the treatment of neonatal sepsis. *J. Matern. Fetal. Neonatal. Med.* **2018**, *31*, 2299–2303. [CrossRef]
88. Slominski, A.T.; Semak, I.; Fischer, T.W.; Kim, T.K.; Kleszczyński, K.; Hardeland, R.; Reiter, R.J. Metabolism of melatonin in the skin: Why is it important? *Exp. Dermatol.* **2017**, *26*, 563–568.

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Editorial

Melatonin Inhibits COVID-19-induced Cytokine Storm by Reversing Aerobic Glycolysis in Immune Cells: A Mechanistic Analysis

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The pathogenesis of a COVID-19 respiratory infection, in a major way, is related to what is referred to as the cytokine storm [cytokine storm syndrome (CSS, hypercytokinemia, etc.], i.e., it is a hyper-inflammatory response. During this response, an explosive production of proinflammatory cytokines such as TNF-α IL-1β, and others occurs, greatly exaggerating the generation of molecule-damaging reactive oxygen species (free radicals) [1]. In severe cases, the cytokine storm is responsible for the most obvious signs of a COVID-19 infection including fever, lung injury which causes cough and shortness of breath (and the long-term complication, lung fibrosis) and in death.

A causative factor related to the hyper-inflammatory state of immune cells is their ability to dramatically change their metabolism. Similar to cancer cells in many solid tumors, immune cells such as macrophages/monocytes under inflammatory conditions abandon mitochondrial oxidative phosphorylation for ATP production in favor of cytosolic aerobic glycolysis (also known as the Warburg effect) [2]. This switch is driven by the transcription factor HIF-1α (hypoxia inducible factor-1α) and the serine/threonine kinase, mTOR (mammalian target of rapamycin) and other proteins The change to aerobic glycolysis allows immune cells to become highly phagocytic, accelerate ATP production, intensify their oxidative burst and to provide the abundant metabolic precursors required for enhanced cellular proliferation and increased synthesis and release of cytokines (Fig. 1).

A number of drugs have been proposed as treatments to prevent or reduce the severity of a COVID-19 infection. One agent that has been suggested to be potentially useful in this regard is the endogenously synthesized molecule, melatonin [3–7]. Melatonin was initially discovered in and thought to be exclusively a product of the vertebrate pineal gland. However, in consideration of the identification of melatonin in prokaryotic bacteria [8], from which mitochondria evolved (the endosymbiotic theory) and the uncommonly high levels of assayable melatonin in mitochondria [9], it was speculated and eventually documented that this indoleamine is synthesized in this organelle [10]. Given that most cells (a few exceptions) contain mitochondria, it is now believed that melatonin production occurs in most cells in all organisms. This has also been specifically demonstrated in human lung monocytes/macrophages [11].

In healthy cells, including macrophages, melatonin synthesis in mitochondria is maintained by the entrance of pyruvate, a glucose metabolite, into the mitochondria where it is metabolized to acetyl-coenzyme A by the enzyme, pyruvate dehydrogenase complex (PDC). Acetyl-coenzyme A feeds the citric acid cycle and supports ATP synthesis, but it is also a required co-factor/substrate for the rate limiting enzyme in melatonin synthesis, arylalkylamine N-acetyltransferase (AANAT) (Fig. 1). Thus, when mitochondria adopt aerobic glycolysis, pyruvate in mitochondria is no longer converted to acetyl-coenzyme A because PDC is inhibited by the enzyme pyruvate dehydrogenase kinase (PDK); Therefore, as a consequence of a COVID-19 infection the macrophage mitochondria cannot synthesize melatonin [12].

Because of melatonin's potent antioxidant and anti-inflammatory activities, it would normally reduce the highly proinflammatory cytokine storm and neutralize the generated free radicals thereby preserving cellular integrity and preventing lung damage. In the absence of acetyl-coenzyme A, mitochondrial melatonin is no longer available to combat the inflammatory response or to neutralize the generated reactive oxygen species and the massive damage that occurs in the respiratory tree resulting in the primary signs of COVID-19 disease. Importantly, endogenous melatonin production diminishes markedly with age especially in frail older individuals. This is consistent with the more serious nature of a COVID-19 infection in the elderly.

Aerobic glycolysis is an important feature of highly proinflammatory state since it ensures the necessary high levels of ATP and the abundant supply of biomolecules to ensure synthesis and release of the damaging molecules that constitute the cytokine storm. This increased aerobic glycolysis coupled with the absence of locally-produced melatonin provides the optimal environment (the perfect “cytokine storm”) for the massive tissue damage that occurs in COVID-19 disease.

Given the above information, the use of supplemental melatonin as a treatment to overcome a COVID-19 infection is justified. Exogenously administered melatonin reverses aerobic glycolysis by repressing both HIF-1α and mTOR thereby disinhibiting PDC activity and allowing acetyl-coenzyme A synthesis which also ensures locally-produced melatonin production [13]. The functionally re-instated mitochondria-generated melatonin in combination with the parenteral melatonin provides a formidable weapon to reduce the cytokine storm as well as its damaging consequences thereby relieving the signs of a COVID-19 infection.

The anti-inflammatory and antioxidant actions of melatonin in protecting the lungs from damage in many experimental models that involve inflammation or oxidative stress (or both) is well documented [14]. Moreover, melatonin has anti-viral actions against viruses other than

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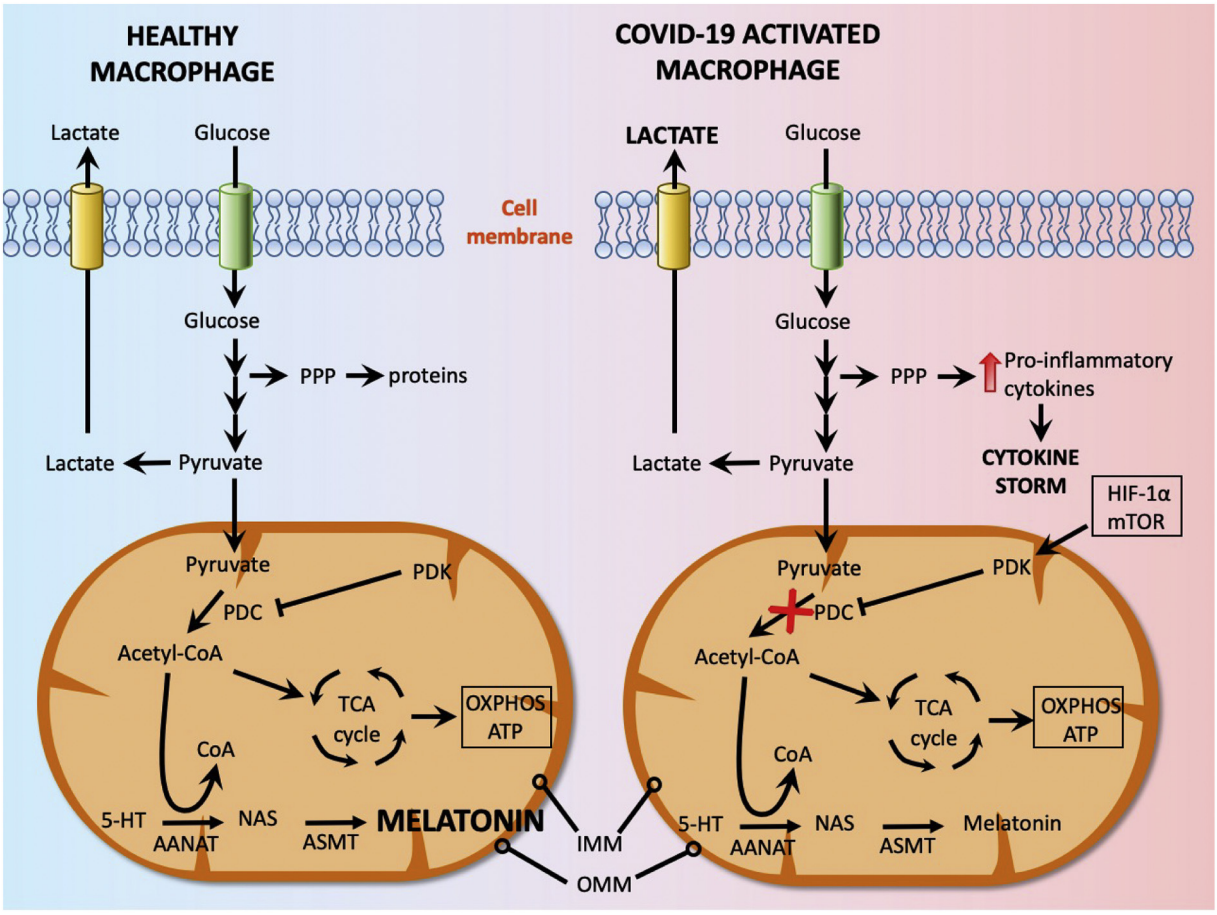

Fig. 1. This figure illustrates the differential glucose metabolism in a healthy macrophage and in a COVID-19-activated macrophage. In a healthy macrophage, pyruvate, a glucose metabolite, enters the mitochondria where it is enzymatically converted to acetyl-coenzyme A by the enzyme pyruvate dehydrogenase complex (PDC). Acetyl-coenzyme A feeds the tricarboxylic acid cycle (TCA) and supports oxidative phosphorylation (OXPHOS). Additionally, acetyl-coenzyme A is an essential co-factor/substrate for the rate limiting enzyme in melatonin synthesis, arylalkylamine N-acetyltransferase (AANAT). This allows for melatonin to be regularly produced in healthy macrophages; melatonin functions intracellularly and is released into the cellular microenvironment, but not into the blood. In COVID-19-activated mitochondria, via HIF-1α, mTOR, etc., the enzyme pyruvate dehydrogenase kinase (PDK) is strongly upregulated and inhibits PDC (red X). Thus, acetyl-coenzyme A is not synthesized and mitochondrial OXPHOS falters with ATP synthesis occurring in the cytosol via aerobic glycolysis (Warburg effect). Similarly, mitochondrial melatonin production is shut down so the cell is deprived of an essential antioxidant, anti-inflammatory agent and of an immune-enhancer so the elevated synthesis of proinflammatory cytokines goes uncontested and the cytokine storm is a result. 5-HT = serotonin; ASMT = acetylserotonin methyltransferase; CoA = coenzyme A; IMM = inner mitochondrial membrane; HIF-1α = hypoxia inducible factor-1α; mTOR = mammalian target of rapamycin; NAS = N-acetylserotonin; OMM = outer mitochondrial membrane; PPP = pentose phosphate pathway.

COVID-19 [15,16]. The collective data, in addition to its very high safety profile, indicate that melatonin would be effective as a treatment for COVID-19 and support the recommendation of the published reports that encourage its use for this purpose [3–7]. Melatonin is inexpensive, non-toxic over a very wide dose range, has a long shelf-life and can be self-administered which is a major advantage when large numbers of individuals are involved. Thus, the use of melatonin to mitigate the COVID-19 pandemic would be feasible and a socially-responsible measure to attempt.

Acknowledgments

Not applicable.

Conflict of Interest

The authors declare no conflict of interest.

Authors' contributions

All authors participated in discussions related to melatonin and COVID-19. The first draft of the manuscript was written by RJR; the paper was then reviewed and edited by all co-authors. The final version of the report was read and approved by all co-authors.

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References

[1] Lake MA. What we know so far: COVID-19 current clinical knowledge and research. Clin Med. 2020;20:124–7.
[2] Bar-Or D, Carrick M, Tanner A, Lieser MJ, Rael LT, Brody E. Overcoming the Warburg effect: is it the key to survival in sepsis? J Crit Care. 2018;43:197–201.
[3] Zhang R, Wang X, Ni L, Di X, Ma B, Niu S, et al. COVID-19: Melatonin as a potential adjuvant treatment. Life Sci. 2020;250:117583.

[4] Zhou Y, Hou Y, Shen J, Huang Y, Martin W, Cheng F. Network-based drug repurposing for human coronavirus. Cell Disc. 2020;6:14.
[5] Peschechera E, Veronesi PA. Injectable melatonin: an anticancer and antiviral treatment option. Melatonin Res. 2020;3:77–80.
[6] Tan DX, Hardeland R. Potential utility of melatonin in deadly infectious diseases related to the overreaction of innate immune response and destructive inflammation: focus on COVID-19. Melatonin Res. 2020;3:120–43.
[7] Shneider A, Kudrivtsev A, Vakhrusheva A. Can melatonin reduce the severity of COVID-19 pandemic? Int J Immunology. 2020. https://doi.org/10.1080/08830185.2020.1756284.
[8] Manchester LC, Poeggeler B, Alvares FL, Ogden GB, Reiter RJ. Melatonin immunoreactivity in the photosynthetic prokaryote Rhodospirillum rubrum: implications for an ancient antioxidant system. Cell Mol Biol Res. 1995;41:391–5.
[9] Venegas C, Garcia JA, Escames G, Ortiz F, Lopez AD, Doerrier C, et al. Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations. J Pineal Res. 2012;52:217–27.
[10] Suofu Y, Jean-Alphonse FG, Jia J, Khattar NK, Li J, Baranov SV, et al. Dual role of mitochondria in producing melatonin and driving GPCR signaling to block cytochrome c release. Proc Natl Acad Sci U S A. 2017;114:E7997–8006.
[11] Muxel JM, Pires-Lapa MA, Morteiro AW, Cecon E, Tamura EK, Flaeten-Winter LM, et al. NF-kB drives the synthesis of melatonin in RAW 264.7 macrophages by inducing the transcription of the arylalkylamine-N-acetyltransferase (AA-NAT) gene. PLoS One. 2012;7(12):e52010.
[12] Reiter RJ, Sharma R, Ma Q, Rosales-Corral S, Acuna-Castroviejo D, Escames G. Inhibition of mitochondrial pyruvate dehydrogenase kinase: a proposed mechanism by which melatonin causes cancer cells to overcome cytosolic glycolysis, reduce tumor biomass and reverse insensitivity to chemotherapy. Melatonin Res. 2019;2:105–19.
[13] Acuna-Castroviejo D, Noguiera-Navarro MT, Reiter RJ. Melatonin actions in the heart: more than a hormone. Melatonin Res. 2018;1:21–6.
[14] Colunga Biancatelli RML, Berrill M, Mohammed YH, Marik PE. Melatonin for the treatment of sepsis: the scientific rationale. J Thorac Dis. 2020;12(Suppl. 1):S54–65.
[15] Boga JA, Coto-Montes A, Rosales-Corral SA, Tan DX, Reiter RJ. Beneficial actions of melatonin in the management of viral infections: a new use for this “molecular handyman?”. Rev Med Virol. 2012;22:323–38.
[16] Reiter RJ, Ma Q, Sharma R. Treatment of Ebola and other infectious diseases: melatonin “goes viral.”. Melatonin Res. 2020;3:43–57.

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Research Paper

COVID-19: Rational discovery of the therapeutic potential of Melatonin as a SARS-CoV-2 main Protease Inhibitor

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Abstract

The SARS-CoV-2 spread quickly across the globe. The World Health Organization (WHO) on March 11 declared COVID-19 a pandemic. The mortality rate, hospital disorders and incalculable economic and social damages, besides the unproven efficacy of the treatments evaluated against COVID-19, raised the need for immediate control of this disease. Therefore, the current study employed *in silico* tools to rationally identify new possible SARS-CoV-2 main protease (Mpro) inhibitors. That is an enzyme conserved among the coronavirus species; hence, the identification of an Mpro inhibitor is to make it a broad-spectrum drug. Molecular docking studies described the binding sites and the interaction energies of 74 Mpro-ligand complexes deposited in the Protein Data Bank (PDB). A structural similarity screening was carried out in order to identify possible Mpro ligands that show additional pharmacological properties against COVID-19. We identified 59 hit compounds and among them, melatonin stood out due to its prominent immunomodulatory and anti-inflammatory activities; it can reduce oxidative stress, defence cell mobility and efficiently combat the cytokine storm and sepsis. In addition, melatonin is an inhibitor of calmodulin, an essential intracellular component to maintain angiotensin-converting enzyme 2 (ACE-2) on the cell surface. Interestingly, one of the most promising hits in our docking study was melatonin. It revealed better interaction energy with Mpro compared to ligands in complexes from PDB. Consequently, melatonin can have response potential in early stages for its possible effects on ACE-2 and Mpro, although it is also promising in more severe stages of the disease for its action against hyper-inflammation. These results definitely do not confirm antiviral activity, but can rather be used as a basis for further preclinical and clinical trials.

Key words: Melatonin, COVID-19, Rational Design, Docking, hyper-inflammation

Introduction

The recent outbreak of COVID-19 has become a pandemic with millions of infected patients and tens of thousands of deaths worldwide [1]. COVID-19 is an infectious disease caused by the novel coronavirus classified as 2019-nCoV and named Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2 or merely CoV-2), a highly contagious RNA virus [2]. The severity of COVID-19 symptoms can range from very mild, cold-like symptoms to most people, to a severe disease that requires hospitalization and oxygen support to approximately 10% of those infected, with 5% requiring admission to an intensive care unit [3,4].

Currently, there is no specific drug treatment for COVID-19. Some therapeutic options are antiviral drugs (lopinavir [5-7], danoprevir [8], ritonavir [5, 9], remdesivir [10, 11], umifenovir [12, 13]), immuno-suppressants (iltuximab [14], baricitinib [15], meplazumab [16], bevacizumab [17, 18], tocilizumab [19-22], glucocorticoids [23-26]), chloroquine and

hydroxychloroquine [27-31], antipyretics and mechanical respiratory support [32]. Clinical trials investigate the efficacy of these drugs and seek to define the most appropriate moment, therapeutic doses and patients to initiate the drug intervention [33].

Up to the present date, no drugs or vaccines have confirmed their clinical efficacy against COVID-19. The results indicated therapeutic ineffectiveness in most clinical studies or low resolution of prognosis, such as mortality and length of hospital stay [5, 34]. The lack of double-blind, randomized, placebo-controlled trials with a correct sample size necessary to get accurate and inference worthy results are other observed problems [35, 36]. Research groups, biotech start-ups and large industries have come together or individually as part of an international collaboration to help hasten the availability of a vaccine against COVID-19 [37]. However, a vaccine for general use takes time to develop and it is not likely to be available to the entire population before January 2021.

One factor that negatively influences success in drug discovery and development is the absence of a rational design of biologically active molecules against possible therapeutic targets identified in the viral life cycle of CoV-2 [38]. Among the various therapeutic targets, the SARS-CoV-2 main protease (Mpro, also called chymotrypsin-like protease, 3CLpro) was structured and repositioned in the Protein Data Bank (PDB) [39, 40]. It stands out as a potential target for the inhibition of CoV replication, but it still lacks rational studies to search for possible inhibitors.

There is an urgent need for effective treatment and prevention strategies to contain the progress of cases and, consequently, of mortality. Therefore, we present a rational search for Mpro inhibitors, which used molecular modelling tools and results from previous clinical and preclinical studies.

Materials and Methods

Figure 1 summarizes the workflow of the methodology used in this study as detailed later.

Analyses and validation of CoV-2 main protease complexed with ligand

The protein-ligand complexes for Mpro were taken from PDB (http://www.rcsb.org). We evaluated the amount of amino acid residues, presence and localization of gaps and resolution (Å) of deposited complexes. MolProbity Server calculated bond lengths and bond angles of standard protein residues, too-close contacts, Ramachandran outliers and rotamer outliers of 74 complexes of Mpro present in the Protein Data Bank [41].

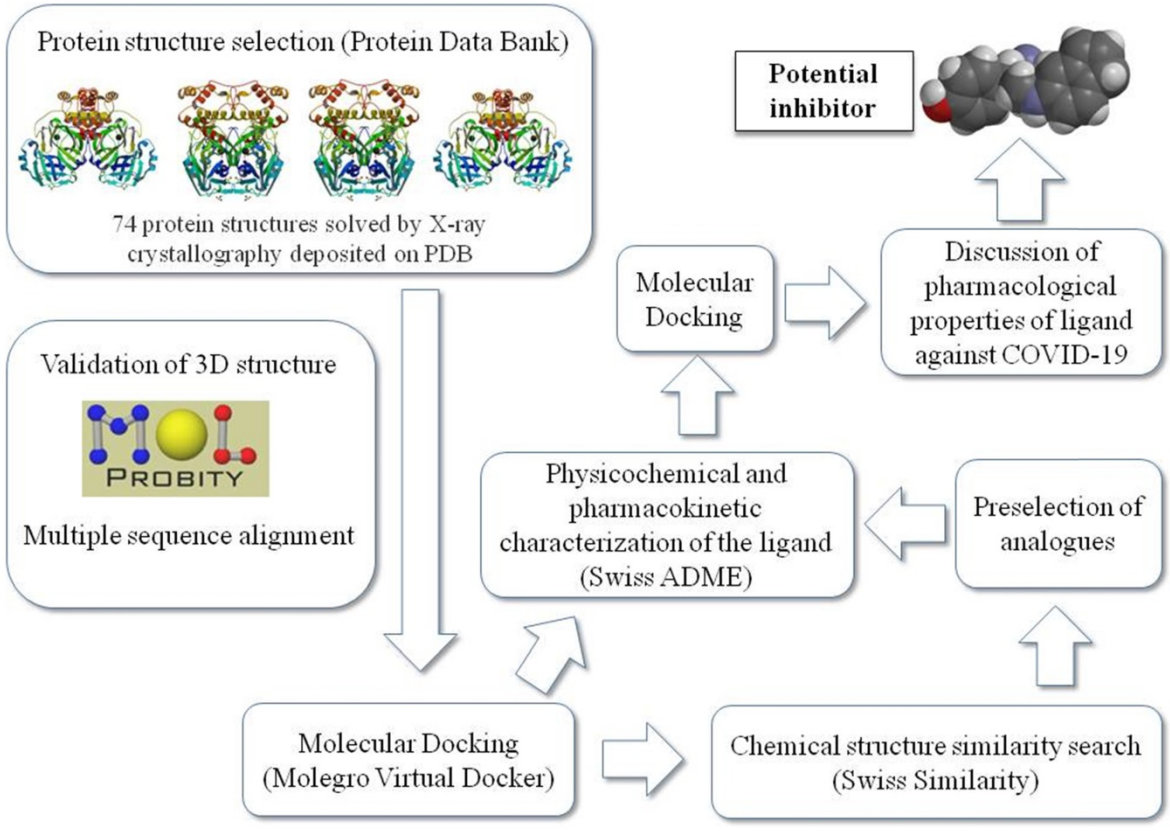

Figure 1. Schematic representation of the work sequence followed in this study.

Molecular docking

Initially, a subset of 74 poised fragments was retrieved from its corresponding PDB entries of ligand-Mpro complexes for structure-based docking.

WaveFunction Spartan 14 (v1. 1.4) was employed to analyze and correct possible errors in the structure of binders, partial charges and electrostatic surfaces. The Molegro Virtual Docker (MVD, version 5.5) and Discovery Studio (DS, version 3.5, Accelrys Software Inc., San Diego) pieces of software were utilized to calculate the partial charges of protein–ligand complexes, add hydrogen atoms, optimize hydrogen bonds and remove atomic clashes [42].

The size and position of the Mpro potential binding sites (also referred to as cavities or active sites) and physicochemical properties of protein surface were identified using the built-in cavity detection algorithm with atom probe size of 1.2Å. The positioning of binders in the crystal served as a reference as possible binding sites. Internal electrostatic interactions (ES), hydrogen bond, intra-molecular torsion energies and MolDock Optimizer algorithm were used to measure the interaction energies (Kcal / mol). The default settings for the docking search algorithm were applicable for the other parameters.

The docking complexes were ranked based on the energy of interaction, Table 1. The ligand-Mpro interactions, changes in the volume of the binding site and amino acids present in the interaction site were evaluated three-dimensionally with the MVD software and in 2D with the DS software. Specific amino acids and chain side of ligand involved in ligand/protein interactions were also confirmed and explored on a structure activity relationship (SAR).

After identifying the residues of the interaction site within a 9Å radius, we searched for likely proteins with high density and similarity in the active site, aiming at understanding how conserved the residues around the ligand are. For so, the alignment of the FASTA sequence of all the Mpro was assessed by the Blast and T-COFFEE servers and the identity score generated by T-COFFEE was used as the measuring parameter.

Similarity search

The search for structural similarity used the 10 hit molecules that presented the best interaction energies (Kcal/mol) measured in the docking study among all 74 ligand-Mpro complexes from PDB. For the similarity search for ligands of Mpro, we used the Swisssimilarity database [43, 44]. The cutoff criteria for the selection were molecules with available *in vivo* studies, clinical studies and similarity index greater than 0.7, on a scale determined by the Swiss Similarity server up to 1.0. In order to determine the drug-likeness of the hits identified, molecular hydrophobicity parameter LogP, molecular weight (MW), the total polar surface area (TPSA) and Lipinski's rule of five (Ro5) were calculated using Swiss ADME [44].

Molecular docking of hits and selected analogues

The selected hits (top 10 best-scored compounds identified by previous docking study), as well as their respective similar binders, were docked into SARS-CoV-2 main protease (Mpro) with unliganded active site (PDB id: 6Y84). Once again, the docking simulations were performed with MVD using the same protocol previously optimized in terms of scoring function, flexible ligand, binding site and radius of the binding site (6 Å), with the presence of crystallographic water within the binding site.

Results and Discussion

The majority (92%) of the 72 complexes studied were characterized as high-resolution structures (< 2 Å). We have more confidence in the location of atoms in structures with these resolution values. In all complexes, the gaps were located only in the protease loop structures, away from the binding site and the total residues were between 304 and 306 conserved amino acids solved by the X-ray diffraction (XRD) method. MolProbit results confirmed acceptable characteristics of complexes, i.e., in Ramachandran plot, more than 97.6% residues were in a favoured region, which is close to the requisite 98% for validating a 3D structure. The parameters of rotamers, Cβ deviations, Cis Prolines and CaBLAM outliers also reinforced the tridimensional quality of proteins [45].

From the docking results, we were able to identify the binding energy (Kcal/mol) between Mpro and ligands (**Table 1**) and active-site residues of amino acids. These results recognized top 10 complexes (highlighted in bold) with greatest binding energy and their respective ligands were characterized as potential hits.

The ligands were designated according to their PDB origin complex; for example, the 5RF7 complex ligand is to be mentioned as L5RF7. One can see in the structure of the molecules with better interaction energies (**Figure 2**) an amphoteric molecular pattern composed by the presence of aromatic rings (e.g. pyrrole, phenyl, indole and naphthalene) and by groups that can make electrostatic interactions (e.g. acetamide, oxopyrrolidine, hydroxyphenyl and pyrimidine). The aromaticity enables several interactions, such as *pi*-stacking (π-π stacking) with other aromatic rings or *pi*-alkyl and *pi*-anion, which is

important in light of the presence of 4 histidines (His41, 163, 172 and 164), one phenylalanine140 and 2 methionines (Met49 and Met165) around the aromatic functional groups.

Properties such as the molecular hydrophobicity parameter LogP, molecular weight (MW) and the total polar surface area (TPSA) have been broadly used in modern rational drug discovery and design [46]. The MW of the top 10 compounds (L5RF7, L5RFL, L5R7Z, L5REN, L5RFJ, L5REX, L5RFQ, L5RFW, L5RFA, L5RFE; **Figure 2**) varies in a short range from 209.24 to 268.35 g/mol. Conversely, the topological polar surface area (TPSA) values are from 23.55 Å^2 to 79.46 Å^2 and LogPs are between 0.68 and 2.70. Three compounds are lipophilic (L5R7Z, L5REX, L5REN); only compound L5RFA is rather hydrophilic with LogP 0.68 and PSAs of 56.16 Å^2 [47]. Among the many rules employed for pre-selection in the drug discovery phase, the most prominent one is Lipinski's rule-of-five (RO5) [48]. It analyses molecular mass, LogP, number of hydrogen-bond donors and acceptors and the sum of nitrogen and oxygen atoms to predict absorption or permeation of a substance. None of the compounds violated RO5, which shows a promising characteristic as small molecule hits for these ligands and their analogs.

The docked pose of L5RF7 into the binding pocket of Mpro (**Figure 3A**) shows that the 4-methyl-piperazine group binds in Met49, His41 and Met165, while a greater amount of interactions occur with aromatic moiety (pyrrolopyridine). Pi-anion and Pi-alkyl are formed between Glu166 and Cys145 with pyridine and pyrrol rings, respectively. Phe140, Leu141, Asn142, Ser144, His163 and His172 take part in a network of van der Waals interactions with pyrrolopyridine fragment. Hydrogen bonds are formed between Glu166 and the carbonyl oxygen atom from the linker.

Table 1. Docking calculations depicting the PDB code, binding site volume (Å) and binding energy (calculated by the Moldock Score Grid algorithm, Kcal/mol) obtained from MVD for 74 ligand-Mpro complexes. The 10 complexes that showed the best interaction energy (more negative) are highlighted in bold. The complexes highlighted in the gap with the asterisk (*) bore their ligands out of the interaction site of the protease, with interaction below -60 Kcal/mol.

PDB code	Cavity volume	Binding energy	PDB code	Cavity volume	Binding energy	PDB code	Cavity volume	Binding energy
5RFL	**120.32**	**-113.01**	5RE9	73.72	-92.70	5REV	143.87	-80.45
5RF7	**112.64**	**-109.99**	5REM	135.68	-91.95	5RFY	126.97	-80.33
5R7Z	**122.36**	**-109.31**	5RFI	115.20	-91.55	5RFU	84.48	-80.21
5REN	**172.03**	**-109.12**	5REL	136.70	-91.51	5RFP	93.18	-79.85
5RFJ	**114.68**	**-103.08**	5REO	138.24	-91.37	5R83	146.94	-79.73
5REX	**162.30**	**-100.61**	5REW	134.65	-90.77	5R7Y	107.52	-79.33
5RFW	**125.44**	**-99.75**	5RFS	132.60	-90.74	5RF6	92.67	-78.50
5RFQ	**98.30**	**-99.53**	5RFM	167.42	-90.48	5RER	142.33	-76.91
5RFA	**16.89**	**-99.15**	5RFF	125.44	-89.97	5REP	96.76	-76.49
5RFE	**124.00**	**-98.88**	5RFT	158.72	-89.24	5RF1	119.80	-73.86
5RFH	133.12	-95.66	5RET	115.20	-86.62	5REB	96.00	-71.81
5REZ	157.18	-95.49	5RFR	152.57	-86.43	5RG0	92.00	-70.34
5R81	45.05	-94.53	5RFV	98.30	-85.19	5RFX	112.34	-69.64
5RFN	136.70	-94.40	5RFG	167.42	-84.02	5RFZ	86.00	-62.84
5RFK	128.00	-93.22	5R80	142.33	-83.96	5RE4	123.90	-62.21
5REY	167.93	-93.11	5R82	113.15	-83.87	5RF3	99.32	-55.54
5REU	112.64	-92.95	5RES	137.72	-83.72	5RFO	129.53	-46.70
5REJ	123.39	-82.37						
***5RE5, 5RE6, 5RE7, 5RE8, 5REA, 5REC, 5RED, 5REF, 5REG, 5REI, 5REK, 5RF8, 5RF9, 5RF4, 5RF5, 5RFB, 5RFC, 5RFD, 5RF0, 5RF4**								

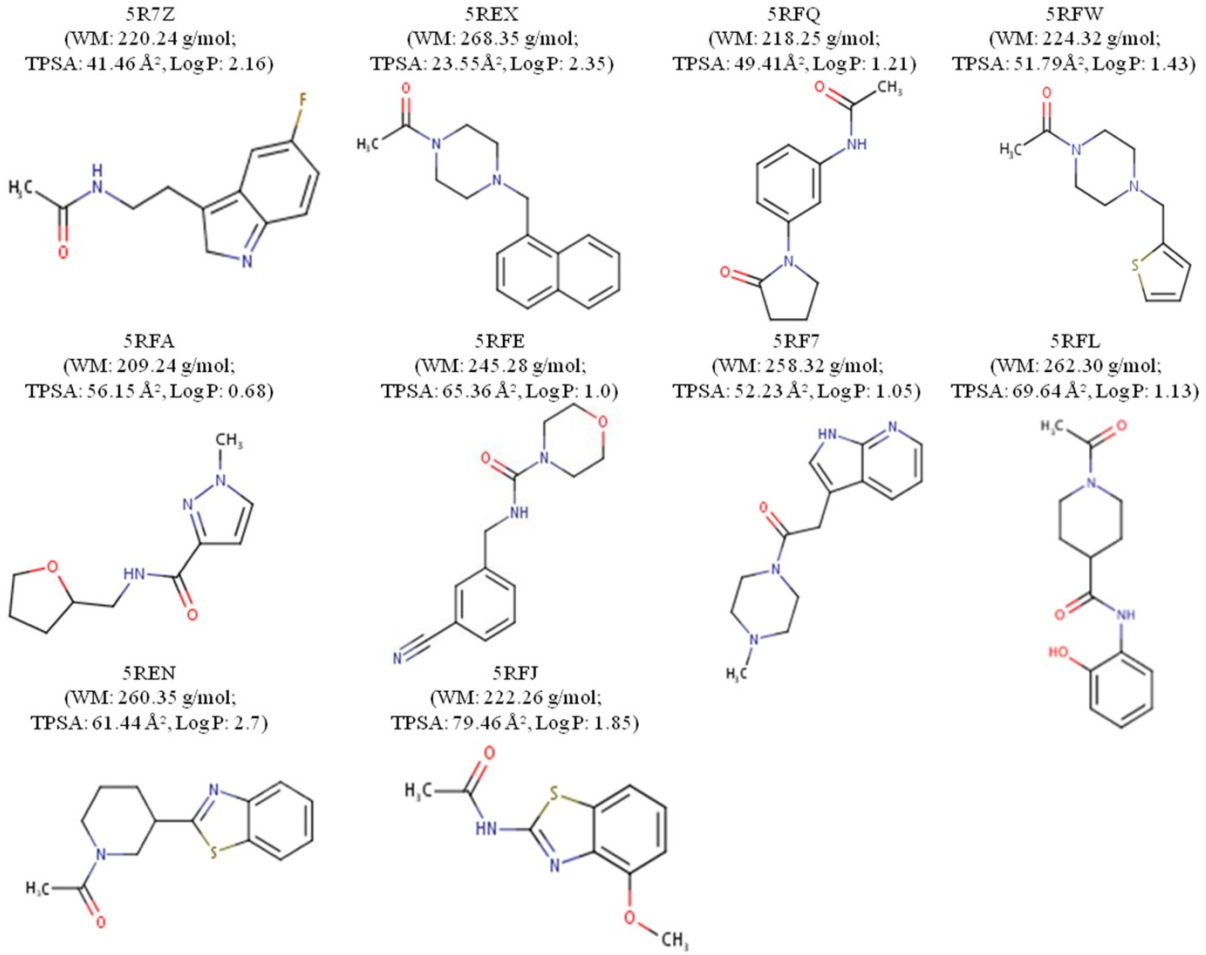

Figure 2. 2D chemical structures and drug-likeness properties of the best 10 ligands measured through MVD.

The linker L5RFL (**Figure 3B**) showed an unfavourable bump with molecule HOH799 when docked with all structural water molecules. However, when removing the structural waters and redoing the docking study, there were no significant changes in the interaction energy. Hydrogen bonds exist between the Gly143, Cys145 and 1-acetyl-N groups, and between Asp142 and hydroxyphenyl. Despite the polar characteristic of the Thr25, Ser46, Ser144 and Hist163 residues, the ligand predominantly made van der Waals interactions due to the lack of groups capable to perform electrostatic interactions. Thus, the polar side chains of these residues were positioned across the ligand, *e.g.* Thr25, Ser46 and Ser144. The intramolecular interaction of 2-hydroxyphenyl and 4-carboxamide harms the potential to attract these polar residues to possible dipole-dipole or ion-dipole interactions, what must be analysed in future cases of structural planning.

L5R7Z (**Figure 3C**) showed an indole moiety that stacks with His41. This interaction is important because it brought together other residues of the binding site, i.e. Met49, Met165 and His164, which interacted positively with L5R7Z. One can see the interaction of the 5-fluorine group with histidines (His41 and His164) and that is due to the electrostatic potential generated by the inductive effects and by the resonance of the fluorine upon the indolic ring of the ligand and the imidazole ring of histidine. Leu 167, Pro168, Gln189 and Gln192 are involved in hydrophobic interactions with the linker (ethanamide group).

The Mpro amino-acid residues of CoV-2 show a high structural similarity with that of other viral ones present in different species of coronavirus, e.g. feline (PDB: 5EU8) and porcine (PDB: 4F49), or with previous COVIDs which overtook human beings (CoV-1, PDB: 4WME). The analyses made by our Blast and T-COFFEE servers have confirmed the high identity among these enzymes, with global score of 87 for the entire sequence analysed and 96 only for the restudies present in a 9Å radius of the Mpro binding cavities. Therefore, one can likely foresee that the COVID protease inhibitors are to have broad action spectra and with activity potential over future species of SARSr-CoV.

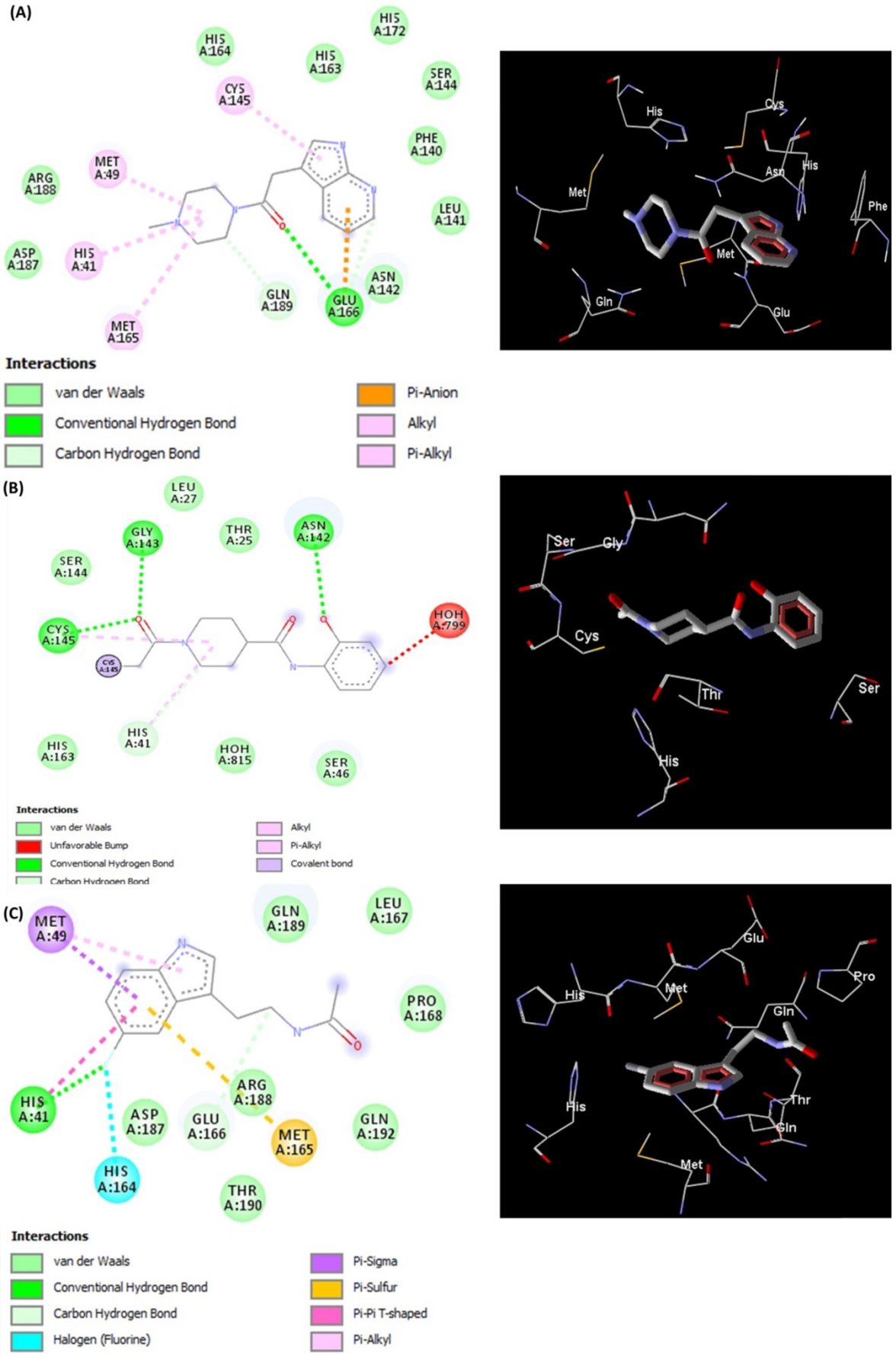

Figure 3. Representations of 2D and 3D interactions between Mpro (PDB id: 6Y84) residues and (**A**) L5RF7, (**B**) L5RFL, (**C**) L5R7Z ligands in the docked complex.

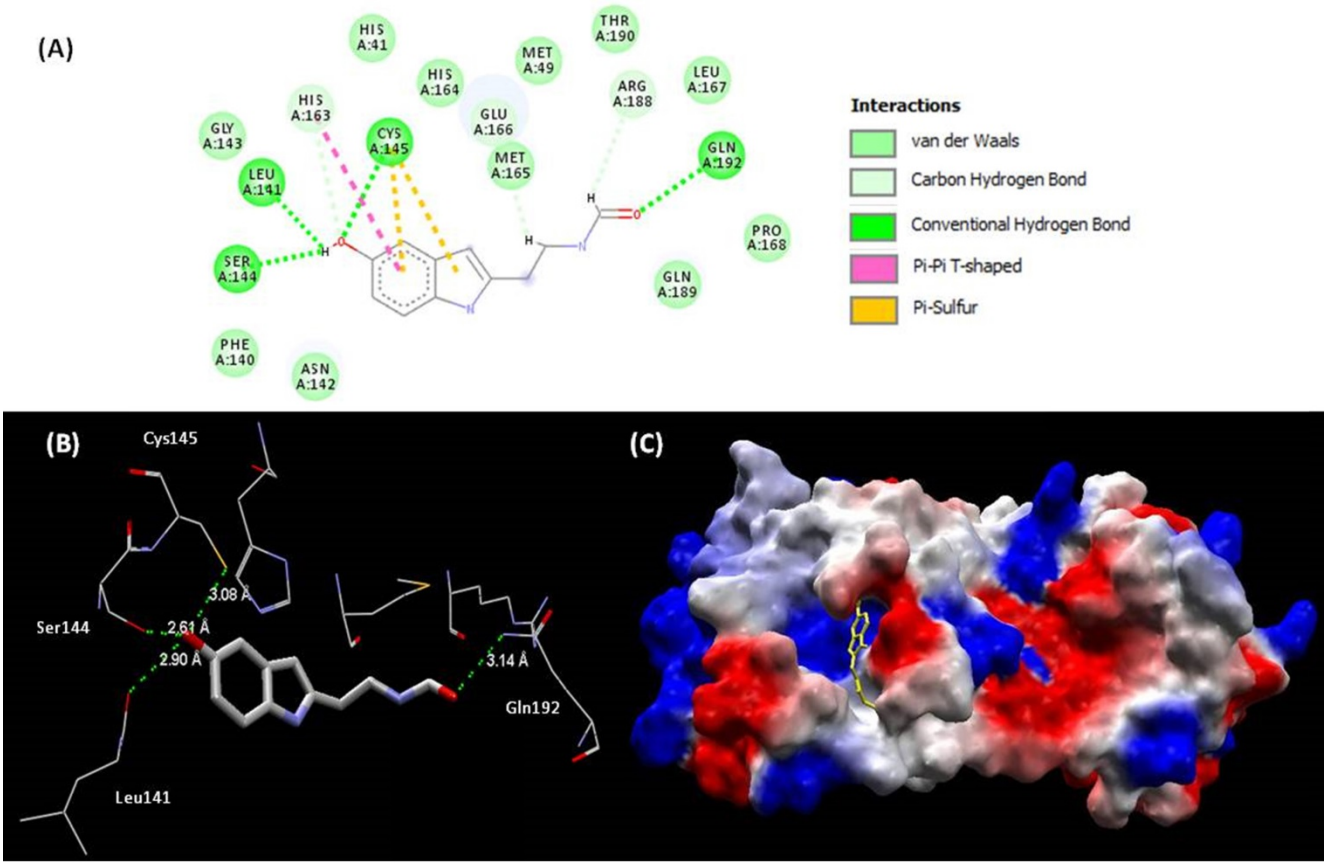

Figure 4. (**A**) Representations of 2D interactions between Mpro (PDB id: 6Y84) residues and melatonin in the docked complex. (**B**) Distance between melatonin and residues of Mpro and (**C**) mapping surface of electrostatic potential of this complex Mpro-melatonin (ligand highlighted in yellow).

Rational search for new protease inhibitor analogues with therapeutic potential against COVID-19

Molecular structure similarity searching used the cutoff criteria described in the method section and found 59 hit molecules from SwissSimilarity. These molecules have been prepared using Spartan and docking studies were performed on MVD and DS software on Mpro with unliganded active site (PDB: 6Y84).

All the 59 analogues bound inside the interaction site and 31 of them had better interaction energies than the 10 best evaluated complexes of PDB. Based on the docking energy results and previous studies of the bioactive characteristics of these analogues, one of them (melatonin) stood out and was selected for further analysis. The interaction between melatonin and Mpro (**Figure 4**) improved the values of binding energy and created a new perspective for a molecule with high therapeutic potential over the COVID-19 pathology to act, so far, only in more severe cases of the disease. The inhibitory effect over the SARS-CoV-2 main protease may be characterized as a big step towards the introduction of melatonin in the front line for the treatment.

Relationship between the infectious condition and the therapeutic potential of melatonin against SARS-CoV-2

To understand the need to clinically evaluate melatonin against Cov-2, we should make a brief introduction to infectious and physiopathological characteristics related mainly to the viral cycle and host immune response in the COVID-19 (**Figure 5**). As an emerging disease, these steps are not fully understood [49]. It has been shown that the infection by SARS-CoV-2 is triggered by similar mechanisms of SARS-CoV and MERS-CoV, and it is amplified by dysfunctional immune responses [50].

The initial event of COVID-19 involves the infection of airway epithelial cells, alveolar epithelial cells, vascular endothelial cells, type-II pneumocytes and macrophages in the lung by SARS-CoV-2. Coronavirus glycosylated spike (S) protein binds to its cellular angiotensin-converting enzymes 2 (ACE2) receptor leading to the membrane fusion between the virus and human plasma membrane [51]. Besides that, this invasion process is facilitated by a transmembrane serine protease 2 (TMPRSS2) produced by the host cell. These interactions are considered as critical for cell entry and future viral

replication [52, 53]. After membrane fusion, the viral RNA genome is released into the cytoplasm and is translated in two polyproteins (pp1a and pp1ab from ORF1a and 1b), which are cleaved by Mpro and papain-like protease (PLpro). This cleavage results in 15 new non-structural proteins (nsp) that compose the replication-transcription complex. Then, the viral genome begins to replicate and generate individual sub-genomic mRNA templates needed for the translation of the viral structural and accessory proteins. The newly formed RNA, nucleocapsid proteins and envelope glycoproteins assemble to form viral particles mediated by the endoplasmic reticulum and Golgi apparatus. Finally, vesicles containing the virus particles are exocytosed from the host cell to continue the infection cycle, resulting in the death of surrounding cells and tissue injury [54].

Effective host immune response against CoV-2 infection begins when viral RNAs, as pathogen-associated molecular patterns (PAMPs), are recognized by the pattern recognition receptors (PRRs) in residing antigen-presenting cells, such as macrophages and dendritic cells. Thus, PAMPs are sensed by PRRs, such as the RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs), initiating a downstream signalling cascade and activating the transcription factors IRF3, IRF7 and NF-kB. This leads to a broad induction of pro-inflammatory genes, particularly those encoding Type I interferon (IFNs), cytokines and chemokines. Moreover, NF-kB also induces the expression of a large number of genes involved in inflammation, stress, proliferation and apoptotic responses, such as cyclooxygenase 2 (COX-2), nitric oxide synthase 2 (NOS-2), vascular endothelial growth factor (VEGF), adhesion molecules, immune-receptors and growth factors. This inflammatory state stimulates local upregulation of adhesion molecules and the creation of chemotactic gradients, and also increases vascular permeability (mediated by VEGF), which together promotes the extravascular recruitment and activation of defence cells [55]. Subsequently, lymphocytes (such as NK, CD4+ T, CD8+ T and B cells) are recruited into the lung to eradicate the pathogen by killing virus-infected cells and producing virus-specific antibodies [56]. The overproduction of these chemokines and cytokines may contribute to the control of infection or to the development of critical prognostics.

Although most of the cases (about 80%) are asymptomatic or mild, clinical manifestations include fever, dry cough, shortness of breath, muscle ache, dizziness, headache, sore throat, rhinorrhoea, chest pain, diarrhoea, nausea and vomiting. Nevertheless, an uncontrolled immune response can trigger a cytokine storm and a large amount of free radicals, resulting in severe damages to the lungs, kidneys, heart, followed by possible sepsis, multiple organ failure and death [57]. Patients with severe COVID-19 cases exhibit elevated serum levels of pro inflammatory cytokines, i.e., interleukins- (IL-6, -1β, -2, -8, -17, -10 and -4), interferon γ (IFN-α/β), granulocyte colony-stimulating factor (G-CSF),

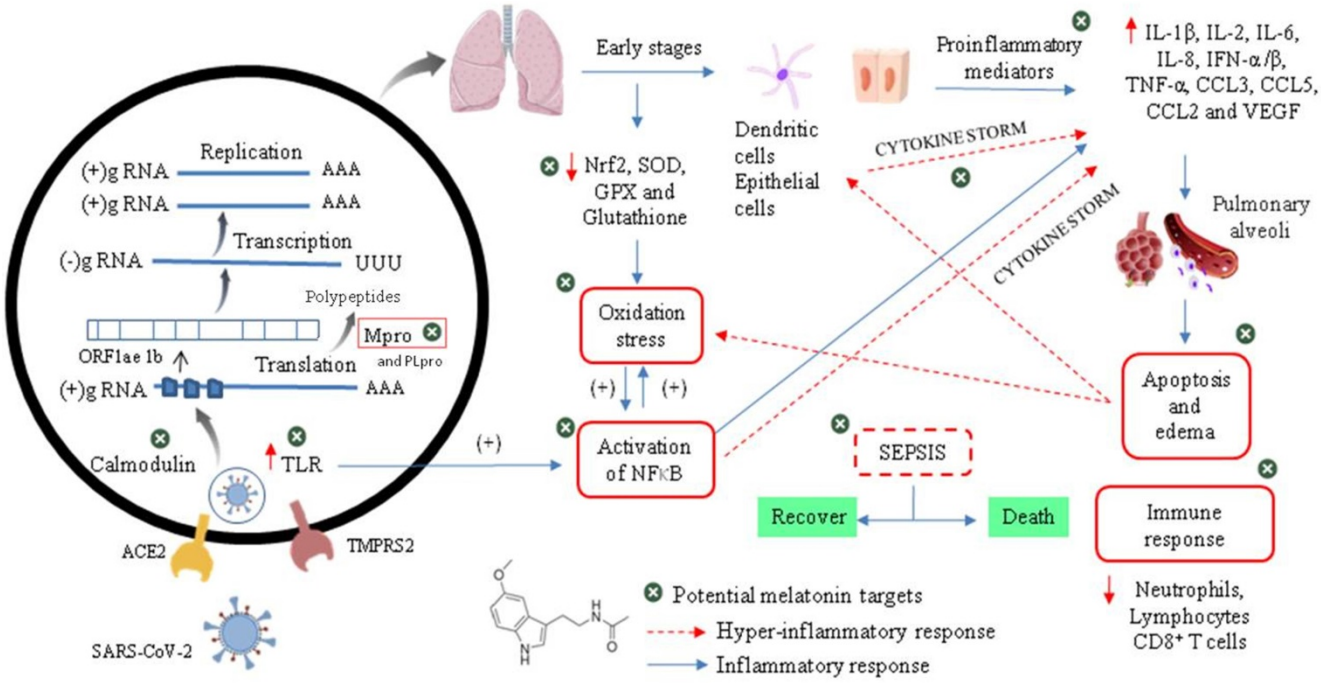

Figure 5. Viral cycle of CoV-2 and probable therapeutic melatonin targets.

granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-inducible protein (IP-10), monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory proteins 1α (MIP1α) and tumour necrosis factor α (TNF-α). In this intensity of disease, lymphopenia is a common feature, with drastically reduced numbers of CD4+ T cells, CD8+ T cells, B cells and natural killer (NK) cells, as well as a reduced percentage of monocytes, eosinophils and basophils. An increase in neutrophil-to-lymphocyte ratio may indicate poor clinical outcome.

There is also a massive infiltration of neutrophils and macrophages in the lung and excess of fluid into the alveoli, resulting in severe acute respiratory syndrome (SARS). During this advanced stage of infection, there may be a reduction in the levels of surfactant protein transcription, which generates increased pulmonary surface tension, reduced respiratory capacity and increased risk of respiratory collapse during expiration [58]. In addition, pathogenic examination of the lung showed proteinaceous exudate with globules, inflammatory cellular infiltration, bilateral diffuse alveolar damage with edema, pneumocyte desquamation and hyaline membrane formation.

There is a correlation between serum levels of cytokines and the severity of the disease indicating that mortality might be due to virally-driven hyper-inflammation. Interestingly, some COVID-19 patients, in spite of being negative for the viral nucleic acid test, have a high level of inflammation, which indicates that this factor is even more important than the viral load to define the patient's prognosis. Hyper-inflammation promotes cellular apoptosis and necrosis in various tissues, which would further trigger inflammation, followed by the release of more inflammatory factors, increased vascular permeability, mobility and accumulation of monocytes, macrophages and neutrophils. This indicates that if an exacerbated inflammatory process is not controlled, a vicious circle is created, capable of activating the "cytokine storm" and a possible evolution to sepsis [59].

In addition to the overwhelming immune response, SARS-CoV-2 can target organs that express ACE2, including the heart, kidney and gastrointestinal tract, aggravating the case. High levels of NOS-2-derived nitric oxide (NO), superoxide and other products of inflammation drive the body to a hemodynamic collapse, including impaired vascular permeability, vasodilatation, tissue hypoperfusion and untreatable hypotension. Thus, tissue hypoxia and the elevated production of reactive oxygen species (ROS) compromise mitochondrial function and activate cell death pathways, increasing tissue damage and the formation of fibrosis in target tissues, which express ACE2, including the heart, kidney and gastrointestinal tract [60-62]. Therefore, the unsolved infection becomes a life-threatening condition evolving to sepsis and even death.

Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous molecule, the final metabolite of the melatoninergic pathway from serotonin to N-acetylserotonin (NAS) by arylalkylamine N-acetyl-serotonin (AA-NAT), with NAS then converted to melatonin by hydroxyindole O-methyltransferase (HIOMT). For a long time, science has only discussed the properties of melatonin in the control of the circadian cycle in the absence of light and its biosynthesis by the pineal gland. Therefore, it is called as the hormone of darkness [63]. Nevertheless, it is noteworthy that melatonin is also produced by immune cells after NF-KB activation, a transcription factor involved in controlling the expression of several genes linked to the inflammatory response. This observation included melatonin as an integral part of the host defense system, where it has the autocrine and paracrine action that dampens immune responsiveness, while increasing the anti-inflammatory and phagocytic response in immune cells [64, 65].

It has been proven that there is a positive correlation between aging and the reduction of plasmatic melatonin, what may affect sleep quality as well as the suitable control of inflammatory processes and thus a higher risk of hyper-inflammation [66]. The beneficial effect of melatonin on acute lung injury states, sepsis and hyper-inflammatory processes caused by viruses, bacteria or radiation has been previously documented [67-70]. Accordingly, it is possible to imagine that melatonin can be indicated as adjuvant therapy for the treatment of COVID-19 due to its potential cytoprotective, anti-inflammatory, immunosuppressive and, probably, antiviral mechanisms [64]. Another relevant topic is that the drug interaction of melatonin with the main drugs currently available can reduce the side effects such as renal damage and oxidative stress of the lopinavir/ritonavir association, potentiate the antiviral response of ribavirin and reduce oedema when associated with methylprednisolone [66].

Anti-inflammatory effect of melatonin occurs through a range of mechanisms and mediators. During inflammatory and immunological processes, the intracellular mediator NF-KB (activated by the degradation of its inhibitor IKB by ROS pro-inflammatory cytokines) plays a key role in regulating multiple aspects of the innate and adaptive

immune response, and acts as a signal for the expression of pro-inflammatory reaction, inflammasomes and immune cells (e.g. macrophages). These cells are elevated during hyper-inflammation and are pivotal in the production of pro-inflammatory cytokines and chemokines involved with a higher inflammatory response and worse prognosis. Melatonin could modulate inflammation by decreasing the activation of both NF-κB and inflammasomes NLRP3 (nucleotide-binding domain leucine-rich repeat (NLR) and pyrin domain-containing receptor 3). Therefore, there will be a reduction in cell membrane damage and the inflammatory release of cell content to the extracellular space [66]. The mechanism of action as inhibitor of NF-κB has not yet been fully clarified but it seems to involve reducing oxidative stress via NF-E2-related factor (Nrf2), reducing the binding ability of NF-κB via acetylation of the p50 subunit [71-73] and toll-like receptors (TLRs). It inhibits, under inflammatory conditions, the expression of TLR2, TLR4 and TLR9, key mediators of the innate immune response that are massively activated when the virus reacts with its endosomal receptors or in pro-inflammatory processes [65].

Melatonin can up-regulate Nrf2, an important transcription factor activated by oxidative stress. It has a role in the regulation of the expression of detoxifying and antioxidant genes, inducing phase II protective gene, such as heme oxygenase-1 (HO-1), glutathione-S-transferase (GST) and UDP-glucuronosyltransferase (UGT) 1A1, which mediate cell survival and mitochondrial dose-dependent damage. Concomitantly, a more intense induction of inflammatory biomarkers was observed in Nrf2 KO mice, i.e. IL-1β, IL-6, TNFα and pro-inflammatory enzymes iNOS and COX2. Therefore, the increase in Nrf2 by melatonin is crucial in tissue protection, such as pulmonary, hepatocytes, renal and cardiac in patients positive for SARS-CoV-2 [74, 75].

Asadi-Pooya & Simani [76] highlighted that coronavirus infections have been associated with neurological manifestations (e.g. febrile seizures, convulsions, change in mental status and encephalitis), causing inflammation and de-myelination. The effect of melatonin in experimental autoimmune encephalomyelitis has demonstrated a therapeutic role by ameliorating the clinical severity and restricting the infiltration of inflammatory Th17 cells into the CNS. Moreover, another study reinforces this potential therapeutic by confirming an enhancement of IL-10, a multifunctional cytokine with expression of antiviral and cytoprotective properties [77] and, in contrast, that also suppresses IFN-γ, IL-17, IL-6, CCL20 and T cell proliferation in the CNS [78].

Severe COVID-19 patients may develop sepsis that is characterized by uncontrolled increase in oxidative stress, mitochondrial dysfunction, cellular energy failure, vasodilatation with hypo-responsiveness to drugs and multi-organ failure. Melatonin acts as a free radical scavenger and stimulates anti-oxidative enzymes (e.g., superoxide dismutase) and HO-1 (via activation of Nrf2), and also impairs pro-oxidative enzymes (e.g. iNOS) with a consequent reduction of peroxynitrite formation [79]. Several studies have demonstrated the therapeutic efficacy of melatonin in controlling septic conditions induced by toxic drugs or infection [80-82].

Another important anti-inflammatory effect is the inhibition of bradikinin-induced vasodilation, as well as the reduction of vascular permeability. It occurs since the melatonin treatment suppresses the vascular endothelial growth factor (VEGF) mRNA and decreases hypoxia-inducible factor (HIF)-1alpha protein levels and expression [82]. These changes added to the reduction of adhesion molecules (by inhibition of NF-κB) allow one to predict a lesser leukocyte adhesion to endothelial cells and to decrease migration into tissues, especially alveoli [65].

Moreover, melatonin has possible anti-infectious activity through different mechanisms. It can promote the formation of the neutrophil extracellular trap (NET), increase levels of the generally beneficial cells Th1 and NK cells. Previous studies with virus-infected animal models support the indication of melatonin as an antiviral after observing a reduction in viral load, improving the functions of the infected organ, decreased mortality and viremia. However, despite the reported viral load reductions, these studies have not discussed the possible mechanism involved in these outcomes. Supported by our results presented in the possible inhibition of Mpro and interaction with calmodulin (previous studies), we propose two new possible mechanisms in addition to the properties already reported to control hyper-inflammation and thus control COVID-19 [67].

ACE2 is a type I transmembrane metallo-peptidase with an extracellular ectodomain containing its zinc-coordinating catalytic site that is expressed and active in most tissues, mainly lung, heart, brain and kidney. Cell membranes richer of ACE2 become more susceptible to CoV-2 infection. Regulation of its expression at the cell surface is therefore of prime importance to control of virus-induced cell fusion by host [83, 84].

Calmodulin (CaM) is considered as the major regulator of Ca^{2+}-dependent signalling in all eukaryotic cells. CaM regulated the surface expression and retention of ACE2 in the plasma membrane. Inhibitors of this calcium binding protein

enhance the release of the ACE2 ectodomain and decrease the association between CaM and ACE2 in a dose- and time-dependent manner [85]. *In vitro* studies have demonstrated that melatonin binds to CaM (Kd of 188 pM) and inhibits it in a Ca^{2+}-dependent pocket [86, 89]. As a consequence of this interaction, melatonin can be classified as an indirect inhibitor of ACE2-CoV2 coupling during viral particle fusion [89]. Both, melatonin and CaM, are phylogenetically preserved. Therefore, their interaction probably stands out as an important pathway for the regulation of cell physiology [90], including protection against CoV-2 infection, autophagy, cell proliferation and apoptosis via inflammatory process, etc. [91].

Viral proteases are responsible for vital processing polyproteins, leading to the formation of structural and functional viral proteins. Since Mpro is unique in the virus and not found in the host cells, this protein is a prominent target for the development of antivirals against coronavirus infections. Mpro is a key CoV enzyme, which plays a crucial role by cleaving viral polyproteins during coronavirus replication and transcription [92]. The inhibition of viral protease is a strategy used successfully in many viral infections, mainly against the human immunodeficiency virus (HIV). It results in the incapacity of the new viral particles formed to replicate, producing, after the budding stage, only non-infectious virions.

Mpro is composed by 3 domains (I-chymotrypsin, II-picornavirus 3C protease-like and III-globular cluster involved in protein dimerisation). We can observe in this study that melatonin interacts in the site between domains I and II, blocking the access to the catalytic cysteine 145 (Fig. 4B) [92]. The binding site of melatonin on SARS-CoV-2 Mpro is considered as highly conserved, 96% identical (98% similar) in the aminoacid sequence to that of SARS-CoV Mpro. After comparing the binding site of melatonin to Mpro active site of SARS-CoV-2 and other coronavirus, we can confirm that melatonin is a potential inhibitor of this protease (Fig, 4A). The residues His41, Phe140, Ser144, Cys145, His163, His164, Glu 166, Gln189 and Thr190 are important fo the interaction of inhibitors of SARS-CoV Mpro [93] and are also present on the binding site of melatonin. Inhibitory actions on calmodulin and Mpro can further expand the spectrum of action of melatonin [92] and help to explain some antiviral results.

Viruses cause host mitochondrial dysfunction, which can reduce melatonin biosynthesis. The reduced serum melatonin cannot upregulate the expression of antioxidant enzymes, control the immune response, oxidative stress and the outcome of hyperinflammation. Therefore, the administration of melatonin has been successfully used to treat different viral infections in animal models, *e.g.* parvovirus, Venezuelan equine encephalomyelitis virus, LP-BM5 retrovirus, respiratory syncytial virus and rabbit hemorrhagic disease virus [65, 94].

A recent study determined the efficacy and tolerability of high-dose melatonin (36 mg/day to 72 mg/day per os (p.o.) in 4 divided doses) as adjuvant therapy, in addition to standard and/or empirical therapy [95]. All the patients were admitted with flu-like symptoms and chest imaging findings of ground glass opacities highly suggestive of COVID19 pneumonia. The 10 patients given melatonin had high-risk features determined for age (> 60 years) or/and established comorbidities. No significant side-effects were noted except for drowsiness. Benefits of time were observed for clinical improvement (reduction of symptoms, stabilization and/or regression of lung infiltrates, decrease in pro-inflammatory markers), as well as the need for mechanical ventilation, duration of hospital stay and outcome (death, or recovery and discharge) [95]. These results may encourage new greater trial studies.

In addition to its therapeutic properties on hyper-inflammation, oedema, immune dysregulation and possible sepsis in moderate-advanced stages of the disease, this drug may also be evaluated for its antiviral action in more recent stages of COVID-19 infection. Therapeutic regimes for prophylaxis and COVID-19 treatment using melatonin either as monotherapy or associated were proposed, i.e., 50- and 300-mg daily doses twice a day for 7 days for the control of both light and severe cases of COVID-19, respectively [96,97]. They have considered only the therapeutic potential over the modulation of the immune and inflammatory responses, not mentioning the new mechanisms proposed in the present study on the inhibition of calmodulin and Mprotease. Those doses still have to be validated to measure the efficacy and clinical outcomes.

Conclusion

We are dealing with one of the most potentially deadly pandemics in terms of economic and social damages of the last century. In the absence of readily accessible and efficient medicines, there is an urgent need for the development of some treatment. Therefore, finding a substance with therapeutic potential, low toxicity and cost, with mechanisms of action described, is certainly a great step in the therapeutic advance thanks to its wide use.

The results presented provide the first indication that the therapeutic administration of melatonin can be an effective Mpro inhibitor to prevent viral replication by binding to viral protease. Thus, we

suggest that melatonin could play an adjunct therapeutic role in treating COVID-19 in different stages of the disease. Therefore, clinical studies on the possible therapeutic value of melatonin on this infection should be performed in the near future.

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Competing Interests

The authors have declared that no competing interest exists.

References

1. Cucinotta D, Vanelli M. WHO declares COVID-19 a pandemic. Acta Biomed. 2020; 91 (1): 157-160. https://doi.org/10.23750/abm.v91i1.9397.
2. Weiss P, Murdoch DR. Clinical course and mortality risk of severe COVID-19. Lancet. 2020; 395: 1014-1015. https://doi.org/10.1016/S0140-6736(20)30633-4.
3. Fauci AS, Lane HC, Redfield RR. Covid-19 — navigating the uncharted. N Engl J Med. 2020; 382: 1268-1269. https://doi.org/10.1056/NEJMe2002387.
4. Mahase E, Kmietowicz Z. Covid-19: doctors are told not to perform CPR on patients in cardiac arrest. BMJ. 2020; 368: 1-2. https://doi.org/10.1136/bmj.m1282.
5. Cao B, Wang Y, Wen D, *et al*. A trial of lopinavir–ritonavir in adults hospitalized with severe Covid-19. N Engl J Med. 2020: 1-13, https://doi.org/10.1056/NEJMoa2001282.
6. Lim J, Jeon S, Shin HY, *et al*. Case of the index patient who caused tertiary transmission of COVID-19 infection in Korea: the application of lopinavir/ritonavir for the treatment of COVID-19 infected pneumonia monitored by quantitative RT-PCR. J Korean Med Sci. 2020; 35 (6): 1-6. https://doi.org/10.3346/jkms.2020.35.e79.
7. Liu F, Xu A, Xuan W, *et al*. Patients of COVID-19 may benefit from sustained lopinavir-combined regimen and the increase of eosinophil may predict the outcome of COVID-19 progression. Int J Infect Dis. 2020; 95: 183-191. https://doi.org/10.1016/j.ijid.2020.03.013.
8. Chen H, Xhang Z, Wang L, *et al*. First clinical study using HCV protease inhibitor danoprevir to treat naive and experienced COVID-19 patients. MedRxiv. 2020. https://doi.org/10.1101/2020.03.22.20034041.
9. Bhatnagar T, Murhekar MV, Soneja M, *et al*. Lopinavir/ritonavir combination therapy amongst symptomatic coronavirus disease 2019 patients in India: Protocol for restricted public health emergency use. Indian J Med Res. 2020; 151 (2): 184-189. https://doi.org/10.4103/ijmr.IJMR_502_20.
10. Cao YC, Deng QX, Dai SX. Remdesivir for severe acute respiratory syndrome coronavirus 2 causing COVID-19: An evaluation of the evidence. Travel med. infect. dis. 2020; 2: 1-6. https://doi.org/10.1016/j.tmaid.2020.101647.
11. Wit E, Feldmann F, Cronin J, *et al*. Prophylactic and therapeutic remdesivir (GS-5734) treatment in the rhesus macaque model of MERS-CoV infection. Proc Natl Acad Sci. 2020; 117: 6771-6776. https://doi.org/10.1073/pnas.1922083117.
12. Zhu Z, Lu Z, Xu T, *et al*. Arbidol monotherapy is superior to lopinavir/ritonavir in treating COVID-19. J Infect. 2020; 21: 1-3. https://doi.org/10.1016/j.jinf.2020.03.060.
13. Deng L, Li C, Zeng Q, *et al*. Arbidol combined with LPV/r versus LPV/r alone against Corona Virus Disease 2019: A retrospective cohort study. J Infect. 2020; 16: 1-5. https://doi.org/10.1016/j.jinf.2020.03.002.
14. An Observational Case-control study of the use of siltuximab in ARDS patients diagnosed with COVID-19 infection (SISCO). SISCO Study (NCT04322188): https://www.clinicaltrials.gov/ct2/show/NCT04322188.
15. Baricitinib in symptomatic patients infected by COVID-19: an open-label, pilot study. (BARI-COVID). SISCO Study (NCT04320277): https://www.clinicaltrials.gov/ct2/show/NCT04320277.
16. Clinical study of anti-CD147 humanized meplazumab for injection to treat with 2019-nCoV pneumonia. SISCO Study (NCT04275245): https://clinicaltrials.gov/ct2/show/NCT04275245.
17. Bevacizumab in severe or critically severe patients with COVID-19 Pneumonia-RCT (BEST-RCT). SISCO Study (NCT04305106): https://clinicaltrials.gov/ct2/show/NCT04305106.
18. bevacizumab in severe or critical patients with COVID-19 pneumonia (BEST-CP). SISCO Study (NCT04275414): https://clinicaltrials.gov/ct2/show/NCT04275414.
19. Favipiravir combined with tocilizumab in the treatment of corona virus disease 2019. SISCO Study (NCT04310228): https://clinicaltrials.gov/ct2/show/NCT04310228.
20. Tocilizumab for SARS-CoV2 (COVID-19) severe pneumonitis. SISCO Study (NCT04315480): https://clinicaltrials.gov/ct2/show/NCT04315480.
21. Anti-IL6 treatment of serious COVID-19 disease with threatening respiratory failure (TOCIVID). SISCO Study (NCT04322773): https://www.clinicaltrials.gov/ct2/show/NCT04322773.
22. Tocilizumab vs CRRT in management of cytokine release syndrome (CRS) in COVID-19 (TACOS). SISCO Study (NCT04306705): https://clinicaltrials.gov/ct2/show/NCT04306705.
23. The efficacy of different hormone doses in 2019-nCoV severe pneumonia. SISCO Study (NCT04263402): https://clinicaltrials.gov/ct2/show/NCT04263402.
24. Glucocorticoid therapy for COVID-19 critically ill patients with severe acute respiratory failure. SISCO Study (NCT04244591): https://clinicaltrials.gov/ct2/show/NCT04244591.
25. Efficacy and safety of corticosteroids in COVID-19. SISCO Study (NCT04273321): https://clinicaltrials.gov/ct2/show/NCT04273321.
26. Efficacy of methylprednisolone for patients with COVID-19 severe acute respiratory syndrome (MP-C19). SISCO Study (NCT04323592): https://clinicaltrials.gov/ct2/show/NCT04323592.
27. Gautret P, Lagier JC, Parola P, *et al*. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial. nt. J Antimicrob Agents. 2020: 105949-105973. https://doi.org/10.1016/j.ijantimicag.2020.105949.
28. Sinha N, Balayla G. Hydroxychloroquine and COVID-19. Postgrad. Med. J. 2020: 1-6. https://doi.org/10.1136/postgradmedj-2020-137785.
29. Sharma A. Chloroquine paradox may cause more damage than help fight COVID-19. Microbes Infect. 2020: 1-3. https://doi.org/10.1016/j.micinf.2020.04.004.
30. Sarma P, Kaur H, Kumar H, et al. Virological and clinical cure in COVID-19 patients treated with hydroxychloroquine: A systematic review and meta-analysis. J. Med. Virol. 2020. https://doi.org/10.1002/jmv.25898.
31. Sturrock BRH, Chevassut TJT. Chloroquine and COVID-19 - a potential game changer? Clin Med. 2020. https://doi.org/10.7861/clinmed.2020-0129.
32. Costanzo M, Giglio MAR, Roviello GN. SARS CoV-2: Recent reports on antiviral therapies based on lopinavir/ritonavir, darunavir/umifenovir, hydroxychloroquine, remdesivir, favipiravir and other drugs for the treatment of the new coronavirus. Curr Med Chem. 2020. https://doi.org/10.2174/0929867327666200416131117.
33. Sapp JL, Alqarawi W, MacIntyre CJ, *et al*. Guidance on minimizing risk of drug-induced ventricular arrhythmia during treatment of COVID-19: a statement from the canadian heart rhythm society. Can J Cardiol. 2020: 1-4. https://doi.org/10.1016/j.cjca.2020.04.003.
34. Chen C, Zhang Y, Huang J, et al. Favipiravir versus arbidol for COVID-19: A randomized clinical. MedRxiv. 2020; 10: 1-30. https://doi.org/10.1101/2020.03.17.20037432.
35. Chen Z, Hu J, Zhang Z, *et al*. Efficacy of hydroxychloroquine in patients with COVID-19: results of a randomized clinical trial. MedRxiv. 2020. https://doi.org/10.1101/2020.03.22.20040758.
36. Molina JM, Delaugerre C, Goff J. L, *et al*. No evidence of rapid antiviral clearance or clinical benefit with the combination of hydroxychloroquine and azithromycin in patients with severe COVID-19 infection. Med Maladies Infect. 2020: 30085-30088. https://doi.org/10.1016/j.medmal.2020.03.006.
37. Mandal H. Mobilizing the research ecosystem for scientific advances towards positive impact in the context of the COVID-19 pandemic. Turk J Med Sci. 2020; 50: 485-488. https://doi.org/10.3906/sag-2004-180.
38. Garofalo M, Grazioso G, Cavalli A, *et al*. How computational chemistry and drug delivery techniques can support the development of new anticancer drugs. Molecules. 2020; 25 (7): 1756. https://doi.org/10.3390/molecules25071756.
39. Qamar TM, Alqahtanic SM, Mubarak A, *et al*. Structural basis of SARS-CoV-2 3CLpro and anti-COVID-19 drug discovery from medicinal plants. J Pharm Anal. 2020. https://doi.org/10.1016/j.jpha.2020.03.009.
40. Wu Y, Liub Y, Yang P, *et al*. Analysis of therapeutic targets for SARS-CoV-2 and discovery of potential drugs by computational methods. Acta Pharm Si B. 2020: 1-23. https://doi.org/10.1016/j.apsb.2020.02.008.
41. Chen VB, Arendall WB, Headd JJ, *et al*. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. 2010; 66: 12-21. https://doi.org/10.1107/S0907444909042073.
42. Bitencourt-Ferreira G, Azevedo WF. Molegro virtual docker for docking. In: Docking screens for drug discovery. Human. 2019; 2053: 149-67. https://doi.org/10.1007/978-1-4939-9752-7_10.
43. Zoete V, Daina A, Bovigny C, *et al*. SwissSimilarity: a web tool for low to ultra-high throughput ligand-based virtual screening. J Chem Inf Model. 2016; 56: 1399-1404. https://doi.org/10.1021/acs.jcim.6b00174.
44. Daina A, Michielin O, Zoete V. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci Rep. 2017; 7: 42717. https://doi.org/10.1038/srep42717.
45. Yadav S, Pandey SK, Singh VK, *et al*. Molecular docking studies of 3-bromopyruvate and its derivatives to metabolic regulatory enzymes: Implication in designing of novel anticancer therapeutic strategies. PloS one. 2017; 12: 1-15. https://doi.org/10.1371/journal.pone.0176403.
46. Seidel T, Schutez DA, Garon A, *et al*. The pharmacophore concept and its applications in computer-aided drug design. Prog Chem Org Nat Prod. 2019; 110: 99-141. https://doi.org/10.1007/978-3-030-14632-0_4.

47. Zhong HA, Victoria M, Theodor WA, *et al*. Understanding the molecular properties and metabolism of top prescribed drugs. Curr Top Med Chem. 2013; 13: 1290-1307. https://doi.org/10.2174/15680266113139990034.
48. Lipinski CA, Lombardo F, Dominy BW, *et al*. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev. 1997; 23: 3-25. https://doi.org/10.1016/s0169-409x(00)00129-0.
49. Tobaiqy M, Qashqary M, Dahery, SA, *et al*. Therapeutic management of COVID-19 patients: A systematic review. Infect Prevent Pract. 2020: 100061. https://doi.org/10.1016/j.infpip.2020.100061.
50. Zheng J. SARS-CoV-2: An emerging coronavirus that causes a global threat. Int J Biol Sci. 2020; 16: 1678. https://doi.org/10.7150/ijbs.45053.
51. Bonavia A, Zelus BD, Wentworth DE, *et al*. Identification of a receptor-binding domain of the spike glycoprotein of human coronavirus HCoV-229E. J Virol. 2003; 77: 2530-2538. https://doi.org/10.1128/jvi.77.4.2530-2538.2003.
52. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. Methods Mol Biol. 2015: 1-23. https://doi.org/10.1007/978-1-4939-2438-7_1.
53. Li SW, Wang CY, Jou YJ, Huang SH, Hsiao LH, Wan L, Lin YJ, Kung SH, Lin CW. SARS coronavirus papain-like protease inhibits the TLR7 signaling pathway through removing Lys63-linked polyubiquitination of TRAF3 and TRAF6**.** Int J Mol Sci. 2016; 17: 678-688. https://doi.org/10.3390/ijms17050678.
54. Shereen MA, Khan S, Kazmi A, Bashir N, Siddique R. COVID-19 infection: Origin, transmission, and characteristics of human coronaviruses**.** J Adv Res. 2020; 24: 91-98. https://doi.org/10.1016/j.jare.2020.03.005.
55. Chen Y, Li L. SARS-CoV-2: virus dynamics and host response. Lancet Infect Dis. 2020. https://doi.org/10.1016/S1473-3099(20)30235-8.
56. Chen J, Lau YF, Lamirande EW, Paddock CD, Bartlett JH, Zaki SR, Subbarao K. Cellular immune responses to severe acute respiratory syndrome coronavirus (SARS-CoV) infection in senescent BALB/c mice: CD4+ T cells are important in control of SARS-CoV infection**.** J Virol. 2010; 84: 1289-1301. https://doi.org/10.1128/JVI.01281-09.
57. Zimmermann P, Curtis N. Coronavirus infections in children including COVID-19: an overview of the epidemiology, clinical features, diagnosis, treatment and prevention options in children. Ped Infect Dis J. 2020; 39: 355-368. https://doi.org/10.1097/inf.0000000000002660.
58. Gralinski LE, Baric RS. Molecular pathology of emerging coronavirus infections. J Pathol. 2015; 235: 185-195. https://doi.org/10.1002/path.4454.
59. Tufan A, Güler AA, Matucci-Cerinic M. COVID-19, immune system response, hyperinflammation and repurposing anti-rheumatic drugs. Turk J Med Sci. 2020; 50: 620-632. https://doi.org/10.3906/sag-2004-168.
60. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, Goor HV. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Path. 2004; 203: 631-637. https://doi.org/10.1002/path.1570.
61. Bertram S, Heurich A, Lavender H, Gierer S, Danisch S, Perin P, Lucas JM, Nelson PS, Pöhlmann S, Soilleux EJ. Influenza and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts. PloS one. 2012; 7: 35876, https://doi.org/10.1371/journal.pone.0035876.
62. Netland J, Meyerholz DK, Moore S, Cassell M, Perlman S. Severe acute respiratory syndrome coronavirus infection causes neuronal death in the absence of encephalitis in mice transgenic for human ACE2. J Virol. 2008; 82: 7264-7275. https://doi.org/10.1128/jvi.00737-08.
63. Amaral FG, Cipolla-Neto J. A brief review about melatonin, a pineal hormone. Arch. Endocrinol. Metab. 2018; 62: 472-479. https://doi.org/10.20945/2359-3997000000066.
64. Zhang R, Wang X, Ni L, Di X, Ma B, Niu S, Liu C, Reiter RJ. COVID-19: Melatonin as a potential adjuvant treatment. Life Sci. 2020; 250: 117583. https://doi.org/10.1016/j.lfs.2020.117583.
65. Tan DX, Hardeland R. Potential utility of melatonin in deadly infectious diseases related to the overreaction of innate immune response and destructive inflammation: focus on COVID-19. Melatonin Res. 2020; 3: 120-143. doi.10.32794/mr11250052.
66. Shneider A, Kudriavtsev A, Vakhrusheva A. Can melatonin reduce the severity of COVID-19 pandemic? Int Rev Immunol. 2020; 29: 1-10. doi: 10.1080/08830185.2020.1756284.
67. Acuña-Castroviejo D, Rahim I, Acuña-Fernández C, Fernández-Ortiz M, Solera-Marín J, Sayed RKA., Díaz-Casado ME, Rusanova I, López LC, Escames G. Melatonin, clock genes and mitochondria in sepsis. Cellular and Molecular Life Sci. 2017; 74: 3965-3987. https://doi.org/10.1007/s00018-017-2610-1.
68. Henderson R, Kim S, Lee E. Use of melatonin as adjunctive therapy in neonatal sepsis: a systematic review and meta-analysis. Complement Ther Med; 2018; 39: 131-136. https://doi.org/10.1016/j.ctim.2018.06.002.
69. D'angelo G, Marseglia L, Reiter RJ, Buonocore G, Gitto E. Melatonin and neonatal sepsis: a promising antioxidant adjuvant agent. Am J Perinatol, 2017; 34: 1382-1388, https://doi.org/10.1055/s-0037-1604244.
70. Srinivasan V, Mohamed M, Kato H. Melatonin in bacterial and viral infections with focus on sepsis: a review. Recent Pat Endocr Metab Immune Drug Discov. 2012; 6: 30-39. https://doi.org/10.2174/187221412799015317.
71. Choi EY, Jin JY, Lee JY, Choi JI, Choi IS, Kim SJ. Melatonin inhibits prevotella intermedia lipopolysaccharide-induced production of nitric oxide and interleukin-6 in murine macrophages by suppressing NF-κB and STAT1 activity. J Pineal Res. 2010; 50: 197-206. https://doi.org/10.1111/j.1600-079x.2010.00829.x.
72. Lee S, Le NH, Kang D. Melatonin alleviates oxidative stress-inhibited osteogenesis of human bone marrow-derived mesenchymal stem cells through AMPK activation. Int J Med Sci 2018; 15(10): 1083-1091. https://doi:10.7150/ijms.26314.
73. Shi D, Xiao X, Wang J, Liu L, Chen W, Fu L, Xie F, Huang W, Deng W. Melatonin suppresses proinflammatory mediators in lipopolysaccharide-stimulated CRL1999 cells via targeting MAPK, NF-κB, c/EBPβ, and p300 signaling. J Pineal Res. 2012; 53: 154-165. https://doi.org/10.1111/j.1600-079x.2012.00982.x.
74. Ali T, Rehman SU, Shah FA, Kim MO. Acute dose of melatonin via Nrf2 dependently prevents acute ethanol-induced neurotoxicity in the developing rodent brain. J Neuroinflammation Res. 2018; 15: 119-138. https://doi.org/10.1186/s12974-018-1157-x.
75. Fang J, Yan Y, Teng X, Wen X, Li N, Peng S, Liu W, Donadeu F.X, Zhao S, Hua J. Melatonin prevents senescence of canine adipose-derived mesenchymal stem cells through activating NRF2 and inhibiting ER stress. Aging Res. 2018; 10: 2954-2972. https://doi.org/10.18632/aging.101602.
76. Asadi-Pooya AA, Simani L. Central nervous system manifestations of COVID-19: a systematic review. J Neurological Sciences Res. 2020; 413: 116832-116836. https://doi.org/10.1016/j.jns.2020.116832.
77. Rojas JM, Avia M, Martín V, Sevilla N. IL-10: a multifunctional cytokine in viral infections. J Immunology Res. 2017; 2017: 1-14. https://doi.org/10.1155/2017/6104054.
78. Chen SJ, Huang SH, Chen JW, Wang KC, Yang YR, Liu PF, Lin GJ, Sytwu HK. Melatonin enhances interleukin-10 expression and suppresses chemotaxis to inhibit inflammation in situ and reduce the severity of experimental autoimmune encephalomyelitis. I Immunopharmacology Res. 2016; 31: 169-177. https://doi.org/10.1016/j.intimp.2015.12.020.
79. Prauchner CA. Oxidative stress in sepsis: pathophysiological implications justifying antioxidant co-therapy. Burns Res. 2017; 43: 471-485. https://doi.org/10.1016/j.burns.2016.09.023.
80. Srinivasan V, Pandi-Perumal SR, Spence DW, Kato H, Cardinali DP. Melatonin in septic shock: some recent concepts. J Critical Care Res. 2010; 25: 656-656. https://doi.org/10.1016/j.jcrc.2010.03.006.
81. Biancatelli RMLC, Berrill M, Mohammed YH, Marik PE. Melatonin for the treatment of sepsis: the scientific rationale. J Thoracic Disease Res. 2020; 12: 54-65. https://doi.org/10.21037/jtd.2019.12.85.
82. Xu L, Zhang W, Kwak M, Zhang L, Lee PCW, Jin JO. Protective effect of melatonin against polymicrobial sepsis is mediated by the anti-bacterial effect of neutrophils. F Immunology Res. 2019; 10: 1371. https://doi.org/10.3389/fimmu.2019.01371.
83. Kandemir YB, Konuk E, Behram M, Sindel M. Effect of melatonin on the expression of VEGF-A and on the degeneration of follicle reserve in rat ovary. The Euro J Medicine Res. 2018; 50 (3): 160-163. https://doi.org/10.5152/eurasianjmed.2018.17361.
84. Leung JM, Yang CX, Tam A, Shaipanich T, Hackett TL, Singhera GK, Dorscheid DR, Sin DD. ACE-2 expression in the small airway epithelia of smokers and COPD patients: implications for COVID-19: Implications for COVID-19. Euro Res J Res. 2020; 2000688-2000698. https://doi.org/10.1183/13993003.00688-2020.
85. Thomson G. COVID-19: social distancing, ACE 2 receptors, protease inhibitors and beyond? Int J Clinical Practice Res. 2020; 1-3 https://doi.org/10.1111/ijcp.13503.
86. Lambert DW, Clarke NE, Hooper NM, Turner AJ. Calmodulin interacts with angiotensin-converting enzyme-2 (ACE2) and inhibits shedding of its ectodomain. Febs Letters Res. 2007; 582: 385-390. https://doi.org/10.1016/j.febslet.2007.11.085.
87. Romero MP, García-Pergañeda A, Guerrero JM, Osuna C. Membrane-bound calmodulin in Xenopus laevis oocytes as a novel binding site for melatonin. The FASEB J Res. 1998; 12: 1401-1408. https://doi.org/10.1096/fasebj.12.13.1401.
88. Benítez-King G, Ríos A, Martínez A, Antón-Tay F. *In vitro* inhibition of Ca2+/calmodulin-dependent kinase II activity by melatonin. Biochim Biophys Acta. 1996; 1290: 191-196. https://doi.org/10.1016/0304-4165(96)00025-6.
89. Río BD, Pedrero JM, Martínez-Campa C, Zuazua P, Lazo PS, Ramos S. Melatonin, an endogenous-specific inhibitor of estrogen receptor α via calmodulin. J Bio Chemistry Res. 2004; 279: 38294-38302, https://doi.org/10.1074/jbc.m403140200.
90. Benítez-King G, Huerto-Delgadillo L, Antón-Tay F. Binding of 3H-melatonin to calmodulin. Life Sci Res. 1993; 53: 201-207. https://doi.org/10.1016/0024-3205(93)90670-x.
91. Berchtold MW, Villalobo A. The many faces of calmodulin in cell proliferation, programmed cell death, autophagy, and cancer. Bio et Bio Acta (BBA)-Mol Cell Res. 2014; 1843: 398-435. https://doi.org/10.1016/j.bbamcr.2013.10.021.
92. Ui-Tei K, Nagano M, Sato S, Miyata Y. Calmodulin-dependent and-independent apoptosis in cells of a drosophila neuronal cell line. Apoptosis. 2000; 5: 133-140. https://doi.org/10.1023/a:1009676528805.
93. Jin Z, Du X, Xu Y, et al. Structure of Mpro from SARS-CoV-2 and discovery of its inhibitors. Nature. 2020; 582: 289–293. https://doi.org/10.1038/s41586-020-2223-y.
94. Zhang L, Lin D, Kusov Y, et al. α ketoamides as broad-spectrum inhibitors of coronavirus and enterovirus replication: Structure-based design, synthesis,

and activity assessment. J Med Chem. 2020, 63: 4562−4578. https://doi:10.1021/acs.jmedchem.9b01828.
95. Tan DX, Hardeland R. Potential utility of melatonin in deadly infectious diseases related to the overreaction of innate immune response and destructive inflammation: focus on COVID-19. Melatonin Research. 2020; 3: 120-143. https://doi.org/10.32794/mr11250052.
96. Castillo RR, Quizon GRA, Juco MJM, et al. Melatonin as adjuvant treatment for coronavirus disease 2019 pneumonia patients requiring hospitalization (MAC-19 PRO): a case series. Melatonin Res. 2020; 3: 297-310. https://doi.org/10.32794/mr11250063.
97. Reiter RJ, Abnreu-Gonzalez P, Marik PE, Dominguez-Rodriguez A. Therapeutic algorithm for the use of melatonin in patients with COVID-19. Front Med. 2020; 7: 1-7. https://doi.org/10.3389/fmed.2020.00226.

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The Role of Vitamin C as Adjuvant Therapy in COVID-19

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Abstract

Background and objective

The anti-inflammatory properties of vitamin C (VC) and the promising results it has shown in the treatment for common cold have prompted clinicians to use it as adjuvant therapy in the treatment of COVID-19. The purpose of this study was to find out the role of VC as adjunctive therapy in coronavirus disease 2019 (COVID-19).

Methodology

This study was conducted from March to July 2020 in the COVID-19 unit of a tertiary care hospital in Karachi. In this randomized controlled trial (RCT), one group received the intervention [50 mg/kg/day of intravenous (IV) VC] along with the standard therapy, and the other group received standard therapy only. Data such as age, gender, vitals, and biochemical values as well as outcomes including the number of days required for treatment, hospital stay, need for ventilation, and mortality were compared between the two groups and recorded using a self-structured questionnaire.

Results

COVID-19 patients who received IV VC became symptom-free earlier (7.1 ± 1.8 vs. 9.6 ± 2.1 days, p-value: <0.0001) and spent fewer days in the hospital (8.1 ± 1.8 vs. 10.7 ± 2.2 days, p-value: <0.0001) compared to those who received standard therapy only. However, there was no significant difference in the need for mechanical ventilation (p-value: 0.406) and mortality (p-value: 0.31) between the two groups.

Conclusion

VC can significantly improve clinical symptoms in patients affected with COVID-19; however, it had no impact on mortality and the need for mechanical ventilation. More large-scale studies are required to further assess the role of VC in the treatment of COVID-19.

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Introduction

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by a newly discovered strain of coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Most infected people will develop mild to moderate illnesses with the most common symptoms being fever, dry cough, and fatigue, and recover without hospitalization [1]. Vitamin C (VC), otherwise known as ascorbic acid, is known to boost immunity and acts as a potent antioxidant. Therefore, VC can be beneficial in resolving infection and inflammation. A randomized controlled trial (RCT) has demonstrated a measurable benefit of VC supplementation in reducing cold episodes in young men with low to average VC status [2]. In a recent meta-analysis, VC supplementation was shown to reduce serum C-reactive protein (CRP) levels, particularly in younger subjects with higher CRP baseline levels, at a lower dosage, and with intravenous (IV) administration [3].

Potential benefits of VC have sparked an interest regarding finding its use in the treatment of COVID-19. In a case series involving VC administration to COVID-19 patients, there was a significant decrease in inflammatory markers, indicating that the use of IV VC in patients with moderate to severe COVID-19 disease may be clinically feasible [4]. Administration of high doses of VC as a therapeutic agent can

favorably impact severely ill COVID-19 patients with viral pneumonia and acute respiratory distress syndrome (ARDS) by decreasing inflammation, pathogen infectiveness, and virulence, and also by optimizing immune defense, reducing tissue and organ injuries, and improving the overall outcome of the disease [5].

During the ongoing global outbreak of COVID-19, there are many studies and trials underway to figure out the therapeutic role of VC in COVID-19 patients. Moreover, there is so much more yet to be learned about this vitamin itself. We believe that our study will contribute significantly towards the efforts to find out if VC could be beneficial as adjunctive therapy in the treatment of COVID-19.

Materials And Methods

This prospective, open-label RCT was conducted in the COVID-19 unit of a tertiary care hospital in Karachi, Pakistan. Data of patients who were admitted with severe COVID-19 infection from March to July 2020 were included in the study. Patients were diagnosed with severe COVID-19 based on the national health guidelines of Pakistan [6]. Patients who needed mechanical ventilation within 12 hours of admission were excluded from the study. All subjects provided informed consent for inclusion before they participated in the study.

Patients were randomized to the interventional arm or placebo arm using a randomizer software. The interventional arm received 50 mg/kg/day of IV VC in addition to standard therapy for COVID-19 infection. The placebo arm received only the standard therapy for COVID-19. Standard therapy included antipyretics, dexamethasone, and prophylactic antibiotics and was comparable between both groups. Data were compared between the patients who received IV VC vs. those who did not receive it. Patients' gender, age, and vitals and biochemical values such as respiratory rate, oxygen saturation, CRP, and lactate dehydrogenase (LDH) levels were recorded using a self-structured questionnaire. The number of days required for the disappearance of symptoms, number of days spent in the hospital, need for ventilation, and mortality were also noted and compared for both groups.

The collected data were analyzed using SPSS Statistics version 21.0 (IBM Corp, Armonk, NY). Mean and standard deviations (SD) were calculated for numerical data. Frequency and percentages were calculated for categorical data. Frequencies were compared using a chi-squared test. Independent t-test and chi-square test were used as appropriate. A p-value of less than 0.05 indicated that there was a significant difference between the two groups and the null hypothesis was void.

Results

A total of 150 patients were included in the study; 75 of them were randomized to the interventional arm and received IV VC in addition to standard therapy for COVID-19 infection, and 75 were in the placebo group and received only standard care for COVID-19 infection. There were 99 (56.9%) males and 76 (43.1%) females in the study. The differences in age, respiratory rate, levels of CRP, and LDH levels between the two groups were not statistically significant (Table *1*).

Characteristics	SOC + IV vitamin C (n=75), mean + SD	SOC without IV vitamin C (n=75), mean + SD	P-value
Age (in years)	52 ± 11	53 ± 12	0.59
Respiratory rate (BPM)	30.2 ± 5.7	29.8 ± 4.9	0.64
CRP (mg/L)	118.2 ± 16.2	116.2 ± 17.2	0.46
LDH (IU)	315.2 ± 87.6	311.3 ± 89.3	0.78
Oxygen saturation (%)	87.2 ± 4.6	86.1 ± 4.9	0.15

TABLE 1: Comparison of demographics and clinical characteristics between the two groups

CRP: C-reactive protein; LDH: lactate dehydrogenase; SOC: standard of care; SD: standard deviation; IV: intravenous

COVID-19 patients who received IV VC became symptom-free earlier (7.1 ± 1.8 vs. 9.6 ± 2.1 days, p-value: <0.0001) and spent fewer days in the hospital (8.1 ± 1.8 vs. 10.7 ± 2.2 days, p-value: <0.0001) compared to those who received standard therapy only. However, the overall difference regarding the need for mechanical ventilation and mortality between the interventional arm and the placebo group was not statistically significant (Table *2*).

Outcome	SOC + IV vitamin C (n=75)	SOC without IV vitamin C (n=75)	P-value
Days to be symptom-free, mean + SD	7.1 ± 1.8	9.6 ± 2.1	<0.0001*
Days spent in the hospital, mean + SD	8.1 ± 1.8	10.7 ± 2.2	<0.0001*
Need for mechanical ventilation, n (%)	12 (16%)	15 (20%)	0.406**
Overall death, n (%)	7 (9.3%)	11 (14.6%)	0.31**

TABLE 2: Comparison of outcomes between the two groups

*Statistically significant; **Statistically not significant

SOC: standard of care; SD: standard deviation; IV: intravenous

Discussion

SARS-CoV-2 primarily targets the respiratory system, leading to the development of severe ARDS and even respiratory failure. Symptoms manifested depend on the organ system involved and range from high-grade fever, dry cough, shortness of breath, sore throat, and fatigue to diarrhea, confusion, seizures, and impairment of taste and smell [7].

The infection caused by SARS-CoV-2 and its progression to respiratory failure is driven by a strong and dysregulated immune-inflammatory response, which leads to elevated levels of pro-inflammatory cytokines including interleukin 6 (IL-6) and endothelin-1 (ET-1) in the body and a “cytokine storm” causing accumulation of neutrophils within the lungs, destroying alveolar capillaries. VC exerts its effects by reducing the secretion of these pro-inflammatory cytokines from the immune effector cells and preventing neutrophils activation and accumulation and the formation of neutrophil extracellular traps, which is a biological event of alveolar vascular injury caused by neutrophil activation [8,9]. In China, 50 patients with moderate to severe COVID-19 were given 10-20 g/day of VC. The oxygenation index of the patients improved, and all the patients were eventually cured and discharged [10]. An RCT conducted in the United States (US) showed that administration of ~15 g/day of IV VC for four days may decrease mortality in patients with sepsis and ARDS [11]. In a recent meta-analysis of nine RCTs, 0.7-0.8 g/day dose of VC improved symptoms and reduced the duration of infection and time of indoor confinement in patients with common cold virus infection [12]. In another meta-analysis of eight RCTs, supplementation of 0.5-2 g/day of VC in 3,153 patients reduced the duration of the upper respiratory tract infection by 1.6 days, suggesting its potential role in the treatment of COVID-19 [13]. However, very few studies have been conducted to correctly predict the role of VC as adjuvant therapy for COVID-19, but those conducted have shown promising results related to high doses of VC due to its favorable side-effect profile [8].

The present study was conducted to analyze the benefit of using VC as an adjuvant therapy to treat COVID-19 patients presenting to a tertiary care hospital in Pakistan. This study also concluded that patients who received IV VC along with standard therapy for SARS-CoV-2 infection recovered earlier (7.1 ± 1.8 vs. 9.6 ± 2.1 days, p-value: <0.0001) and spent fewer days in the hospital (8.1 ± 1.8 vs. 10.7 ± 2.2 days, p-value: <0.0001) when compared to patients who only received standard therapy for SARS-CoV-2 infection. However, the overall difference in the need for mechanical ventilation and mortality rate between them was not significant. This was in contrast with the meta-analysis by Hemilä et al., which reported that high doses of IV VC shortened the length of mechanical ventilation by 14% in critically ill patients, accompanied by a significant reduction in the mortality rate [14,15]. An RCT conducted on 56 critical COVID-19 patients concluded that the difference was not significant in invasive mechanical ventilation-free days in 28 days (IMVFD28) between groups that received high-dose IV VC and the group that received bacteriostatic water infusion. However, the study reported reduced 28-day mortality in severe COVID-19 patients who received high-dose IV VC [16].

This study, to the best of our knowledge, is the first study to be conducted in Pakistan to analyze the role of VC as an adjunct therapy for COVID-19 patients admitted to hospitals. However, the study has its limitations. Since it was a single-center study, the sample was not diverse and care should be taken when extrapolating the results on a larger scale.

Conclusions

Based on our findings, VC can significantly improve clinical symptoms of patients affected with SARS-CoV-2 and reduce days spent in the hospital; however, VC supplementation had no impact on mortality and the need for mechanical ventilation. Nevertheless, VC has been proven to improve immunity in various forms of virus infections, and more studies on a larger scale are needed to further assess the role of VC in the

treatment of COVID-19.

Additional Information

Disclosures

Human subjects: Consent was obtained by all participants in this study. Ghulam Muhammad Mahar Medical College IRB issued approval GMMMC/20/03-4 (electronic). This study was approved by Ghulam Muhammad Mahar Medical College IRB. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

References

1. World Health Organization: coronavirus . (2020). Accessed: October 22, 2020: https://www.who.int/health-topics/coronavirus.
2. Johnston CS, Barkyoumb GM, Schumacher SS: Vitamin C supplementation slightly improves physical activity levels and reduces cold incidence in men with marginal vitamin C status: a randomized controlled trial. Nutrients. 2014, 6:2572-2583. 10.3390/nu6072572
3. Jafarnejad S, Boccardi V, Hosseini B, Taghizadeh M, Hamedifard Z: A meta-analysis of randomized control trials: the impact of vitamin C supplementation on serum CRP and serum hs-CRP concentrations. Curr Pharm Des. 2018, 24:3520-3528. 10.2174/1381612824666181017101810
4. Hiedra R, Lo KB, Elbashabsheh M, et al.: The use of IV vitamin C for patients with COVID-19: a case series . Expert Rev Anti Infect Ther. 2020, 18:1259-1261. 10.1080/14787210.2020.1794819
5. Hoang BX, Shaw G, Fang W, Han B: Possible application of high-dose vitamin C in the prevention and therapy of coronavirus infection. J Glob Antimicrob Resist. 2020, 23:256-262. 10.1016/j.jgar.2020.09.025
6. NHSRC: clinical management guideline for COVID-19 infections . (2020). Accessed: Nov 29, 2020: http://www.nhsrc.gov.pk/SiteImage/Misc/files/20200704%20Clinical%20Management%20Guidelines%20for%20COVID-19%20infecti....
7. Mao L, Jin H, Wang M, et al.: Neurologic manifestations of hospitalized patients with coronavirus disease 2019 in Wuhan, China. JAMA Neurol. 2020, 77:683-690. 10.1001/jamaneurol.2020.1127
8. Feyaerts AF, Luyten W: Vitamin C as prophylaxis and adjunctive medical treatment for COVID-19? . Nutrition. 2020, 79:110948. 10.1016/j.nut.2020.110948
9. Colunga Biancatelli RML, Berrill M, Catravas JD, Marik PE: Quercetin and vitamin C: an experimental, synergistic therapy for the prevention and treatment of SARS-CoV-2 related disease (COVID-19). Front Immunol. 2020, 11:1451. 10.3389/fimmu.2020.01451
10. Cheng RZ: Can early and high intravenous dose of vitamin C prevent and treat coronavirus disease 2019 (COVID-19)?. Med Drug Discov. 2020, 5:100028. 10.1016/j.medidd.2020.100028
11. Fowler AA 3rd, Truwit JD, Hite RD, et al.: Effect of vitamin C infusion on organ failure and biomarkers of inflammation and vascular injury in patients with sepsis and severe acute respiratory failure: the CITRIS-ALI randomized clinical trial. JAMA. 2019, 322:1261-1270. 10.1001/jama.2019.11825
12. Ran L, Zhao W, Wang J, Wang H, Zhao Y, Tseng Y, Bu H: Extra dose of vitamin C based on a daily supplementation shortens the common cold: a meta-analysis of 9 randomized controlled trials. Biomed Res Int. 2018, 2018:1837634. 10.1155/2018/1837634
13. Vorilhon P, Arpajou B, Vaillant Roussel H, Merlin É, Pereira B, Cabaillot A: Efficacy of vitamin C for the prevention and treatment of upper respiratory tract infection. A meta-analysis in children. Eur J Clin Pharmacol. 2019, 75:303-311. 10.1007/s00228-018-2601-7
14. Hemilä H, Chalker E: Vitamin C may reduce the duration of mechanical ventilation in critically ill patients: a meta-regression analysis. J Intensive Care. 2020, 8:15. 10.1186/s40560-020-0432-y
15. Marik PE, Khangoora V, Rivera R, Hooper MH, Catravas J: Hydrocortisone, vitamin C, and thiamine for the treatment of severe sepsis and septic shock: a retrospective before-after study. Chest. 2017, 151:1229-1238. 10.1016/j.chest.2016.11.036
16. Zhang J, Rao X, Li Y, et al.: High-dose vitamin C infusion for the treatment of critically ill COVID-19 [PREPRINT]. Res Square. 2020, 10.21203/rs.3.rs-52778/v1

Global campaign makes plea for vitamin C and COVID-19

17-Dec-2020 By Shane Starling

An international consortium of vitamin C advocates wants regulators to bring vitamin C into the therapeutic bag of measures in the battle against COVID-19.

Mitchell Felder Feb 14, 2021, 4:30 PM

to William, me, Mitchell

Global campaign makes plea for vitamin C and COVID-19

By Shane Starling
17-Dec-2020 - Last updated on 18-Dec-2020 at 14:04 GMT

https://www.nutraingredients.com/Article/2020/12/17/Global-campaign-makes-plea-for-vitamin-C-and-COVID-19

RELATED TAGS: Vitamin c, COVID-19, HFMA

An international consortium of vitamin C advocates wants regulators to bring vitamin C into the therapeutic bag of measures in the battle against COVID-19.

The **VitaminC4Covid** campaign, so far backed by about 350 leading vitamin C researchers, doctors, healthcare professionals and nutritionists along with 5000+ signatories from about 60 countries, says evidence shows injected or orally taken high doses of vitamin C can halt the onset of severe COVID-19 symptoms.

The UK-led campaign cites a **review of 100+ vitamin C trials** just published in *Nutrients* – some of which are specific to COVID-19; some specific to sepsis and broader respiratory issues – to back its case.

Lead author of that review and VitaminC4Covid campaign director, Patrick Holford, told NutraIngredients vitamin C data was as strong as that which existed for vitamin D and had the potential to reduce COVID-19 symptoms at low cost and *"suppress viral replication in the early stages".*

"The critical phase of COVID-19 is a direct result of an inflammatory reaction by the body's immune system, akin to sepsis, that only occurs if there is a large quantity of dead virus particles," said Holford, who is the founder of the Food for the Brain Foundation and has written 45 nutrition-focused books.

"Early suppression and rapid resolution of symptoms, as shown in cold studies giving 4 to 8 grams of vitamin C in the first 24 hours of infection, has the potential to stop conversion to critical COVID, thus taking the pressure off the National Health Service."

The *Nutrients* review has been presented to the British Health Minister Matt Hancock as well as the New and Emerging Respiratory Virus Threats Advisory Group (NERVTAG), the Scientific Advisory Committee on Nutrition (SACN) and the National Institute for Clinical Evidence

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Editorial

Be well: A potential role for vitamin B in COVID-19

Coronavirus disease (COVID-19) is caused by the SARS-CoV-2 virus. In January 2020, the World Health Organization declared COVID-19 as a Public Health Emergency of International Concern and in March 2020, COVID-19 was characterized as a global pandemic that is responsible for infecting over 20 million and more than 700,000 deaths. COVID-19 symptoms are fever, cough, fatigue, headache, diarrhea, arthromyalgias, serious interstitial pneumonia that can lead to acute respiratory distress syndrome, sepsis-induced coagulopathy and multi-organ dysfunction [1]. In addition, the severe progression of COVID-19 results in cytokine storm with excessive production of pro-inflammatory cytokines [2]. Previously, outbreaks of similar viruses which belong to the β-coronavirus family occurred in 2002–2004 and 2012–2014, as severe acute respiratory syndrome (SARS) and as the Middle East respiratory syndrome (MERS), respectively [3,4].

Currently, there is no approved drug treatment or vaccine against the SARS-CoV-2 virus. Until these become available, one must include adequate and balanced nutrition for proper body functioning and boosting of the immune system. Micronutrients, vitamin C and vitamin D have gained much attention during the pandemic because of their anti-inflammatory and immune-supporting properties. Low levels of vitamins D and C result in coagulopathy and suppress the immune system, causing lymphocytopenia. Evidence has shown that the mortality rate is higher in COVID-19 patients with low vitamin D concentrations. Further, vitamin C supplementation increases the oxygenation index in COVID-19 infected patients [5]. Similarly, vitamin B deficiency can significantly impair cell and immune system function, and lead to inflammation due to hyperhomocysteinemia.

There is a need to highlight the importance of vitamin B because it plays a pivotal role in cell functioning, energy metabolism, and proper immune function [6]. Vitamin B assists in proper activation of both the innate and adaptive immune responses, reduces pro-inflammatory cytokine levels, improves respiratory function, maintains endothelial integrity, prevents hypercoagulability and can reduce the length of stay in hospital [7,8]. Therefore, vitamin B status should be assessed in COVID-19 patients and vitamin B could be used as a non-pharmaceutical adjunct to current treatments (Fig. 1).

1. Can vitamin B be used to manage COVID-19?

1.1. Vitamin B_1 (Thiamine)

Thiamine is able to improve immune system function and has been shown to reduce the risk of type-2 diabetes, cardiovascular disease, aging-related disorders, kidney disease, cancer, mental disorders and neurodegenerative disorders [6]. Thiamine deficiency affects the cardiovascular system, causes neuroinflammation, increases inflammation and leads to aberrant antibody responses [6]. As antibodies, and importantly T-cells, are required to eliminate the SARS-CoV-2 virus, thiamine deficiency can potentially result in inadequate antibody responses, and subsequently more severe symptoms. Hence, adequate thiamine levels are likely to aid in the proper immune responses during SARS-CoV-2 infection. In addition, the symptoms of COVID-19 are very similar to altitude sickness and high-altitude pulmonary edema. Acetazolamide is commonly prescribed to prevent high-altitude sickness and pulmonary edema through inhibition of the carbonic anhydrase isoenzymes and subsequently increases oxygen levels. Thiamine also functions as a carbonic anhydrase isoenzyme inhibitor [9]; hence, high-doses of thiamine given to people at early stages of COVID-19 could potentially limit hypoxia and decrease hospitalization. Further research is required to determine whether administration of high thiamine doses could contribute to the treatment of patients with COVID-19.

1.2. Vitamin B_2 (Riboflavin)

Riboflavin together with UV light cause irreversible damage to nucleic acids such as DNA and RNA, rendering microbial pathogens unable to replicate. Riboflavin and UV light has been shown to be effective against the MERS-CoV virus, suggesting that it could also be helpful against SARS-CoV-2 [10]. In fact, riboflavin-UV decreased the infectious titer of SARS-CoV-2 below the limit of detection in human blood [10] and in plasma and platelet products [11]. This could alleviate some of the risk of transfusion transmission of COVID-19 and as well as reducing other pathogens in blood products for critically ill COVID-19 patients.

1.3. Vitamin B_3 (Nicotinamide, Niacin)

Niacin acts as a building block of NAD and NADP, both vital during chronic systemic inflammation [12]. NAD^+ acts as a coenzyme in various metabolic pathways and its increased levels are essential to treat a wide range of pathophysiological conditions. NAD^+ is released during the early stages of inflammation and has immunomodulatory properties, known to decrease the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α. [13–15]. Recent evidence indicates that targeting IL-6 could help control the inflammatory storm in patients with COVID-19 [16]. Moreover, niacin reduces neutrophil infiltration and exhibits an anti-inflammatory effect in patients with ventilator-induced lung injury. In hamsters, niacin and nicotinamide prevents lung tissue damage [17]. In addition, nicotinamide reduces viral replication (vaccinia virus, human immunodeficiency virus, enteroviruses, hepatitis B virus) and

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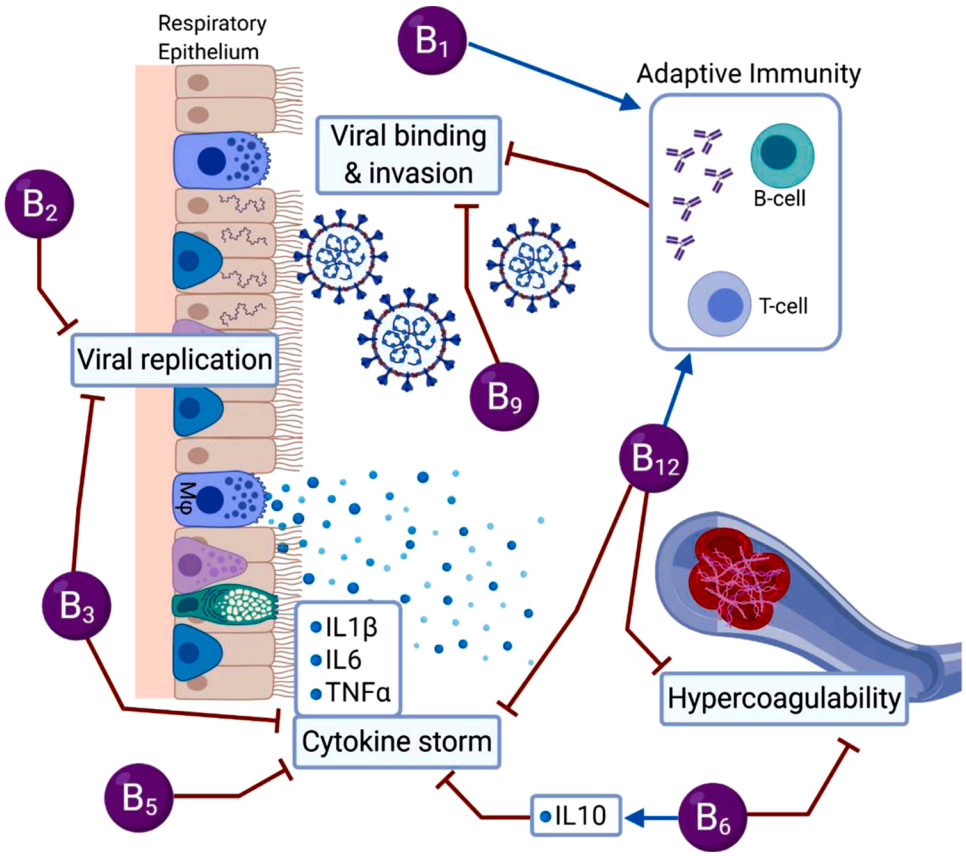

Fig. 1. Summary of the different roles vitamin B can play during COVID-19.

strengthens the body's defense mechanisms. Taking into account the lung protective and immune strengthening roles of niacin, it could be used as an adjunct treatment for COVID-19 patients [8,18].

1.4. Vitamin B_5 (Pantothenic acid)

Pantothenic acid has a number of functions, including cholesterol- and triglyceride-lowering properties, improves wound healing, decreases inflammation and improves mental health [6]. Even though there are limited studies demonstrating the effects of pantothenic acid on the immune system, it is a viable vitamin for future investigation.

1.5. Vitamin B_6 (Pyridoxal 5′-phosphate, Pyridoxine)

Pyridoxal 5′-phosphate (PLP) is an active form of pyridoxine, and is an essential cofactor in various inflammatory pathways with deficiency leading to immune dysregulation. PLP has an inverse relationship with plasma IL-6 and TNF-α in chronic inflammatory conditions. During inflammation, the utilization of PLP increases results in its depletion, suggesting that COVID-19 patients with high inflammation may have deficiency. Low PLP levels have been noted in patients with type-2 diabetes, cardiovascular disease and in the elderly [19–21], groups who are at higher risk of poorer COVID-19 outcomes. Dysregulation of immune responses and increased risk of coagulopathy have also been noted among COVID-19 patients. In a recent preprint it is suggested that PLP supplementation mitigates COVID-19 symptoms by regulating immune responses, decreasing pro-inflammatory cytokines, maintaining endothelial integrity and preventing hypercoagulability [22]. In fact, it was shown three decades ago that PLP levels reduce abnormalities in platelet aggregation and blood clot formation [23]. Recently researchers at Victoria University reported that vitamin B_6 (as well as B_2 and B_9) upregulated IL-10, a powerful anti-inflammatory and immunosuppressive cytokine which can deactivate macrophages and monocytes and inhibit antigen-presenting cells and T cells [24]. COVID-19 patients often respond to the virus by mounting an excessive T cell response and secretion of pro-inflammatory cytokines. It may be that PLP is able to contribute to dampening the cytokine storm and inflammation suffered by some COVID-19 patients.

1.6. Vitamin B_9 (folic acid, folate)

Folate is an essential vitamin for DNA and protein synthesis and in the adaptive immune response. Furin is an enzyme associated with bacterial and viral infections and is a promising target for treatment of infections. Recently, it was noted that folic acid was able to inhibit furin, preventing binding by the SARS-CoV-2 spike protein, preventing cell entry and virus turnover. Therefore it was suggested that folic acid could be beneficial for the management of COVID-19-associated respiratory disease in the early stages [25]. A recent preprint report that folic acid and its derivatives tetrahydrofolic acid and 5-methyl tetrahydrofolic acid have strong and stable binding affinities against the SARS-CoV-2, through structure-based molecular docking. Therefore, folic acid may be used as a therapeutic approach for the management of COVID-19 [26].

1.7. Vitamin B_{12} (cobalamin)

Vitamin B_{12} is essential for red blood cell synthesis, nervous system health, myelin synthesis, cellular growth and the rapid synthesis of DNA. The active forms of vitamin B_{12} are hydroxo-, adenosyl- and methylcobalamin. Vitamin B_{12} acts as a modulator of gut microbiota and low levels of B_{12} elevate methylmalonic acid and homocysteine, resulting in

increased inflammation, reactive oxygen species and oxidative stress [15]. Hyperhomocysteinemia causes endothelial dysfunction, activation of platelet and coagulation cascades, megaloblastic anemia, disruption of myelin sheath integrity and decreased immune responses [27–30]. However, SARS-CoV-2 could interfere with vitamin B_{12} metabolism, thus impairing intestinal microbial proliferation. Given that, it is plausible that symptoms of vitamin B_{12} deficiency are close to COVID-19 infection such as elevated oxidative stress and lactate dehydrogenase, hyperhomocysteinemia, coagulation cascade activation, vasoconstriction and renal and pulmonary vasculopathy [28,31]. In addition, B_{12} deficiency can result in disorders of the respiratory, gastrointestinal and central nervous systems [30]. Surprisingly, a recent study showed that methylcobalamin supplements have the potential to reduce COVID-19-related organ damage and symptoms [32]. A clinical study conducted in Singapore showed that COVID-19 patients who were given vitamin B_{12} supplements (500 μg), vitamin D (1000 IU) and magnesium had reduced COVID-19 symptom severity and supplements significantly reduced the need for oxygen and intensive care support [33].

2. What is the outcome?

Vitamin B not only helps to build and maintain a healthy immune system but it could potentially prevent or reduce COVID-19 symptoms or treat SARS-CoV-2 infection. Poor nutritional status predisposes people to infections more easily; therefore, a balanced diet is necessary for immuno-competence. There is a need for safe and cost-effective adjunct or therapeutic approaches, to suppress aberrant immune activation, which can lead to a cytokine storm, and to act as anti-thrombotic agents. Adequate vitamin intake is necessary for proper body function and strengthening of the immune system. In particular, vitamin B modulates immune response by downregulating pro-inflammatory cytokines and inflammation, reducing breathing difficulty and gastrointestinal problems, preventing hypercoagulability, potentially improving outcomes and reducing the length of stay in the hospital for COVID-19 patients.

Contributors

Hira Shakoor contributed to the writing and revision of this editorial.
Jack Feehan contributed to the revision of this editorial.
Kathleen Mikkelsen contributed to the revision of this editorial.
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Habiba I Ali contributed to the revision of this editorial.
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Leila Cheikh Ismail contributed to the revision of this editorial.
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Vasso Apostolopoulos conceptualized the editorial and contributed to the writing, revision and approval of the final version of this editorial.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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References

[1] Z.Y. Zu, M.D. Jiang, P.P. Xu, W. Chen, Q.Q. Ni, G.M. Lu, L.J. Zhang, Coronavirus disease 2019 (COVID-19): a perspective from China, Radiology (2020), 200490.
[2] C. Zhang, Z. Wu, J.-W. Li, H. Zhao, G.-Q. Wang, The cytokine release syndrome (CRS) of severe COVID-19 and interleukin-6 receptor (IL-6R) antagonist tocilizumab may be the key to reduce the mortality, Int. J. Antimicrob. Agents (2020), 105954.
[3] A. Zumla, D.S. Hui, S. Perlman, Middle East respiratory syndrome, The Lancet 386 (9997) (2015) 995–1007.
[4] D. Schoeman, B.C. Fielding, Coronavirus envelope protein: current knowledge, Virol. J. 16 (1) (2019) 1–22.
[5] H. Shakoor, J. Feehan, S.A. Al Dhaheri, I.H. Ali, C. Platat, C.L. Ismail, V. Apostolopoulos, L. Stojanovska, Immune boosting role of vitamins D, C, E, zinc, selenium and omega-3 fatty acids: could they help against COVID-19? Maturitas (2020).
[6] K. Mikkelsen, V. Apostolopoulos, Vitamin B.1, B2, B3, B5, and B6 and the immune system, Nutr. Immunity (2019) 115–125.
[7] C.A. Michele, B. Angel, L. Valeria, M. Teresa, C. Giuseppe, M. Giovanni, P. Ernestina, B. Mario, Vitamin supplements in the era of SARS-Cov2 pandemic, GSC Biol. Pharm. Sci. 11 (2) (2020) 007–019.
[8] L. Zhang, Y. Liu, Potential interventions for novel coronavirus in China: a systematic review, J. Med. Virol. 92 (5) (2020) 479–490.
[9] Z.O. Ozdemir, M. Senturk, D. Ekinci, Inhibition of mammalian carbonic anhydrase isoforms I, II and VI with thiamine and thiamine-like molecules, J. Enzyme Inhib. Med. Chem. 28 (2) (2013) 316–319.
[10] I. Ragan, L. Hartson, H. Pidcoke, R. Bowen, R. Goodrich, Pathogen reduction of SARS-CoV-2 virus in plasma and whole blood using riboflavin and UV light, Plos One 15 (5) (2020), e0233947.
[11] S.D. Keil, I. Ragan, S. Yonemura, L. Hartson, N.K. Dart, R. Bowen, Inactivation of severe acute respiratory syndrome coronavirus 2 in plasma and platelet products using a riboflavin and ultraviolet light-based photochemical treatment, Vox Sang. (2020).
[12] Y. Boergeling, S. Ludwig, Targeting a metabolic pathway to fight the flu - boergeling - 2017, FEBS J. - Wiley Online Library (2020).
[13] K. Mikkelsen, V. Apostolopoulos, B vitamins and ageing, biochemistry and cell biology of ageing: part I biomedical science, Springer (2018) 451–470.
[14] K. Mikkelsen, L. Stojanovska, V. Apostolopoulos, The effects of vitamin B in depression, Curr. Med. Chem. 23 (38) (2016) 4317–4337.
[15] K. Mikkelsen, L. Stojanovska, M. Prakash, V. Apostolopoulos, The effects of vitamin B on the immune/cytokine network and their involvement in depression, Maturitas 96 (2017) 58–71.
[16] B. Liu, M. Li, Z. Zhou, X. Guan, Y. Xiang, Can we use interleukin-6 (IL-6) blockade for coronavirus disease 2019 (COVID-19)-induced cytokine release syndrome (CRS)? J. Autoimmun. (2020), 102452.
[17] A. Nagai, H. Matsumiya, M. Hayashi, S. Yasui, H. Okamoto, K. Konno, Effects of nicotinamide and niacin on bleomycin-induced acute injury and subsequent fibrosis in hamster lungs, Exp. Lung Res. 20 (4) (1994) 263–281.
[18] M. Mario, J. Nina, S. Urs, Nicotinamide riboside—the current State of research and therapeutic uses, Nutrients 12 (6) (2020) 1616.
[19] C. Merigliano, E. Mascolo, R. Burla, I. Saggio, F. Vernì, The relationship between vitamin B6, diabetes and cancer, Front. Genet. 9 (2018) 388.
[20] C.O. Lengyel, S.J. Whiting, G.A. Zello, Nutrient inadequacies among elderly residents of long-term care facilities, Can. J. Diet. Pract. Res. 69 (2) (2008) 82–88.
[21] W.A. Nix, R. Zirwes, V. Bangert, R.P. Kaiser, M. Schilling, U. Hostalek, R. Obeid, Vitamin B status in patients with type 2 diabetes mellitus with and without incipient nephropathy, Diabetes Res. Clin. Pract. 107 (1) (2015) 157–165.
[22] J. Desbarats, Pyridoxal 5'-Phosphate to Mitigate Immune Dysregulation and Coagulopathy in COVID-19, 2020.
[23] V. Van Wyk, H.G. Luus, Ad.P. Heyns, The in vivo effect in humans of pyridoxal-5′-phosphate on platelet function and blood coagulation, Thrombosis Res. 66 (6) (1992) 657–668.
[24] K. Mikkelsen, M.D. Prakash, N. Kuol, K. Nurgali, L. Stojanovska, V. Apostolopoulos, Anti-tumor effects of vitamin B2, B6 and B9 in promonocytic lymphoma cells, Int. J. Mol. Sci. 20 (15) (2019).
[25] Z. Sheybani, M.H. Dokoohaki, M. Negahdaripour, M. Dehdashti, H. Zolghadr, M. Moghadami, S.M. Masoompour, A.R. Zolghadr, The Role of Folic Acid in the Management of Respiratory Disease Caused by COVID-19, 2020.
[26] V. Kumar, M. Jena, In Silico Virtual Screening-Based Study of Nutraceuticals Predicts the Therapeutic Potentials of Folic Acid and Its Derivatives Against COVID-19, 2020.
[27] N. Nemazannikova, K. Mikkelsen, L. Stojanovska, G.L. Blatch, V. Apostolopoulos, Is there a link between vitamin B and multiple sclerosis? Med. Chem. 14 (2) (2018) 170–180.

[28] W. Sabry, M. Elemary, T. Burnouf, J. Seghatchian, H. Goubran, Vitamin B12 deficiency and metabolism-mediated thrombotic microangiopathy (MM-TMA), Transfusion Apheresis Sci. 59 (1) (2020), 102717.
[29] M.M. Stipp, SARS-CoV-2: micronutrient optimization in supporting host immuno-competence, Int. J. Clin.Case Rep. Rev. 2 (2) (2020).
[30] B.H.R. Wolffenbuttel, H.J.C.M. Wouters, M.R. Heiner-Fokkema, M.M. Van der klauw, The many faces of cobalamin (vitamin B12) deficiency, Mayo Clin. Proc.: Innov. Quality Outcomes 3 (2) (2019) 200–214.
[31] S. Grangé, S. Bekri, E. Artaud-Macari, A. Francois, C. Girault, A.-L. Poitou, Y. Benhamou, C. Vianey-Saban, J.-F. Benoist, V. Châtelet, Adult-onset renal thrombotic microangiopathy and pulmonary arterial hypertension in cobalamin C deficiency, Lancet (London, England) 386 (9997) (2015) 1011.
[32] L.M.J. dos Santos, Can vitamin B12 be an adjuvant to COVID-19 treatment? GSC Biol. Pharm. Sci. 11 (3) (2020) 1–5.
[33] C.W. Tan, L.P. Ho, S. Kalimuddin, B.P.Z. Cherng, Y.E. Teh, S.Y. Thien, H.M. Wong, P.J.W. Tern, J.W.M. Chay, C. Nagarajan, A cohort study to evaluate the effect of combination vitamin D, magnesium and vitamin B12 (DMB) on progression to severe outcome in older COVID-19 patients, medRxiv (2020).

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Review

Vitamin B_{12} in Health and Disease

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Abstract: Vitamin B_{12} is essential for DNA synthesis and for cellular energy production. This review aims to outline the metabolism of vitamin B_{12}, and to evaluate the causes and consequences of sub-clinical vitamin B_{12} deficiency. Vitamin B_{12} deficiency is common, mainly due to limited dietary intake of animal foods or malabsorption of the vitamin. Vegetarians are at risk of vitamin B_{12} deficiency as are other groups with low intakes of animal foods or those with restrictive dietary patterns. Malabsorption of vitamin B_{12} is most commonly seen in the elderly, secondary to gastric achlorhydria. The symptoms of sub-clinical deficiency are subtle and often not recognized. The long-term consequences of sub-clinical deficiency are not fully known but may include adverse effects on pregnancy outcomes, vascular, cognitive, bone and eye health.

Keywords: vitamin B_{12}; physiology; nutrition; adults; chronic disease

Vitamin B_{12} deficiency was first described in 1849, and was considered to have a fatal outcome until 1926 when a diet of liver, high in vitamin B_{12}, was shown to slow the disease process. Much is now known about the biochemistry and metabolism of vitamin B_{12}, however, the diagnosis of its deficiency has become more complicated with the classification of a "sub-clinical" deficiency category, characterized by serum vitamin B_{12} concentrations that were once considered to be adequate. Vitamin B_{12} deficiency was previously thought to take many years to develop, and only in strict vegetarians or those with pernicious anaemia. More recent research has suggested that there are disease implications associated with sub-clinical B_{12} deficiency, which develop most commonly due to malabsorption or dietary inadequacy. The rates of sub-clinical deficiency of vitamin B_{12} are high in

developing countries, in the elderly, and in vegetarian populations. The long term consequences are not fully known but may include adverse effects on pregnancy outcomes and aspects of ageing.

1. Vitamin B_{12} Function

Vitamin B_{12} also known as cobalamin, comprises a number of forms including cyano-, methyl-, deoxyadenosyl- and hydroxy-cobalamin. The cyano form, which is used in supplements, is found in trace amounts in food [1]. The other forms of cobalamin can be converted to the methyl- or 5-deoxyadenosyl forms that are required as co factors for methionine synthase and L-methyl-malonyl-CoA mutase.

Methionine synthase is essential for the synthesis of purines and pyrimidines. The reaction depends on methyl cobalamin as a co-factor and is also dependent on folate, in which the methyl group of methyltetrahydrofolate is transferred to homocysteine to form methionine and tetrahydrofolate. A deficiency of vitamin B_{12} and the interruption of this reaction leads to the development of megaloblastic anaemia. Folate deficiency independent of vitamin B_{12} also causes megaloblastic anaemia [2]. Methylmalonyl CoA mutase converts methylmalonyl CoA to succinyl CoA, with 5-deoxy adenosyl cobalamin required as a cofactor. It is a defect in this reaction, and the subsequent accumulation of methylmalonyl CoA that is thought to be responsible for the neurological effects in vitamin B_{12} deficiency [2].

Serum vitamin B_{12} is bound to proteins known as transcobalamins (TC). The majority of the vitamin, approximately 80%, is transported on the inactive TCI (also called haptocorrin). The active transport protein for vitamin B_{12} is transcobalamin II (TCII), which caries about 20% of the vitamin in the circulation [3]. Holo-transcobalamin (holo-TC) is TCII with attached cobalamin, which delivers vitamin B_{12} to cells. A low serum vitamin B_{12} concentration can be associated with a deficiency of TCI, while TCII levels and so vitamin B_{12} status remain adequate [4].

2. Biochemical Assessment of Vitamin B_{12} Status

Traditionally vitamin B_{12} status is assessed by its concentrations in serum, however, concerns have been raised about the use of serum vitamin B_{12} measurements alone. Although low serum vitamin B_{12} concentrations are a sensitive indicator of vitamin B_{12} deficiency and high vitamin B_{12} concentrations generally indicate sufficiency, the interpretation of the intermediate range of vitamin B_{12} concentrations is unclear [4].

Methylmalonic acid (MMA) and homocysteine (tHcy) are recognized indicators of vitamin B_{12} status. Their measurement has highlighted the existence of sub-clinical deficiency, the consequences of which are still being elucidated. MMA is considered to be the specific indicator of cobalamin metabolism, and tHcy is raised in vitamin B_{12} deficiency along with deficiencies of folate and vitamin B_6. These biomarkers can be confounded by physiological or environmental conditions. Plasma tHcy concentrations are elevated also with renal impairment, polymorphisms in methylenetetrahydrofolate reductase (MTHFR), or the use of some medication. Plasma MMA concentrations are elevated also in renal insufficiency, common in older people [4,5].

It has been recommended by some authors [6,7] that measuring serum vitamin B_{12} concentrations and following up low values with MMA measurements is an appropriate strategy for the assessment of

vitamin B_{12} status. However, the threshold of vitamin B_{12} at which further testing should occur is controversial. A study of serum vitamin B_{12}, MMA and tHcy concentrations indicates that if a lower limit of normal (200 ng/L or 147 pmol/L) is used, patients with increased MMA would be missed, however, if higher values (500 ng/L or 370 pmol/L) are used, most patients would need follow-up MMA tests which may be within the normal range [8]. Carmel recommends a composite criteria based on serum vitamin B_{12} < 148 pmol/L, or 148–258 pmol/L and MMA > 0.30μmol/L, or tHcy > 13 nmol/L (females) and >15 nmol/L (males) be used to define inadequate vitamin B_{12} status [9].

Studies that have assessed the use of holo-TC as a marker of vitamin B_{12} status show similarity in specificity and sensitivity to serum vitamin B_{12} concentrations. However, when used in combination with vitamin B_{12} the predictive value for determining vitamin B_{12} deficiency is improved [10].

3. Absorption

Vitamin B_{12} is bound to protein in food and is available for absorption after it has been cleaved from protein by the hydrochloric acid produced by the gastric mucosa. The released cobalamin then attaches to R protein and passes into the duodenum where the R protein is removed and free cobalamin binds to Intrinsic Factor (IF). The IF-cobalamin complex is absorbed by the distal ileum and requires calcium [2]. Vitamin B_{12} enters the circulation about 3–4 hours later bound to TC.

Vitamin B_{12} is secreted in bile and reabsorbed via the enterohepatic circulation by ileal receptors which require IF, thus the development of vitamin B_{12} deficiency is likely to be more rapid in patients with pernicious anaemia as IF is lacking [3]. Vitamin B_{12} is excreted via the faeces, which is composed of unabsorbed biliary vitamin B_{12}, gastrointestinal cells and secretions, and vitamin B_{12} synthesised by bacteria in the colon. It is estimated that daily vitamin B_{12} losses are in proportion to body stores with approximately 0.1% excreted per day [11]. Excessive vitamin B_{12} in the circulation, e.g., such as after injections, usually exceeds the binding capacity of TC and is excreted in the urine [3].

Historically, vitamin B_{12} absorption has been measured by a number of methods including whole body counting of radiolabeled vitamin B_{12}, metabolic balance studies [1] or controlled feeding studies in vitamin B_{12}-depleted individuals [12].

It is known that the total amount of vitamin B_{12} that is absorbed increases with vitamin B_{12} intake but that the percentage absorption decreases with increasing doses [13]. One study using crystalline vitamin B_{12} supplements reported that 50% was retained at a 1 μg dose, 20% at a 5 μg dose and 5% at a 25 μg dose, suggesting saturation of the absorption mechanisms [14]. The absorption capacity is thought to recover to baseline levels within 4-6 hours allowing for efficient absorption of the next dose [11]. Approximately 1% of large doses of crystalline vitamin B_{12} found in some supplements (1,000μg), are absorbed through a mass action process, even in the absence of IF [15], indicating crystalline vitamin B_{12} in high doses and food vitamin B_{12} are absorbed by different mechanisms.

The Schilling test was the classical procedure for assessing the absorption of vitamin B_{12} but is now rarely used. As there has been no replacement a number of individual tests must be used to diagnose the cause of vitamin B_{12} deficiency. Tests that diagnose atrophic gastritis, a common cause of vitamin B_{12} malabsorption, include gastroscopy or serum gastrin and pepsinogen levels. Specific tests for pernicious anaemia include IF antibodies and serum gastrin estimation. MMA and tHcy are better markers of vitamin B_{12} status, although they are not appropriate for testing absorption [16]. An

overview of the medical management of vitamin B_{12} deficiency can be found in a recent article by Ralph Carmel [17].

4. Food Sources and Bioavailability of Vitamin B_{12}

Vitamin B_{12} is synthesised by certain bacteria in the gastrointestinal tract of animals and is then absorbed by the host animal. Vitamin B_{12} is concentrated in animal tissues, hence, vitamin B_{12} is found only in foods of animal origin [11]. Foods that are high in vitamin B_{12} (μg/100g) include: liver (26–58), beef and lamb (1–3), chicken (trace-1), eggs (1–2.5) and dairy foods (0.3–2.4).

There are no naturally occurring bioactive forms of vitamin B_{12} from plant sources. Some plant foods contain added vitamin B_{12} and others e.g., seaweed and mushrooms contain vitamin B_{12} analogues that are inactive in humans, although 2 studies suggest certain types of Japanese seaweed (nori) have prevented vitamin B_{12} deficiency in vegans [18]. Some foods that are contaminated or fermented by bacteria e.g., tempeh and Thai fish sauce, have been reported to contain vitamin B_{12} [18], although these may have low affinity with IF and may be poorly absorbed [19].

A number of methods have been used to determine the vitamin B_{12} content of foods. Microbiological assays using vitamin B_{12} requiring bacteria were used, however, they are no longer the reference method as measurement uncertainty is high. Radio isotope dilution assays with labeled vitamin B_{12} and hog IF are used [20]. Further advances are expected with the development of more specific monoclonal antibodies tests using specific binding proteins [21].

The bioavailability of vitamin B_{12} in humans is dependent on an individual's gastrointestinal absorption capacity. As outlined previously, vitamin B_{12} absorption is complex and there are adverse changes with age. In view of the technical challenges and biological factors, there is little data on the bioavailability of dietary vitamin B_{12} in humans. It is thought that 1.5–2.0 μg of synthetic vitamin B_{12} saturates the IF-cobalamin ileal receptors, but other studies have shown higher absorption rates [1,11]. In normal humans the absorption of vitamin B_{12} from foods has been shown to vary depending on the quantity and type of protein consumed [19]. Vitamin B_{12} from foods appear to have different absorption rates with better absorption from chicken and beef as compared to eggs. Studies assessing absorption of food bound vitamin B_{12} from whole foods are described in Table 1.

5. Vitamin B_{12} Requirement

The Recommended Dietary Intake (RDI) is set to prevent megaloblastic anaemia and maintain adequate serum vitamin B_{12} concentrations. It is assumed that 50% of dietary vitamin B_{12} is absorbed. The RDI and estimated average requirement (EAR) do not vary once adulthood is reached. However, the US and Australian Nutrient Reference Values suggest that older adults with atrophic gastritis may require higher intakes of vitamin B_{12}-rich foods, vitamin B_{12} fortified foods or supplements [3,22]. The US Institute of Medicine has recommended that adults over 51 years consume most of their vitamin B_{12} from fortified foods or from supplements, again recognising the high rates of malabsorption due to gastritis that occurs with age. Vitamin B_{12} stores last several years and the development of deficiency is slow, however the combination of malabsorption and inadequate dietary intake will hasten deficiency [3].

Table 1. Bioavailability of vitamin B_{12} from whole foods.

Food Type	Subjects	Vitamin B_{12} content	% Absorption, mean (range)	Analysis method	Reference
Mutton	3 healthy young subjects	0.9 μg in 100g portion	65 (56–77)	Radiolabelled vitamin B_{12} , whole body counting	[11]
	2 healthy young subjects	3.03 μg in 200g portion	83		
	2 healthy young subjects	5.11 μg in 300g portion	53		
Liver pate	6 healthy subjects	38 μg per serve	9.1 (5.1–19.5)	Radiolabelled vitamin B_{12} , whole body counting	[11]
	4 older subjects		4.5 (2.4–6.0)		
	5 subjects with pernicious anaemia		1.8 (0–3.7)		
Chicken	3 healthy subjects	0.4–0.6 μg in 100g portion	65 (58–74)	Radiolabelled vitamin B_{12} , faecal excretion studies	[23]
		0.8–1.3 μg in 200g portion	63 (48–76)		
		1.3–1.9 μg in 300g portion	61 (49–75)		
Fish	3 healthy subjects	2.1 μg in 50g portion	42	Radiolabelled vitamin B_{12} , faecal and urinary excretion studies	[24]
		3.1 μg in 100g portion	38		
		9.2 μg in 200g portion	42		
		13.3 μg in 300g portion	30		
Eggs: boiled, scrambled, fried	18 healthy subjects	0.9–1.4 μg in 100g portion	3.7–9.2	Radiolabelled vitamin B_{12} , faecal and urinary excretion	[25]

6. Vitamin B_{12} Deficiency

Deficiency is usually caused by the malabsorption of vitamin B_{12} although dietary inadequacy is common in the elderly, vegans or ovo-lacto vegetarians with poor diets. Causes can also relate to inadequate IF production, atrophic gastritis, interference with the ileal uptake of vitamin B_{12} due to disease, resection or interference by bacterial overgrowth, drug-nutrient interactions as well as some less common genetic defects [3].

Vegans who consume no foods of animal origin can meet their vitamin B_{12} requirement from fortified foods or supplements. Ovo-lacto vegetarians with only a small intake of dairy foods or eggs, may require supplemental vitamin B_{12}. Pregnant and/or lactating women following vegetarian or vegan diets are at high risk of deficiency due to the increased metabolic demand for vitamin B_{12} and require adequate intake of vitamin B_{12}-containing foods or supplements.

The elderly are at risk of undernutrition in general, predominately due to reduced intake related to illness but also due to physical capacity e.g., difficulties with food preparation, and psychological factors e.g., depression. Protein bound malabsorption is thought to be the most common cause of sub-clinical vitamin B_{12} deficiency in the elderly and is commonly associated with some degree of atrophic gastritis. Gastritis or inflammation of the gastric mucosa increases with age and results in a reduction, or in some cases, complete loss of the acid required to cleave vitamin B_{12} from protein. Synthetic vitamin B_{12} remains available for absorption as it is not protein bound [3,22].

Pernicious anemia is the end stage of an auto-immune gastritis and results in the loss of synthesis of IF. It is this loss of IF that causes vitamin B_{12} deficiency and if untreated, megaloblastic anaemia and neurological complications develop. Pernicious anaemia is treated with vitamin B_{12} injections, or large doses of oral vitamin B_{12}. Vitamin B_{12} deficiency will also develop after gastric antrum resection as this is the site of secretion of IF and acid [3].

Reduced ileal uptake of vitamin B_{12} can be caused by competition for vitamin B_{12} in patients with bacterial overgrowth or parasitic infection. Resection or diseases of the ileum such as Crohn's Disease or other chronic bowel inflammatory conditions also cause malabsorption of vitamin B_{12} [3].

The potential masking of vitamin B_{12} deficiency by folate fortification of the food supply has also raised some safety concerns. When vitamin B_{12} concentrations are low, high doses of folate (supplements or food fortification) allow DNA synthesis to continue, prevent megaloblastic anaemia and potentially "mask" vitamin B_{12} deficiency, potentially allowing homocysteine and MMA concentrations to rise and neurological damage to progress. Neurological damage in the absence of anaemia has been reported in 20-30% of cases of vitamin B_{12} deficiency [4]. In view of this and the effect of vitamin B_{12} deficiency on pregnancy outcomes [26,27], there is discussion of the need to fortify flour with vitamin B_{12}. Vitamin B_{12} fortification of flour is most likely to benefit those with poor dietary intake of vitamin B_{12} and the elderly with food bound malabsorption, but would be inadequate for those with pernicious anaemia, which affects 2–4% of the US population depending on ethnicity [28]. Patients with pernicious anaemia require larger oral supplements (e.g., 500–1,000 μg/d) or intramuscular injections. In developing countries, fortification could potentially have a more significant impact as the population's intake is often low. However, as yet there not enough intervention trials on the effect of different fortification levels of flour in different populations [29].

7. Drug-nutrient Interactions

Some medications are thought to interfere with the absorption or metabolism of vitamin B_{12}. These include proton pump inhibitor (PPI) medications, metformin, nitrous oxide anaesthesia, some epileptic medications and colchicine.

PPI medications are commonly used in the elderly for the treatment of gastro-oesophageal reflux disease. PPI medications act by reducing the secretion of gastric acid and pepsin, theoretically leading to a decrease in the absorption of protein-bound vitamin B_{12}. However, the current literature on PPI usage and vitamin B_{12} status is inconsistent [30-33]. The monitoring of vitamin B_{12} concentrations is recommended for patients undergoing prolonged PPI treatment, in recognition that the bioavailability of food-bound vitamin B_{12} may be compromised [3].

Metformin is a biguanide used for the treatment of non-insulin dependent diabetes and some patients taking this medication develop megaloblastic anaemia [34,35]. This may relate to intestinal

mobility changes or bacterial overgrowth competing for vitamin B_{12} in the gastrointestinal tract. It has also been shown that calcium improves the uptake of vitamin B_{12} in metformin users [35].

Nitrous oxide anesthesia inhibits methionine synthase and L-methylmalonyl–CoA mutase and produces deficiency symptoms despite concentrations of serum vitamin B_{12} in the normal range [36]. Antiepileptic drugs have been associated with low concentrations of vitamin B_{12}, but this is controversial with some studies showing no change and others increased levels of vitamin B_{12} [37].

8. Vitamin B_{12} and Neural Tube Defects (NTD)

NTD include spina bifida, anencephaly, and encephalocele. These are caused by the failure of the neural tube to close during gestation. The aetiology of NTD is not fully understood but risk factors include folate deficiency, genetic and environmental factors [26,27]. A significant reduction in NTD has been reported since folate fortification of the US food supply [38]. As folate has reduced but not eliminated rates of NTD, research is ongoing to determine further strategies to minimise risk. Low vitamin B_{12} status has been postulated as a potential risk factor for NTD [39] since vitamin B_{12} acts as a cofactor for methionine synthase in the folate cycle. When vitamin B_{12} supply is low, the folate needed for DNA synthesis remains trapped in the methylation cycle and cell replication is impaired. Studies assessing the impact of vitamin B_{12} status on NTD are shown in Table 2. The studies consistently report a 2-4 fold increased risk of NTD with low vitamin B_{12} status. The studies were undertaken in a range of population groups including those that are exposed to folate fortified foods, as well and non-fortified populations.

Table 2. Case control and cohort studies of vitamin B_{12} and Neural Tube Defects.

Design and reference	Study Details	Main Outcome
Case control [39]	81 NTD cases and 247 controls	In cases only, plasma vitamin B_{12} and plasma folate affected maternal Red Cell Folate (multiple $r = 0.68$, $p < 0.001$).
Case control [40]	84 NTD pregnancies and 110 controls	Women with lower vitamin B_{12} have increased risk of NTD.
Cohort [41]	Vitamin B_{12} at 15 weeks' gestation	Vitamin B_{12} <185 pmol/L associated with the highest risk of NTD.
Case control [42]	46 NTD pregnancies and 44 controls	Lower serum vitamin B_{12} ($p = 0.005$) in cases compared to controls
Case control [43]	35 NTD neonates and parents vs 24 normal neonates.	Low vitamin B_{12} in both the parents of child with NTD.
Case control [44]	89 NTD and 422 controls	Increased NTD risk with lower holo-TC.
Case control [1][45]	36 NTD vs normal pregnancy.	Low vitamin B_{12} associated with 2-3 x increased risk for NTD
Case control [46]	46 NTD and 73 control mothers	For NTD holo-TC % (holo-TC/total TCII) Q1vs Q4 OR = 5.0 (95% CI:1.3, 19.3).

Table 2. *Cont.*

Case control [1][47]	57 NTD cases and 186 controls	Q1 vs Q5 of vitamin B_{12} OR = 3.0 (95% CI:1.4, 6.3)
Case control [1][48]	45 mothers and NTD children vs 83 controls	Case mothers with vitamin $B_{12} \leq 185$ pmol/L OR = 3.5-fold (95% CI:1.3, 8.9) for NTD risk.
Case control [49]	56 NTD babies and mothers vs 97 control children and mothers.	Low vitamin B_{12} levels increase risk of NTD.
Case control [1][50]	60 NTD cases and 94 controls	NTD for mothers for vitamin B_{12} levels $\leq 5^{th}$ % *vs* $\geq 95^{th}$
Case control [51]	32 NTD pregnancies and 132 control pregnancies.	MMA higher in cases vs controls.

[1]Study performed in folate fortified population, NTD = Neural tube defects, OR (95%CI) = Odds Ratio and 95% confidence interval, Q4 = 4th quartile, Q5 = 5th quintile, RBC = red blood cell, tHcy = total homocysteine concentration, holo-TC = holotranscobalamin II, total TCII = total transcobalamin II, MMA = methylmalonic acid

9. Vitamin B_{12} and Cardiovascular Disease (CVD)

Nutritional risk factors for CVD include hypercholesterolaemia, hypertension and obesity. Elevated tHcy concentrations are also considered a risk factor, however, it is unclear if tHcy is a modifiable risk factor or an independent marker of the disease process. Much of the research into CVD and tHcy is related to the effects of folate supplementation with or without the addition of vitamins B_{12} and B_6. Investigations of the relationship between CVD and vitamin B_{12} per se are limited.

Meta-analyses of prospective studies (Table 3) have consistently shown associations between tHcy and increased risk of CVD. Supplementation with vitamin B_{12} of doses ranging from 0.02–1 mg/d produces approximately 7% reduction in tHcy, while folate produces 10–30% reduction in risk. Vitamin B_6 has been shown to have little effect [52].

Table 3. Meta-analyses of studies assessing vitamin B_{12} and CVD.

Trial Type	**Study Details**	**Main Outcome**
Meta-analysis [53]	9 case-control studies. Assessed associations between tHcy and CVD risk.	5μM tHcy increment associated with increased risk of CAD, OR = 1.6 (95% CI:1.4 to 1.7) for males and 1.8 (95% CI:1.3 to 1.9) for females.
Meta-analysis [54]	30 prospective or retrospective studies assessed tHcy and CVD risk.	25% lower tHcy associated with lower risk of IHD & stroke.
Meta-analysis 7 RCTs [55]	B vitamin supplementation and tHcy lowering, assessed effect of vitamin B_{12} (range 0.02–1.0 mg/day)	Vitamin B_{12} (median dose 0.4 mg/d) - further decrease (-7%) in tHcy

Table 3. *Cont.*

Meta-analysis 12 RCTs [56]	Preexisting CVD or renal disease- included 3 studies of vitamin B_{12} supplementation, with doses 0.4–1.0 mg B_{12}/day.	Reduction in stroke risk in vitamin B_{12} (1 mg/d) intervention OR = 0.76 (95% CI:0.59, 0.96)
Meta-analysis 8 RCTs [57]	4 studies assessed vitamin B_{12} supplementation (0.018–1 mg) and stroke risk	Reduction in stroke greater in longer trials with more tHcy lowering and no stroke history. No specific effect of vitamin B_{12}.
Meta-analysis 24 RCTs [58]	Assessed CIMT: 3 with vitamin B_{12}: 0.4–0.5 mg/d; endothelial function: 5 with B_{12}: 6 μg–1 mg/day	↓ CIMT, ↑ FMD found in short-term not long term trials

μM = micromolar, tHcy = total homocysteine, CAD = coronary artery disease, OR = odds ratio, CI=confidence intervals, CVD = coronary vascular disease, IHD=ischaemic heart disese, CIMT = carotid intima media thickness, FMD = flow mediated dilation

The recent B vitamin supplementation trials investigating the effect of tHcy reduction and CVD did not show the expected reductions in risk of CVD [59-63]. All of these randomised controlled trials (RCTs) included vitamin B_{12} supplementation (ranging from 6 μg-1 mg) in tandem with folate, and it is not possible to determine the individual impact of vitamin B_{12}. A number of reviews have discussed the limitations of these trials [64-66] and identified inadequate treatment with vitamin B_{12} as one of the limitations.

Subgroup analysis of the VISP Trial found that patients with higher baseline vitamin B_{12} concentrations, taking high dose vitamins, had the best outcomes and those with lower baseline vitamin B_{12} taking low-dose vitamins had the poorest outcomes for stroke, death, and coronary events, suggesting higher vitamin B_{12} doses may be needed in some patients [67]. Vitamin B_{12} has been shown to be a major determinant of tHcy concentrations in subjects with adequate folate status [68] and the existence of vitamin B_{12} deficiency could be one reason for the lack of effect of intervention with folate [69].

10. Cognitive Decline

The assessment of vitamin B_{12} status forms part of the screening process for dementia, however, the effects of sub-clinical levels of vitamin B_{12} on cognitive status are unclear. Studies investigating cognitive decline and vitamin B_{12} status using serum vitamin B_{12} concentrations alone, have been inconclusive [70]. Raised MMA concentrations are associated with cognitive decline and Alzheimer's Disease [71]. It has been suggested that holo-TC and MMA and the ratio holo-TC:vitamin B_{12} [72,73] are better correlated with cognition and the rate of cognitive decline in elderly subjects. In older people with low vitamin B_{12} status, a high serum folate concentration was associated with increased odds of cognitive impairment, but in subjects with normal vitamin B_{12} status, high serum folate was found to be protective against cognitive impairment [74].

To date, there are few intervention studies that examine the relationship between vitamin B_{12} and cognitive function. A Cochrane review, based on 2 studies, identified no effect of supplementation with vitamin B_{12} alone on cognitive score in older adults [75]. A meta-analysis and review identified a correlation between tHcy and Alzheimer's Disease, and suggested the effect was due to lower levels of vitamins B_{12}, B_6 and folate [76]. These studies suggest a role for vitamin B_{12} in the prevention of cognitive decline. However, more long-term studies using biomarkers of vitamin B_{12} status and intervention studies from mid-life are needed to determine the effects of B vitamins on cognition.

11. Osteoporosis

Dietary factors associated with the development of osteoporosis include inadequate protein, calcium and vitamin D. More recently, there has been interest in the effect of other nutrients, including vitamin B_{12} on bone health.

Elevated tHcy has been associated with an increased risk of bone fractures, however it is not clear whether this is related to tHcy per se, to the level of vitamins B_{12}, B_6 or folate which are required for its metabolism, or to other causes of elevated tHcy such as environmental factors or underlying disease. A recent systematic review found that there is evidence for the association between tHcy and increased fracture risk, but less conclusive evidence for tHcy and low bone mineral density (BMD) or for the association between vitamin B_{12} and either fracture risk or low BMD [77]. Intervention trials of the association between B vitamin supplementation have also shown mixed results.

Positive effects of the supplementation of B vitamins on BMD have been found in a subgroup of osteoporotic patients with high tHcy and stroke patients at risk for osteoporosis [78,79], but none in a group of healthy older people or from the secondary analysis of the HOPE Trial for CVD reduction [80,81]. Cohort studies with more than 1,000 subjects or smaller intervention trials are summarized in Table 4. Some of the inconsistencies in study results may be due to differences in the study populations [82,83], differences in the cut points used to define vitamin B_{12} status, and the reliance on serum vitamin B_{12} concentrations rather than more specific biomarkers.

Table 4. Studies of vitamin B_{12} and risk of osteoporosis or fracture.

Design and reference	Study Details	Main Outcome	Reduced risk
Cohort [84]	Elderly, fracture risk	Low vitamin B_{12} and/or HHcy: RR = 3.8 (95% CI:1.2, 1.6) males and 2.8 (95% CI:1.3, 5.7) females	Yes
Cohort [85]	Elderly, fracture risk	tHcy > 14, hip fracture HR = 1.49; (95% CI: 0.91, 2.46)	No
Cohort [86]	Hip fracture risk	fracture for high vs low tHcy (≥15 *vs* <9 μM), HR=2.42 (95% CI:1.43, 4.09) in women	Yes
Cohort [87]	Elderly, fracture risk	For 1 SD in tHcy fracture RR =1.4 (95% CI:1.2, 1.6)	Yes

Table 4. *Cont.*

Cohort [88]	Elderly BMD, tHcy, MTHFR polymorphisms	OR for low BMD w HHcy ≥15 μM *vs.* low tHcy OR=1.96 (95% CI:1.40, 2.75) for females.	Yes
Cohort [89]	Elderly BMD and plasma vitamins	Vitamin B_{12} <148 pM had lower BMD at hip (males) and spine (females) $p < 0.05$.	Yes
Cohort [90]	Elderly subjects (n=1550)	Serum vitamin B_{12} <15th percentile: OR of osteoporosis/osteopenia = 2.0 (95% CI:1.0, 3.9).	Yes
RCT [79]	559 subjects:5 mg folate, 1.5 mg vitamin B_{12} or placebo	RR for hip fracture = 0.20 (95% CI: 0.08, 0.50)	Yes
RCT [78]	47 Osteoporotic subjects 2.5 mg folate, 0.5 mg vitamin B_{12} and 25 mg B_6 or placebo.	No changes in BMD or bone metabolism markers.	No
RCT [80]	Healthy older n = 276; folate 1 mg, vitamin B_{12} 0.5 mg, B_6 10 mg or placebo.	No differences in bone markers in vitamin vs placebo groups.	No
CT [81]	5522 subjects with vascular disease, 2.5 mg folic acid, 50 mg B_6, 1 mg vitamin B_{12} or placebo	HR =1.06 (95% CI:0.81, 1.40) for fracture risk in supplemented vs non supplemented	No

HHcy = hyperhomocysteinaemia, tHcy = total homocysteine, CI = confidence intervals, SD = standard deviation, RR = relative risk, OR = odds ratio, HR = hazard ratio.

12. Other Aspects of Vitamin B_{12} and Ageing

Vitamin B_{12} has been associated with the development of age related macular degeneration (AMD) and risk of frailty, both leading causes of disability in the elderly.

AMD is the leading cause of vision loss in the elderly. Risk factors include increasing age, family history, hypertension, smoking, obesity, sunlight exposure and hypercholesterolemia [91]. Some [91,92] but not all [93] cross sectional studies have found lower vitamin B_{12} concentrations in AMD cases. However, a recent RCT with 5205 female health professionals at risk of vascular disease found a 34% reduction in the relative risk of AMD after supplementation with vitamins B_{12}, B_6 and folate (daily doses of 1 mg, 50 mg, 2.5 mg respectively) [94].

Frailty is characterized by muscle wasting, diminished strength, often with weight loss with or without reduced nutritional intake. Frailty is associated with an increased vulnerability to stresses, causing longer and more complicated recovery from illness or surgery [95].

Increased risk of frailty and disability has been associated with poor B vitamin status. Subjects with vitamins B_{12} and B_6 in the lowest quintiles and subjects with elevated MMA and tHcy concentrations, have been found to have increased risk of decline in physical function and the development of frailty [96,97]. Two cross sectional studies found the length of hospital stay was associated with poor vitamin B_{12} status as assessed by MMA and serum vitamin B_{12} concentrations [98,99]. To date there

are limited studies, however, if improvements in nutrition can delay frailty progression, it could significantly enhance the independence of the increasing numbers of older people.

13. Conclusion

Vitamin B_{12} is a particularly important vitamin for women of childbearing age and for older people, however, adequate vitamin B_{12} status over the whole of the lifecycle is needed for optimal health. There has been renewed interest in vitamin B_{12} since the reporting of associations between homocysteine and chronic disease, particularly vascular disease. The effects of sub-clinical deficiency are not fully known and many aspects of vitamin B_{12} absorption, bioavailability and metabolism are yet to be determined. The identification of sensitive biomarkers of vitamin B_{12} status will help elucidate the relationships between vitamin B_{12} and chronic disease, and help to identify those at risk of clinical and sub-clinical deficiency.

References

1. Scott, J.M. Bioavailability of vitamin B12. *Eur. J. Clin. Nutr.* **1997**, *51,* S49-53.
2. Gibson, R.S. *Principles of Nutritional Assessment*. 2nd ed.; Oxford University Press: New York, NY, USA, 2005.
3. Food and Nutrition Board: Institute of Medicine, Vitamin B12. In *Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin and choline*; National Research Council, Ed. National Academy Press: Washington, DC, USA, 1998; pp. 306-356.
4. Carmel, R. Mild transcobalamin I (haptocorrin) deficiency and low serum cobalamin concentrations. *Clin. Chem.* **2003**, *49*, 1367-1374.
5. Carmel, R.; Green, R.; Rosenblatt, D.S.; Watkins, D. Update on cobalamin, folate, and homocysteine. *Hematology Am. Soc. Hematol. Educ. Program* **2003**, 62-81.
6. Pfeiffer, C.M.; Caudill, S.P.; Gunter, E.W.; Osterloh, J.; Sampson, E.J. Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999-2000. *Am. J. Clin. Nutr.* **2005**, *82*, 442-450.
7. Klee, G.G. Cobalamin and folate evaluation: measurement of methylmalonic acid and homocysteine vs vitamin B(12) and folate. *Clin. Chem.* **2000**, *46*, 1277-1283.
8. Holleland, G.; Schneede, J.; Ueland, P.M.; Lund, P.K.; Refsum, H.; Sandberg, S. Cobalamin deficiency in general practice. Assessment of the diagnostic utility and cost-benefit analysis of methylmalonic acid determination in relation to current diagnostic strategies. *Clin. Chem.* **1999**, *45*, 189-198.
9. Carmel, R.; Sarrai, M. Diagnosis and management of clinical and subclinical cobalamin deficiency: advances and controversies. *Curr. Hematol. Rep.* **2006**, *5*, 23-33.
10. Miller, J.W.; Garrod, M.G.; Rockwood, A.L.; Kushnir, M.M.; Allen, L.H.; Haan, M.N.; Green, R. Measurement of total vitamin B12 and holotranscobalamin, singly and in combination, in screening for metabolic vitamin B12 deficiency. *Clin. Chem.* **2006**, *52*, 278-285.

11. Heyssel, R.M.; Bozian, R.C.; Darby, W.J.; Bell, M.C. Vitamin B12 turnover in man. The assimilation of vitamin B12 from natural foodstuff by man and estimates of minimal daily requirements. *Am. J. Clin. Nutr.* **1966**, *18*, 176-184.
12. Dagnelie, P.C.; van Staveren, W.A.; van den Berg, H. Vitamin B-12 from algae appears not to be bioavailable. *Am. J. Clin. Nutr.* **1991**, *53*, 695-697.
13. Chanarin, I. *The Megaloblastic Anaemias*, 2nd ed.; Blackwell Scientific: Oxford, UK, 1979.
14. Adams, J.F.; Ross, S.K.; Mervyn, R.L.; Boddy, K.; King, P. Absorption of cyanocobalamin, coenzyme B12, methylcobalamin, and hydroxycobalamin at different dose levels. *Scand. J. Gastroenterol.* **1971**, *6*, 249-252.
15. Berlin, H.; Berlin, R.; Brante, G. Oral treatment of pernicious anamia with high doses of vitamin B12 without intrinsic factor. *Acta. Med. Scand.* **1968**, *184*, 247-257.
16. Schneede, J.; Ueland, P.M. Novel and established markers of cobalamin deficiency: complementary or exclusive diagnostic strategies. *Semin. Vasc. Med.* **2005**, *5*, 140-155.
17. Carmel, R. How I treat cobalamin (vitamin B12) deficiency. *Blood* **2008**, *112*, 2214-2221.
18. Stabler, S.P.; Allen, R.H. Vitamin B12 deficiency as a worldwide problem. *Annu. Rev. Nutr.* **2004**, *24*, 299-326.
19. Watanabe, F. Vitamin B12 sources and bioavailability. *Exp. Biol. Med.* **2007**, *232*, 1266-1274.
20. Casey, P.J.; Speckman, K.R.; Ebert, F.J.; Hobbs, W.E. Radioisotope dilution technique for determination of vitamin B12 in foods. *J. Assoc. Off. Anal. Chem.* **1982**, *65*, 85-88.
21. Blake, C.J. Analytical procedures for water-soluble vitamins in foods and dietary supplements: a review. *Anal. Bioanal. Chem.* **2007**, *389*, 63-76.
22. National Health and Medical Research Council, Nutrient Reference Values for Australia and New Zealand. In Department of Health and Ageing, Ed. Australian Government: Canberra, Australia, 2005; pp. 91-96.
23. Doscherholmen, A.; McMahon, J.; Ripley, D. Vitamin B12 absorption from chicken meat. *Am. J. Clin. Nutr.* **1978**, *31*, 825-830.
24. Doscherholmen, A.; McMahon, J.; Ripley, D. Vitamin B12 absorption from fish. *Proc. Soc. Exp. Biol. Med.* **1981**, *167*, 480-484.
25. Doscherholmen, A.; McMahon, J.; Ripley, D. Vitamin B12 absorption from eggs. *Proc. Soc. Exp. Biol. Med.* **1975**, *149*, 987-990.
26. Li, F.; Watkins, D.; Rosenblatt, D.S. Vitamin B12 and birth defects. *Mol. Genet. Metab.* **2009**, *98*, 166-172.
27. Thompson, M.D.; Cole, D.E.; Ray, J.G. Vitamin B-12 and neural tube defects: the Canadian experience. *Am. J. Clin. Nutr.* **2009**, *89*, 697S-701S.
28. Carmel, R. Prevalence of undiagnosed pernicious anemia in the elderly. *Arch. Intern. Med.* **1996**, *156*, 1097-1100.
29. Allen, L.H. How common is vitamin B-12 deficiency? *Am. J. Clin. Nutr.* **2009**, *89*, 693S-696S.
30. Valuck, R.J.; Ruscin, J.M. A case-control study on adverse effects: H2 blocker or proton pump inhibitor use and risk of vitamin B12 deficiency in older adults. *J. Clin. Epidemiol.* **2004**, *57*, 422-428.

31. Den Elzen, W.P.; Groeneveld, Y.; De Ruijter, V.; Souverijn, J.H.; Le Cessie, S.; Assendelft, W.J.; Gussekloo, J. Long-term use of proton pump inhibitors and vitamin B12 status in elderly individuals. *Aliment. Pharmacol. Ther.* **2008**, *27*, 491-497.
32. O'Leary, F.; Wai, J.; Wormold, L.; Flood, V.; Petocz, P.; Samman, S. No effect of Proton Pump Inhibitor (PPI) medications on vitamin B12 status in elderly rehabilitation patients. Is there a dietary component? *Asia Pac. J. Clin. Nutr.* **2007**, *31*, S96.
33. Dharmarajan, T.S.; Kanagala, M.R.; Murakonda, P.; Lebelt, A.S.; Norkus, E.P. Do acid-lowering agents affect vitamin B12 status in older adults? *J. Am. Med. Dir. Assoc.* **2008**, *9*, 162-167.
34. Filioussi, K.; Bonovas, S.; Katsaros, T. Should we screen diabetic patients using biguanides for megaloblastic anaemia? *Aust. Fam. Physician* **2003**, *32*, 383-384.
35. Bauman, W.A.; Shaw, S.; Jayatilleke, E.; Spungen, A.M.; Herbert, V. Increased intake of calcium reverses vitamin B12 malabsorption induced by metformin. *Diabetes Care* **2000**, *23*, 1227-1231.
36. Schilling, R.F. Is nitrous oxide a dangerous anesthetic for vitamin B12- deficient subjects? *JAMA* **1986**, *255*, 1605-1606.
37. Siniscalchi, A.; Mancuso, F.; Gallelli, L.; Ferreri, I.; Biagio, M.; Sarro, G.D. Increase in plasma homocysteine levels induced by drug treatments in neurologic patients. *Pharmacol. Res.* **2005**, *52*, 367-375.
38. Liu, S.; West, R.; Randell, E.; Longerich, L.; O'Connor, K.; Scott, H.; Crowley, M.; Lam, A.; Prabhakaran, V.; McCourt, C. A comprehensive evaluation of food fortification with folic acid for the primary prevention of neural tube defects. *BMC Pregnancy Childbirth.* **2004**, *4*, 20.
39. Kirke, P.N.; Molloy, A.M.; Daly, L.E.; Burke, H.; Weir, D.G.; Scott, J.M. Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects. *Q. J. Med.* **1993**, *86*, 703-708.
40. Zhang, T.; Xin, R.; Gu, X.; Wang, F.; Pei, L.; Lin, L.; Chen, G.; Wu, J.; Zheng, X. Maternal serum vitamin B12, folate and homocysteine and the risk of neural tube defects in the offspring in a high-risk area of China. *Public Health Nutr.* **2009**, *12*, 680-686.
41. Molloy, A.M.; Kirke, P.N.; Troendle, J.F.; Burke, H.; Sutton, M.; Brody, L.C.; Scott, J.M.; Mills, J.L. Maternal vitamin B12 status and risk of neural tube defects in a population with high neural tube defect prevalence and no folic Acid fortification. *Pediatrics* **2009**, *123*, 917-923.
42. Zhang, H.-Y.; Luo, G.-A.; Liang, Q.-L.; Wang, Y.; Yang, H.-H.; Wang, Y.-M.; Zheng, X.-Y.; Song, X.-M.; Chen, G.; Zhang, T.; Wu, J.-X. Neural tube defects and disturbed maternal folate- and homocysteine-mediated one-carbon metabolism. *Exp. Neurol.* **2008**, *212*, 515-521.
43. Ratan, S.K.; Rattan, K.N.; Pandey, R.M.; Singhal, S.; Kharab, S.; Bala, M.; Singh, V.; Jhanwar, A. Evaluation of the levels of folate, vitamin B12, homocysteine and fluoride in the parents and the affected neonates with neural tube defect and their matched controls. *Pediatr. Surg. Int.* **2008**, *24*, 803-808.
44. Ray, J.G.; Wyatt, P.R.; Thompson, M.D.; Vermeulen, M.J.; Meier, C.; Wong, P.-Y.; Farrell, S.A.; Cole, D.E. Vitamin B12 and the risk of neural tube defects in a folic-acid-fortified population. *Epidemiology* **2007**, *18*, 362-366.
45. Gaber, K.R.; Farag, M.K.; Soliman, S.E.; El-Bassyouni, H.T.; El-Kamah, G. Maternal vitamin B12 and the risk of fetal neural tube defects in Egyptian patients. *Clin. Lab.* **2007**, *53*, 69-75.

46. Afman, L.A.; Van Der Put, N.M.; Thomas, C.M.; Trijbels, J.M.; Blom, H.J. Reduced vitamin B12 binding by transcobalamin II increases the risk of neural tube defects. *Q.J. Med.* **2001**, *94*, 159-166.
47. Suarez, L.; Hendricks, K.; Felkner, M.; Gunter, E. Maternal serum B12 levels and risk for neural tube defects in a Texas-Mexico border population. *Ann. Epidemiol.* **2003**, *13*, 81-88.
48. Groenen, P.M.; van Rooij, I.A.; Peer, P.G.; Gooskens, R.H.; Zielhuis, G.A.; Steegers-Theunissen, R.P. Marginal maternal vitamin B12 status increases the risk of offspring with spina bifida. *Am. J. Obstet. Gynecol.* **2004**, *191*, 11-17.
49. Wilson, A.; Platt, R.; Wu, Q.; Leclerc, D.; Christensen, B.; Yang, H.; Gravel, R.A.; Rozen, R. A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. *Mol. Genet. Metab.* **1999**, *67*, 317-323.
50. van der Put, N.M.; Thomas, C.M.; Eskes, T.K.; Trijbels, F.J.; Steegers-Theunissen, R.P.; Mariman, E.C.; De Graaf-Hess, A.; Smeitink, J.A.; Blom, H.J. Altered folate and vitamin B12 metabolism in families with spina bifida offspring. *Q. J. Med.* **1997**, *90*, 505-510.
51. Adams, M.J., Jr.; Khoury, M.J.; Scanlon, K.S.; Stevenson, R.E.; Knight, G.J.; Haddow, J.E.; Sylvester, G.C.; Cheek, J.E.; Henry, J.P.; Stabler, S.P.; Allen, R.H. Elevated midtrimester serum methylmalonic acid levels as a risk factor for neural tube defects. *Teratology* **1995**, *51*, 311-317.
52. Moens, A.L.; Vrints, C.J.; Claeys, M.J.; Timmermans, J.P.; Champion, H.C.; Kass, D.A. Mechanisms and potential therapeutic targets for folic acid in cardiovascular disease. *Am. J. Physiol. Heart Circ. Physiol.* **2008**, *294*, 1971-1977.
53. Boushey, C.J.; Beresford, S.A.; Omenn, G.S.; Motulsky, A.G. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA* **1995**, *274*, 1049-1057.
54. Homocysteine Studies Collaboration, Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA* **2002**, *288*, 2015-2022.
55. Homocysteine Lowering Trialists' Collaboration, Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. *Am. J. Clin. Nutr.* **2005**, *82*, 806-812.
56. Bazzano, L.A.; Reynolds, K.; Holder, K.N.; He, J. Effect of folic acid supplementation on risk of cardiovascular diseases: a meta-analysis of randomized controlled trials. *JAMA* **2006**, *296*, 2720-2726.
57. Wang, X.; Qin, X.; Demirtas, H.; Li, J.; Mao, G.; Huo, Y.; Sun, N.; Liu, L.; Xu, X. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. *Lancet* **2007**, *369*, 1876-1882.
58. Potter, K.; Hankey, G.J.; Green, D.J.; Eikelboom, J.; Jamrozik, K.; Arnolda, L.F. The effect of long-term homocysteine-lowering on carotid intima-media thickness and flow-mediated vasodilation in stroke patients: a randomized controlled trial and meta-analysis. *BMC Cardiovasc. Disord.* **2008**, 8, 24.
59. Ebbing, M.; Bleie, O.; Ueland, P.M.; Nordrehaug, J.E.; Nilsen, D.W.; Vollset, S.E.; Refsum, H.; Pedersen, E.K.; Nygard, O. Mortality and cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial. *JAMA* **2008**, *300*, 795-804.

60. Toole, J.F.; Malinow, M.R.; Chambless, L.E.; Spence, J.D.; Pettigrew, L.C.; Howard, V.J.; Sides, E.G.; Wang, C.H.; Stampfer, M. Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction, and death: the Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial. *JAMA* **2004**, *291*, 565-575.
61. Lonn, E.; Yusuf, S.; Arnold, M.J.; Sheridan, P.; Pogue, J.; Micks, M.; McQueen, M.J.; Probstfield, J.; Fodor, G.; Held, C.; Genest, J., Jr. Homocysteine lowering with folic acid and B vitamins in vascular disease. *N. Engl. J. Med.* **2006**, *354*, 1567-1577.
62. Bonaa, K.H.; Njolstad, I.; Ueland, P.M.; Schirmer, H.; Tverdal, A.; Steigen, T.; Wang, H.; Nordrehaug, J.E.; Arnesen, E.; Rasmussen, K. Homocysteine lowering and cardiovascular events after acute myocardial infarction. *N. Engl. J. Med.* **2006**, *354*, 1578-1588.
63. Albert, C.M.; Cook, N.R.; Gaziano, J.M.; Zaharris, E.; MacFadyen, J.; Danielson, E.; Buring, J.E.; Manson, J.E. Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial. *JAMA* **2008**, *299*, 2027-2036.
64. Zhou, J.; Austin, R.C. Contributions of hyperhomocysteinemia to atherosclerosis: Causal relationship and potential mechanisms. *Biofactors* **2009**, *35*, 120-129.
65. Antoniades, C.; Antonopoulos, A.S.; Tousoulis, D.; Marinou, K.; Stefanadis, C. Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. *Eur. Heart J.* **2009**, *30*, 6-15.
66. Righetti, M. Protective effect of vitamin B therapy on bone and cardiovascular disease. *Recent Patents Cardiovasc. Drug Discov.* **2009**, *4*, 37-44.
67. Spence, J.D.; Bang, H.; Chambless, L.E.; Stampfer, M.J. Vitamin Intervention For Stroke Prevention trial: an efficacy analysis. *Stroke* **2005**, *36*, 2404-2409.
68. Quinlivan, E.P.; McPartlin, J.; McNulty, H.; Ward, M.; Strain, J.J.; Weir, D.G.; Scott, J.M. Importance of both folic acid and vitamin B12 in reduction of risk of vascular disease. *Lancet* **2002**, *359*, 227-228.
69. Spence, J.D. Homocysteine-lowering therapy: a role in stroke prevention? *Lancet Neurol.* **2007**, *6*, 830-838.
70. Smith, A.D.; Refsum, H. Vitamin B-12 and cognition in the elderly. *Am. J. Clin. Nutr.* **2009**, *89*, 707S-711S.
71. Lewis, M.S.; Miller, L.S.; Johnson, M.A.; Dolce, E.B.; Allen, R.H.; Stabler, S.P. Elevated methylmalonic acid is related to cognitive impairement in older adults enrolled in an elderly nutrition program. *J. Nutr. Elder.* **2005**, *24*, 47-65.
72. Hin, H.; Clarke, R.; Sherliker, P.; Atoyebi, W.; Emmens, K.; Birks, J.; Schneede, J.; Ueland, P.M.; Nexo, E.; Scott, J.; Molloy, A.; Donaghy, M.; Frost, C.; Evans, J.G. Clinical relevance of low serum vitamin B12 concentrations in older people: the Banbury B12 study. *Age Ageing* **2006**, *35*, 416-422.
73. Garrod, M.G.; Green, R.; Allen, L.H.; Mungas, D.M.; Jagust, W.J.; Haan, M.N.; Miller, J.W. Fraction of total plasma vitamin B12 bound to transcobalamin correlates with cognitive function in elderly Latinos with depressive symptoms. *Clin. Chem.* **2008**, *54*, 1210-1217.

74. Morris, M.S.; Jacques, P.F.; Rosenberg, I.H.; Selhub, J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. *Am. J. Clin. Nutr.* **2007**, *85*, 193-200.
75. Malouf, R.; Areosa Sastre, A., Vitamin B12 for cognition. *Cochrane Database Syst. Rev.* **2009,** *1*.
76. Van Dam, F.; Van Gool, W.A. Hyperhomocysteinemia and Alzheimer's disease: A systematic review. *Arch. Gerontol. Geriatr.* **2009**, *48*, 425-430.
77. Herrmann, M.; Peter Schmidt, J.; Umanskaya, N.; Wagner, A.; Taban-Shomal, O.; Widmann, T.; Colaianni, G.; Wildemann, B.; Herrmann, W. The role of hyperhomocysteinemia as well as folate, vitamin B(6) and B(12) deficiencies in osteoporosis: a systematic review. *Clin. Chem. Lab. Med.* **2007**, *45*, 1621-1632.
78. Herrmann, M.; Umanskaya, N.; Traber, L.; Schmidt-Gayk, H.; Menke, W.; Lanzer, G.; Lenhart, M.; Peter Schmidt, J.; Herrmann, W. The effect of B-vitamins on biochemical bone turnover markers and bone mineral density in osteoporotic patients: a 1-year double blind placebo controlled trial. *Clin. Chem. Lab. Med.* **2007**, *45*, 1785-1792.
79. Sato, Y.; Honda, Y.; Iwamoto, J.; Kanoko, T.; Satoh, K. Effect of folate and mecobalamin on hip fractures in patients with stroke: a randomized controlled trial. *JAMA* **2005**, *293*, 1082-1088.
80. Green, T.J.; McMahon, J.A.; Skeaff, C.M.; Williams, S.M.; Whiting, S.J. Lowering homocysteine with B vitamins has no effect on biomarkers of bone turnover in older persons: a 2-y randomized controlled trial. *Am. J. Clin. Nutr.* **2007**, *85*, 460-464.
81. Sawka, A.M.; Ray, J.G.; Yi, Q.; Josse, R.G.; Lonn, E. Randomized clinical trial of homocysteine level lowering therapy and fractures. *Arch. Intern. Med.* **2007**, *167*, 2136-2139.
82. Hong, X.; Hsu, Y.-H.; Terwedow, H.; Tang, G.; Liu, X.; Jiang, S.; Xu, X.; Xu, X. Association of the methylenetetrahydrofolate reductase C677T polymorphism and fracture risk in Chinese postmenopausal women. *Bone* **2007**, *40*, 737-742.
83. Riancho, J.A.; Valero, C.; Zarrabeitia, M.T. MTHFR polymorphism and bone mineral density: meta-analysis of published studies. *Calcif. Tissue Int.* **2006**, *79*, 289-293.
84. Dhonukshe-Rutten, R.A.; Pluijm, S.M.; de Groot, L.C.; Lips, P.; Smit, J.H.; van Staveren, W.A. Homocysteine and vitamin B12 status relate to bone turnover markers, broadband ultrasound attenuation, and fractures in healthy elderly people. *J. Bone Miner. Res.* **2005**, *20*, 921-929.
85. McLean, R.R.; Jacques, P.F.; Selhub, J.; Fredman, L.; Tucker, K.L.; Samelson, E.J.; Kiel, D.P.; Cupples, L.A.; Hannan, M.T. Plasma B vitamins, homocysteine, and their relation with bone loss and hip fracture in elderly men and women. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 2206-2212.
86. Gjesdal, C.G.; Vollset, S.E.; Ueland, P.M.; Refsum, H.; Meyer, H.E.; Tell, G.S. Plasma homocysteine, folate, and vitamin B12 and the risk of hip fracture: the Hordaland Homocysteine Study. *J. Bone Miner. Res.* **2007**, *22*, 747-756.
87. van Meurs, J.B.; Dhonukshe-Rutten, R.A.; Pluijm, S.M.; van der Klift, M.; de Jonge, R.; Lindemans, J.; de Groot, L.C.; Hofman, A.; Witteman, J.C.; van Leeuwen, J.P.; Breteler, M.M.; Lips, P.; Pols, H.A.; Uitterlinden, A.G. Homocysteine levels and the risk of osteoporotic fracture. *N. Engl. J. Med.* **2004**, *350*, 2033-2041.
88. Gjesdal, C.G.; Vollset, S.E.; Ueland, P.M.; Refsum, H.; Drevon, C.A.; Gjessing, H.K.; Tell, G.S. Plasma total homocysteine level and bone mineral density: the Hordaland Homocysteine Study. *Arch. Intern. Med.* **2006**, *166*, 88-94.

89. Tucker, K.L.; Hannan, M.T.; Qiao, N.; Jacques, P.F.; Selhub, J.; Cupples, L.A.; Kiel, D.P. Low plasma vitamin B12 is associated with lower BMD: the Framingham Osteoporosis Study. *J. Bone Miner. Res.* **2005**, *20*, 152-158.
90. Morris, M.S.; Jacques, P.F.; Selhub, J. Relation between homocysteine and B-vitamin status indicators and bone mineral density in older Americans. *Bone* **2005**, *37*, 234-242.
91. Kamburoglu, G.; Gumus, K.; Kadayifcilar, S.; Eldem, B. Plasma homocysteine, vitamin B12 and folate levels in age-related macular degeneration. *Graefes Arch. Clin. Exp. Ophthalmol.* **2005**, 1-5.
92. Rochtchina, E.; Wang, J.J.; Flood, V.M.; Mitchell, P. Elevated serum homocysteine, low serum vitamin B12, folate, and age-related macular degeneration: the Blue Mountains Eye Study. *Am. J. Ophthalmol.* **2007**, *143*, 344-346.
93. Heuberger, R.A.; Fisher, A.I.; Jacques, P.F.; Klein, R.; Klein, B.E.; Palta, M.; Mares-Perlman, J.A. Relation of blood homocysteine and its nutritional determinants to age-related maculopathy in the third National Health and Nutrition Examination Survey. *Am. J. Clin. Nutr.* **2002**, *76*, 897-902.
94. Christen, W.G.; Glynn, R.J.; Chew, E.Y.; Albert, C.M.; Manson, J.E. Folic Acid, Pyridoxine, and Cyanocobalamin Combination Treatment and Age-Related Macular Degeneration in Women The Women's Antioxidant and Folic Acid Cardiovascular Study. *Arch. Intern. Med.* **2009,** *169*, 335-341.
95. Fried, L.P.; Ferrucci, L.; Darer, J.; Williamson, J.D.; Anderson, G. Untangling the concepts of disability, frailty, and comorbidity: implications for improved targeting and care. *J. Gerontol. A. Biol. Sci. Med. Sci.* **2004**, *59*, 255-263.
96. Matteini, A.M.; Walston, J.D.; Fallin, M.D.; Bandeen-Roche, K.; Kao, W.H.; Semba, R.D.; Allen, R.H.; Guralnik, J.; Fried, L.P.; Stabler, S.P. Markers of B-vitamin deficiency and frailty in older women. *J. Nutr. Health Aging* **2008**, *12*, 303-308.
97. Bartali, B.; Semba, R.D.; Frongillo, E.A.; Varadhan, R.; Ricks, M.O.; Blaum, C.S.; Ferrucci, L.; Guralnik, J.M.; Fried, L.P. Low micronutrient levels as a predictor of incident disability in older women. *Arch. Intern. Med.* **2006**, *166*, 2335-2340.
98. O'Leary, F.; Flood, V.; Allman-Farinelli, M.; Petocz, P.; Samman, S. Nutritional status, micronutrient levels and length of stay in an elderly rehabilitation unit. *Asia Pac. J. Clin. Nutr.* **2009**, *33*, 106.
99. O'Leary, F.; Wai, J.; Wormald, L.; Ellis, J.; Flood, V.; Petocz, P.; Samman, S. Vitamin B status and length of stay in elderly rehabilitation patients. *Nutr. Diet.* **2009**, *66*, A43.

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Review

Quercetin, Inflammation and Immunity

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Abstract: *In vitro* and some animal models have shown that quercetin, a polyphenol derived from plants, has a wide range of biological actions including anti-carcinogenic, anti-inflammatory and antiviral activities; as well as attenuating lipid peroxidation, platelet aggregation and capillary permeability. This review focuses on the physicochemical properties, dietary sources, absorption, bioavailability and metabolism of quercetin, especially main effects of quercetin on inflammation and immune function. According to the results obtained both *in vitro* and *in vivo*, good perspectives have been opened for quercetin. Nevertheless, further studies are needed to better characterize the mechanisms of action underlying the beneficial effects of quercetin on inflammation and immunity.

Keywords: quercetin; inflammation; immune function; dietary sources; metabolism

1. Introduction

Quercetin, a flavonoid found in fruits and vegetables, has unique biological properties that may improve mental/physical performance and reduce infection risk [1]. These properties form the basis for potential benefits to overall health and disease resistance, including anti-carcinogenic, anti-inflammatory, antiviral, antioxidant, and psychostimulant activities, as well as the ability to inhibit lipid peroxidation, platelet aggregation and capillary permeability, and to stimulate mitochondrial biogenesis [2]. Therefore, there is a pressing need for well-designed clinical trials to evaluate this novel dietary supplement further. This article reviews effects of quercetin on inflammation and immunity in mental and physical performance and health.

2. Physicochemical Properties of Quercetin

Quercetin is categorized as a flavonol, one of the six subclasses of flavonoid compounds. The name has been used since 1857, and is derived from *quercetum* (oak forest), after *Quercus*. It is a naturally occurring polar auxin transport inhibitor [3]. The International Union of Pure and Applied Chemistry (IUPAC) nomenclature for quercetin is 3, 3′, 4′, 5, 7-pentahydroxyflvanone (or its synonym 3, 3′, 4′, 5, 7-pentahydroxy-2-phenylchromen-4-one). This means that quercetin has an OH group attached at positions 3, 5, 7, 3′, and 4′. Common forms of quercetin were shown in Figure 1.

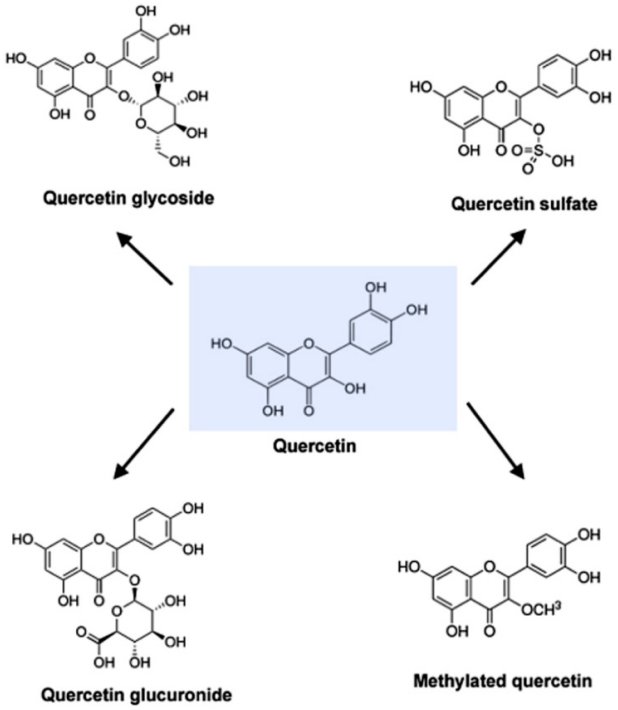

Figure 1. Molecular structure of quercetin, quercetin glycoside, quercetin glucuronide, quercetin sulfate and methylated quercetin.

Quercetin (C15H10O7) is an aglycone, lacking an attached sugar. It is a brilliant citron yellow needle crystal and entirely insoluble in cold water, poorly soluble in hot water, but quite soluble in alcohol and lipids. A quercetin glycoside is formed by attaching a glycosyl group (a sugar such as glucose, rhamnose, or rutinose) as a replacement for one of the OH groups (commonly at position 3). The attached glycosyl group can change the solubility, absorption, and *in vivo* effects. As a general rule of thumb, the presence of a glycosyl group (quercetin glycoside) results in increased water solubility compared to quercetin aglycone [4,5].

A quercetin glycoside is unique by the attached glycosyl group. Generally, the term quercetin should be used to describe the aglycone only; however, the name is occasionally used to refer to quercetin-type molecules, including its glycosides in research and the supplement industry.

3. Dietary Sources of Quercetin

Quercetin-type flavonols (primarily as quercetin glycosides), the most abundant of the flavonoid molecules, are widely distributed in plants. They are found in a variety of foods including apples, berries, Brassica vegetables, capers, grapes, onions, shallots, tea, and tomatoes, as well as many seeds, nuts, flowers, barks, and leaves. Quercetin is also found in medicinal botanicals, including *Ginkgo biloba*, *Hypericum perforatum*, and *Sambucus canadensis* [6–8]. In red onions, higher concentrations of quercetin occur in the outermost rings and in the part closest to the root, the latter being the part of the plant with the highest concentration [9]. One study found that organically grown tomatoes had 79% more quercetin than chemically grown fruit [10]. Quercetin is present in various kinds of honey from different plant sources [11]. Food-based sources of quercetin include vegetables, fruits, berries, nuts, beverages and other products of plant origin [12]. In the determined food, the highest concentration is 234 mg/100 g of edible portion in capers (raw), the lowest concentration is 2 mg/100 g of edible portion in black or green tea (*Camellia sinensis*) [13].

Dietary intake of quercetin was different in several countries. The estimated flavonoid intake ranges from 50 to 800 mg/day (quercetin accounts for 75%), mostly depending on the consumption of

fruits and vegetables and the intake of tea [14]. In the Suihua area of northern China, quercetin intake was reported to be 4.37 mg/day, where the main food sources of flavonol was apples (7.4%), followed by potatoes (3.9%), lettuce (3.8%) and oranges (3.8%) [15], whereas the average quercetin intake was 4.43 mg/day, with apple (3.7%), potato (2.5%), celery (2.2%), eggplant (2.2%), and actinidia (1.6%) being the main food sources of quercetin in Harbin, China [16]. The most recent study showed that quercetin intake is about 18 mg/day for Chinese healthy young males. In the USA, flavonol intake is about 13 mg/day for U.S. adults, while quercetin represents three-quarters of this amount. The mean quercetin intake was approximately 14.90 to 16.39 mg per day. Onions, tea, and apples contained high amounts of quercetin [17]. In Japan, the average and median quercetin intakes were 16.2 and 15.5 mg/day, respectively; the quercetin intake by men was lower than that by women; and the quercetin intakes showed a low correlation with age in both men and women. The estimated quercetin intake was similar during summer and winter. Quercetin was mainly ingested from onions and green tea, both in summer and in winter. Vegetables, such as asparagus, green pepper, tomatoes, and red leaf lettuce, were good sources of quercetin in summer [18]. In Australia, black and green teas were the dominant sources of quercetin. Other sources included onion, broccoli, apple, grape, and beans [19]. Analysis of the 24-h recall data indicated an average adult intake of total flavonoids (>18 years) of 454 mg/day. Apple was the most important source of quercetin until age 16–18 years, after which onion became an increasingly important prominent source [19]. In Spain, the average daily intake of quercetin is 18.48 mg/day, which is significantly higher than that in the United States (9.75 mg/day), based on sources like tea, citrus fruits and juice, beers and ales, wines, melon, apples, onions, berries and bananas [20].

4. Absorption, Bioavailability and Metabolism of Quercetin

The first investigation on the pharmacokinetics of quercetin in humans suggested very poor oral bioavailability after a single oral dose (~2%). The estimated absorption of quercetin glucoside, the naturally occurring form of quercetin, ranges from 3% to 17% in healthy individuals receiving 100 mg. The relatively low bioavailability of quercetin may be attributed to its low absorption, extensive metabolism and/or rapid elimination.

4.1. Absorption

Quercetin glycosides might be differently absorbed based on the type of sugar attached [21]. Available evidence indicates that quercetin glucosides (like those found predominantly in onion or shallot flesh) are far better absorbed than its rutinosides (the major quercetin glycoside in tea). The glucosides are efficiently hydrolyzed in the small intestine by beta-glucosidases to the aglycone form, much of which is then absorbed [22]. Quercetin glucuronic acid and its sulfuric acid derivatives were more easily absorbed than quercetin [22]. Thereafter, its absorption is affected by differences in its glycosylation, the food matrix from which it is consumed, and the co-administration of dietary components such as fiber and fat [23]. Thus different sugar types and sugar group conjugation sites will result in absorption variation.

Quercetin and derivatives are stable in gastric acid; however, there were no reports whether they can be absorbed in stomach. Studies suggest that quercetin is absorbed in the upper segment of small intestinal [24,25].

Among quercetin's derivatives, conjugated forms of its glycosides are better absorbed than quercetin. Purified quercetin glucosides are capable of interacting with the sodium dependent glucose transport receptors in the mucosal epithelium and may therefore be absorbed by the small intestine *in vivo* [21].

4.2. Transformation and Transportation

After absorption, quercetin becomes metabolized in various organs including the small intestine, colon, liver and kidney. Metabolites formed in the small intestine and liver by biotransformation enzymes include the methylated, sulfo-substituted and glucuronidated forms [26,27]. A study

regarding the tissue distribution in rats and pigs has shown that the highest accumulation of quercetin and its metabolites are found in (rat) lung and (pig) liver and kidney [28].

Quercetin and derivatives are transformed into various metabolites (phenolic acid) by enteric bacteria and enzymes in intestinal mucosal epithelial cells. These metabolites are absorbed, transformed or excreted later. Moreover, bacteria ring fission of the aglycon occurs in both the small intestine and colon, resulting in the breakdown of the backbone structure of quercetin and the subsequent formation of smaller phenolics [29].

Quercetin metabolites analyzed in plasma and liver samples have shown that the concentrations of its derivatives in the liver were lower than those in plasma, and the hepatic metabolites were intensively methylated (90%–95%) [30]. Limited studies suggest that quercetin was methylated, vulcanized and glucuronidated in liver [31].

Continuous intake of diet containing quercetin accumulated in blood and significantly increased quercetin concentration in plasma, which was significantly correlated to its dietary content [32]. Quercetin is present in a conjugated form in human blood whose major form is glycoside [33]. While isorhamnetin and glucoside acid-sulfated derivatives of quercetin account for 91.5% of its metabolites, other metabolites include its glucuronoside and methylated form [34]. Boulton also found that quercetin conjugated plasma protein (albumin account for 99.4%), thus decreased its bioavailability in cells [35].

4.3. Excretion

The limited research suggests that quercetin and its metabolites tend to accumulate in the organs involved in its metabolism and excretion, and that perhaps mitochondria might be an area of quercetin concentration within cells [36–42]. Kidney is a major organ of excretion. Quercetin concentration in urine increased with the increasing dose and time after intake of fruit juice was ingested in human [36], perhaps benzoic acid derivatives are common metabolite of quercetin [37]. Human subjects can absorb significant amounts of quercetin from food or supplements, and elimination is quite slow, with a reported half-life ranging from 11 to 28 h [38]. The average terminal half-life of quercetin is 3.5 h [39]. The total recovery of C-quercetin in urine, feces and exhaled air is highly variable, depending on the individual [40]. A high amount of absorbed quercetin is extensively metabolized and eventually eliminated by the lungs [41]. Additional literature suggests that isoquercetin (glycosylated quercetin) is more completely absorbed than quercetin in the aglycone form, and that the simultaneous ingestion of quercetin with vitamin C, folate and additional flavonoids improves bioavailability [38,42].

All of these studies indicate that quercetin glucosides is absorbed in the upper segment of small intestinal, then is methylated, sulfo-substituted and glucuronidated by biotransformation enzymes in the small intestine and liver, and is finally excreted by kidney in urine.

5. Effect of Quercetin on Inflammation and Immune Function

5.1. In Vitro

5.1.1. Anti-Inflammation and Promotion of Immunity

Quercetin was reported as a long lasting anti-inflammatory substance that possesses strong anti-inflammatory capacities [43,44]. It possesses anti-inflammatory potential that can be expressed on different cell types, both in animal and human models [45–53]. It is known to possess both mast cell stabilizing and gastrointestinal cytoprotective activity [54]. It can also play a modulating, biphasic and regulatory action on inflammation and immunity [53]. Additionally, quercetin has an immunosuppressive effect on dendritic cells function [55].

5.1.2. Mechanism of Action

Several studies *in vitro* using different cell lines have shown that quercetin inhibits lipopolysaccharide (LPS)-induced tumor necrosis factor α (TNF-α) production in macrophages [45]

and LPS-induced IL-8 production in lung A549 cells [46]. Moreover, in glial cells it was even shown that quercetin can inhibit LPS-induced mRNA levels of TNF-α and interleukin (IL)-1α, this effect of quercetin resulted in a diminished apoptotic neuronal cell death induced by microglial activation [47]. Quercetin inhibits production of inflammation-producing enzymes (cyclooxygenase (COX) and lipoxygenase (LOX)) [48,49]. It limits LPS-induced inflammation via inhibition of Src- and Syk-mediated phosphatidylinositol-3-Kinase (PI3K)-(p85) tyrosine phosphorylation and subsequent Toll Like Receptor 4 (TLR4)/MyD88/PI3K complex formation that limits activation of downstream signaling pathways in RAW 264.7 cells [50]. It can also inhibit FcεRI-mediated release of pro-inflammatory cytokines, tryptase and histamine from human umbilical cord blood-derived cultured mast cells (hCBMCs); this inhibition appears to involve in inhibition of calcium influx, as well as phospho-protein kinase C (PKC) [51]. The study of quercetin against H_2O_2-induced inflammation showed the protective effects of quercetin against inflammation in human umbilical vein endothelial cells (HUVECs) and indicated that the effect was mediated via the downregulation of vascular cell adhesion molecule 1 (VCAM-1) and CD80 expression [52].

Quercetin significantly induces the gene expression as well as the production of Th-1 derived interferon-γ (IFN-γ) and down-regulates Th-2 derived interleukin 4 (IL-4) by normal peripheral blood mononuclear cells (PBMC). Furthermore, quercetin treatment increased the phenotypic expression of IFN-γ cells and decreased IL-4 positive cells by flow cytometry analysis, which corroborate with protein secretion and gene expression studies. These results suggest that the beneficial immuno-stimulatory effects of quercetin may be mediated through the induction of Th-1 derived cytokine, IFN-γ, and inhibition of Th-2 derived cytokine, IL-4 [56].

Quercetin is able to inhibit matrix metalloproteinases, which are normally inhibited by plasminogen activator inhibitor 1 (PAI-1) in human dermal fibroblasts [57]. IL-1-stimulated IL-6 production from human mast cells is regulated by biochemical pathways distinct from IgE-induced degranulation, and quercetin can block both IL-6 secretion and two key signal transduction steps involved [58].

Quercetin is known to possess both mast cell stabilizing and gastrointestinal cytoprotective activity. A study demonstrates that quercetin has a direct regulatory effect on basic functional properties of immune cells which may be mediated by the extracellular regulated kinase 2 (Erk2) mitogen-activated protein (MAP) kinase signal pathway in human mitogen-activated PBMC and purified T lymphocytes [54].

The property proves inhibitory to a huge panoply of molecular targets in the micromolar concentration range, either by down-regulating or suppressing many inflammatory pathways and functions. Quercetin has shown a biphasic behavior in basophils at nanomolar doses and hence its action on cells involved in allergic inflammation. Quercetin affects immunity and inflammation by acting mainly on leukocytes and targeting many intracellular signaling kinases and phosphatases, enzymes and membrane proteins are often crucial for a cellular specific function. However, the wide group of intracellular targets and the elevated number of natural compounds potentially effective as anti-inflammatory therapeutic agents, asks for further insights and evidence to comprehend the role of these substances in animal cell biology [53].

In vitro treatment of activated T cells with quercetin blocks IL-12-induced tyrosine phosphorylation of JAK2, TYK2, STAT3, and STAT4, resulting in a decrease in IL-12-induced T cell proliferation and Th1 differentiation [59].

Taken as *in vitro* together, the possible pathway of quercetin on inflammation and immune function is as follows (Figure 2).

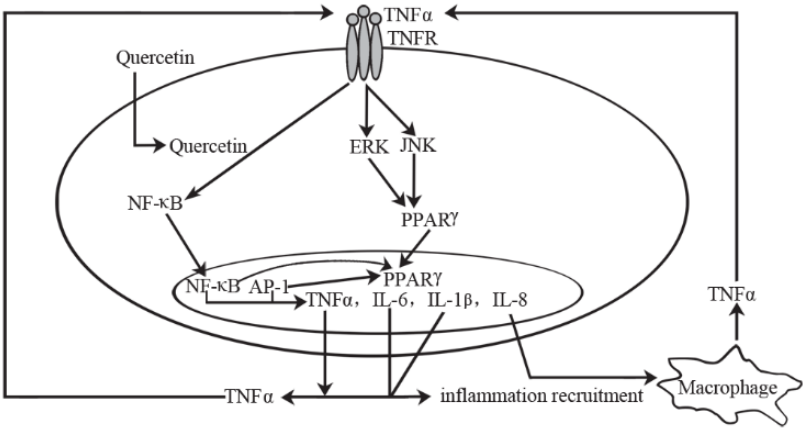

Figure 2. Working model on how quercetin block tumor necrosis factor-α (TNFα)-mediated inflammation. Quercetin prevents TNF-α from directly activating extracellular signal-related kinase (ERK), c-Jun NH_2-terminal kinase (JNK), c-Jun, and nuclear factor-κB (NF-κB), which are potent inducers of inflammatory gene expression and protein secretion. In addition, quercetin may indirectly prevent inflammation by increasing peroxisome proliferator-activated receptor c (PPARγ) activity, thereby antagonizing NF-κB or activator protein-1(AP-1) transcriptional activation of inflammatory genes. Together, these block TNF-α-mediated induction of inflammatory cascades.

The main action of quercetin on inflammation and immune function *in vitro* is summarized in the Table 1.

Table 1. Summary of the main effects of quercetin on inflammation and immune function *in vitro*.

Dosage	Cell Lines	Effect	Mechanism	Reference
Cells from animals				
100 μmol/L	Pulmonary Epithelial Cell (A549)	Anti-inflammation	PARP-1 inhibition and preservation of cellular NAD1 and energy production	[46]
100 μmol/L	N9 microglial cells		Inhibition of TNFα and IL-1α; Reduce of apoptotic neuronal cell death induced by microglial activation	[47]
3 μmol/L	Gunea pig epithelial cells		Inhibition of both cyclooxygenase and lipoxygenase	[48]
15–30 μmol/L	Rat liver epithelial (RLE) cells		Inhibition of arsenite-induced COX-2 expression mainly by blocking the activation of the PI3K signaling pathway	[49]
-	RAW 264.7 cells		Inhibition of Src- and Syk-mediated PI3K-(p85) tyrosine phosphorylation and subsequent TLR4/MyD88/PI3K complex formation that limits activation of downstream signaling pathways	[50]
Cells from human				
10 μmol/L	Human umbilical cord blood-derived cultured mast cells (hCBMCs)	Anti-allergic and anti-inflammation; Protective effects against cell injury; Gastrointestinal cytoprotective action	Inhibition of intracellular calcium influx and PKC theta signaling	[51]
50 or 100 μg	T lymphocyte		Blockage of interleukin-12 signaling through JAK-STAT pathway	[52]
-	Mast cell		Stabilization of mast cell and gastrointestinal cytoprotection via lactone stimulating mucus production, and inhibiting histamine and serotonin release from intestinal mast cells	

Table 1. *Cont.*

Dosage	Cell Lines	Effect	Mechanism	Reference
12.5–25.0 mmol/L	Human inflamed/UV-irradiated skin		Inhibition of MMP-1 and down-regulation of MMP-1 expression via an inhibition of the AP-1 activation	[54]
0–210 μmol/L	Human umbilical vein endothelial cells (HUVECs)		Downregulation of VCAM-1 and CD80 expression	[56]
0.5–50 mmol/L	Human normal peripheral blood mononuclear cells	Beneficial immuno-stimulatory effects	Induction of Th-1 derived cytokine, IFNgamma, and inhibition of Th-2 derived cytokine, IL-4	[57]
1–100 mmol/L	Human umbilical cord blood-derived cultured mast cells (hCBMCs)		Inhibition of IL-1-induced IL-6 secretion, p38 and PKC-theta phosphorylation	[58]
⩾100 mmol/L or ⩽50 mmol/L	Mouse endritic cells (mDCs)	Immunosuppression	Inhibition of DC activation; DC apoptosis; Downregulation of the cytokines and chemokines, disturbance of immunoregulation; Attenuation of LPS-induced DC maturation and limitation of immunostimulatory activity; downregulate of endocytosis and impairment of Ag loading; suppression of DC migration and disconnection of the induction of adaptive immune responses	[55]

5.2. In Vivo

5.2.1. Animal Models

Quercetin exerts inflammation and immune modulating activity in several murine models of autoimmunity. *In vivo*, animal experiments also support an anti-inflammatory effect. Quercetin ameliorates the inflammatory response induced by carrageenan [60] and a high-fat diet [61]. Quercetin reduced visceral adipose tissue TNF-α and nitric oxide production and downregulated nitric oxide synthase (NOS) expression in obese Zucker rats [62]. In chronic rat adjuvant induced arthritis, quercetin decreased clinical signs of arthritis compared to untreated controls [63].

In rats, post-trauma administration of quercetin improves recovery of motor function after acute traumatic spinal cord injury. Intraperitoneal (IP) doses of 5–100 micromoles quercetin/kg body weight resulted in half or more of the animals walking, although with deficit [64]. This ability to promote recovery from spinal cord injury appears to be highly dependent on the dose and frequency of dosing. In this study a lower IP dose was ineffective. In another study, compared to an untreated control group of animals (none of which recovered motor function sufficient to walk), quercetin administration twice daily for three or 10 days resulted in about 50 percent of the animals recovering sufficient motor function to walk. However, when quercetin was injected three times daily, none of the nine animals recovered the ability to walk [65].

5.2.2. Mechanism of Action in Animal

Study has shown that quercetin exerted protective effect against irradiation-induced inflammation in mice through increasing cytokine secretion [66]. Quercetin possesses activity against isoproterenol-induced myocardial oxidative injury and immunity function impairment, and that the mechanism of pharmacological action was related at least in part to the antioxidant activity of quercetin [67]. Quercetin decreased histological signs of acute inflammation in the treated animals in a dose-dependent manner via suppressing leucocyte recruitment, decreasing chemokine levels and levels of the lipid peroxidation end-product malondialdehyde, and increasing antioxidant enzyme activity in experimental rat model [68].

Quercetin ameliorated experimental allergic encephalomyelitis (EAE) by blocking IL-12 signaling and Th1 differentiation [58] and experimental autoimmune myocarditis (EAM) in Dark Agouti rats by interfering with production of pro-inflammatory (TNF-α and IL-17) and/or anti-inflammatory (IL-10) cytokines [69]. Quercetin most likely universally suppresses the accumulation and activation of immune cells, including anti-inflammatory cells, whereas it specifically increased gene expression associated with mitochondrial oxidative phosphorylation in Western diet-induced obese mice. Suppression of oxidative stress and NF-κB activity likely contributed to the prevention of the accumulation and activation of immune cells and resulting chronic inflammation of epididymal adipose tissue in Western diet-induced obese mice [70].

5.2.3. Clinical Studies

Diet supplementation with combinations of resveratrol, pterostilbene, morin hydrate, quercetin, δ-tocotrienol, riboflavin, and nicotinic acid reduces cardiovascular risk factors in humans when used as nutritional supplements with, or without, other dietary changes in healthy seniors and hypercholesterolemic subjects [71].

In a randomized, double-blinded, placebo-controlled trial, 1002 subjects took 500 or 1000 mg/day quercetin or a placebo for 12 weeks. For the group as a whole, quercetin supplementation had no significant influence on rates of upper respiratory tract infections (URTI) compared to placebo. In a subgroup of subjects age 40 or older who self-rated themselves as physically fit, 1000 mg/day quercetin resulted in a statistically significant reduction in total sick days and symptom severity associated with URTI [72]. Female subjects were supplemented with 500 or 1000 mg/day quercetin or placebo for 12 weeks. While quercetin supplementation significantly increased plasma quercetin levels, it had no influence on measure of immune function [73]. Quercetin (100 mg/day) did not alter exercise-induced changes in several measures of immune function following three days of intense exercise in trained athletes, but it significantly reduced URTI incidence (1 of 20 subjects in active *versus* 9 of 20 in placebo group) during the two-week post-exercise period [74]. A similar lack of effect on strenuous exercise-induced immune system perturbation was found in subjects who took 1000 mg/day of quercetin for three weeks before, during, and continuing for two weeks after the 160-km Western States Endurance Run. In this study, however, there were no differences in the post-race illness rates between quercetin and placebo groups [75].

There are several studies in humans investigating the correlation of quercetin and its immunomodulatory effects. Quercetin does indeed reduce illness after intensive exercise. Again, under double-blind conditions, Nieman *et al.* showed that a supplement of 1000 mg of quercetin alone three weeks before, during and two weeks after a three-day period of 3 h of cycling in the winter resulted in a markedly lower incidence of URTI in well-trained subjects in the two weeks after the intensified training, but had no effect on exercise-induced immune dysfunction, inflammation and oxidative stress [76].

The literature is supportive of the anti-pathogenic capacities of quercetin when it is cultured with target cells and a broad spectrum of pathogens including URTI-related rhinoviruses, adenoviruses and coronaviruses. The impact of the co-ingestion of two or more flavonoids increases their bioavailability and the outcomes on immunity. Nieman *et al.* determined the influence of two weeks of 1000 mg/day quercetin compared with placebo supplementation on exercise performance and skeletal muscle mitochondrial biogenesis in untrained, young adult males. It resulted in significantly reduced post-exercise measures for both inflammation and oxidative stress, with a chronic augmentation of granulocyte oxidative burst activity [77]. When taken together, quercetin showed a successful reduction in the illness rates of exercise-stressed athletes as well as a chronic augmentation of their innate immune function.

Most *in vitro* research suggests that quercetin possesses anti-inflammation and immunological improvement. However, the results from a double-blinded, placebo-controlled, randomized trial indicated that quercetin supplementation at 500 and 1000 mg/day for 12 weeks significantly increased plasma quercetin levels but had no influence on measures of innate immune function or inflammation in community-dwelling adult females [73].

The main action of quercetin on inflammation and immune function *in vivo* is summarized in the Table 2.

Table 2. Summary of the main effects of quercetin on inflammation and immune function *in vivo*.

Dosage	Subjects	Effect	Mechanism	Reference
		Animals		
10 mg/kg diet	Rat	Anti-inflammation	Modulation of prostanoid synthesis and cytokine production	[60]
0.8% diet	C57BL/6J mouse		Increase of energy expenditure; Decrease of interferon-γ, interleukin-1α, and interleukin-4	[61]
10 mg/kg of body weight	Zucker rat		Downregulation of visceral adipose tissue inducible nitric oxide synthase expression, increase of endothelial nitric oxide synthase expression	[62]
160 mg/kg body weight (oral administration) 60 mg/kg (intra-cutaneous injections)	Lewis rat		Inhibition of macrophage-derived cytokines and nitric oxide	[63]
10 and 40 mg/kg body weight	Mouse		Increase of cytokine (interleukin-1β and interleukin-6) secretion	[66]
5–100 micromoles /kg body weight (administered intraperitoneally) 25 μmol/kg	Wistar rat	Functional recovery of acute spinal cord injury and motor function	Decrease of secondary damage through iron chelation, No effect	[64,65]
0.05% diet	C57BL/6J mouse		Suppression of the accumulation and activation of immune cells, Suppression of oxidative stress and NFκB activity	[70]
50, 100, 150 mg/kg body weight	Wistar rat	Amelioration of immunity function impairment induced by isoproterenol; Amelioration of brain damage and neuroprotection, experimental allergic encephalomyelitis, experimental autoimmune myocarditis	Increase of activity in aspartate transaminase, creatine kinase, nitric oxide, nitric oxide synthase, interleukin-10, interleukin-1, interleukin-8 and lactate dehydrogenase	[59]
50 mg/kg	Sprague-Dawley (SD) rat		Increase of activity of endogenous antioxidant enzymes and inhibition of free radical generation	[67]
50 or 100 μg	SJL/J mice		Blockage of interleukin-12 signaling and Th1 differentiation	[68]
10 or 20 mg/kg (oral administration)	Dark Agouti rat		Interference of pro-inflammatory (TNF-α and IL-17) and/or anti-inflammatory (IL-10) cytokines production	[69]
		Human		
50 and 100 mg/person	Elderly Human subject	Anti-inflammatory properties	Inhibition of proteasome (nitric oxide, C-reactive protein, γ-glutamyltransferase) activity	[71]
500 and 1000 mg/day	Human subject	Reduction of upper respiratory tract infection and total sick days; Improvement in 12-min treadmill time trial performance	No effect	[72]
1000 mg/day	Human in treadmill		No effect	[76]
500 and 1000 mg/day	Human subject	No effect on innate immune function or inflammation, illness rates	No effect	[73]
1000 mg/day	Human cyclist		No effect	[74]
1000 mg/day	Human runner		No effect	[75]
1000 mg/day	Human cyclist		No effect	[77]

These results suggest that quercetin exhibited anti-inflammation and immune-enhancement *in vitro* (cells) and *in vivo* (animals), however, studies in human did not totally support these results from cells and animals. The effect, in which quercetin acts as an immune booster in humans, needs to be further verified for future broad application.

6. Summary

As a widespread flavonoid, quercetin is a safe and dietary supplement based on its broad range of biological effects in animal. The results of these effects are not consistent, however, and the outcomes need to be carefully evaluated, as they are dependent on the type of subject and their level of health. Taken together, we know definitively that a quercetin glycoside is much more efficient than other forms of quercetin. In the majority of the literature, we find references to the benefits of prolonged supplementation with quercetin.

The future challenge is to investigate optimal benefits of quercetin, especially to the recommendation for the protracted intake. For example, a carbohydrate drink may have a better effect than pure quercetin preparation. The research in this area continues to determine the proper outcomes, dosing regimen and adjuvants that may amplify any perceived bioactive effects of quercetin *in vivo*.

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References

1. Davis, J.M.; Murphy, E.A.; Carmichael, M.D. Effects of the dietary flavonoid quercetin upon performance and health. *Curr. Sports Med. Rep.* **2009**, *8*, 206–213. [CrossRef] [PubMed]
2. Aguirre, L.; Arias, N.; Macarulla, M.T.; Gracia, A.; Portillo, M.P. Beneficial effects of quercetin on obesity and diabetes. *Open Nutraceuticals J.* **2011**, *4*, 189–198.
3. Fischer, C.; Speth, V.; Fleig-Eberenz, S.; Neuhaus, G. Induction of zygotic polyembryos in wheat: Influence of Auxin Polar Transport. *Plant Cell* **1997**, *9*, 1767–1780. [CrossRef] [PubMed]
4. Ross, J.A.; Kasum, C.M. Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **2002**, *22*, 19–34. [CrossRef] [PubMed]
5. Hollman, P.C.; Bijsman, M.N.; van Gameren, Y.; Cnossen, E.P.; de Vries, J.H.; Katan, M.B. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radic. Res.* **1999**, *31*, 569–573. [CrossRef] [PubMed]
6. Häkkinen, S.H.; Kärenlampi, S.O.; Heinonen, I.M.; Mykkänen, H.M.; Törrönen, A.R. Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. *J. Agric. Food Chem.* **1999**, *47*, 2274–2279. [CrossRef] [PubMed]
7. Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **2005**, *81* (Suppl. S1), 243S–255S. [PubMed]
8. Wiczkowski, W.; Romaszko, J.; Bucinski, A.; Szawara-Nowak, D.; Honke, J.; Zielinski, H.; Piskula, M.K. Quercetin from shallots (*Allium cepa* L. var. aggregatum) is more bioavailable than its glucosides. *J. Nutr.* **2008**, *138*, 885–888. [PubMed]
9. Smith, C.; Lombard, K.A.; Peffley, E.B.; Liu, W. Genetic analysis of quercetin in onion (*Allium cepa* L.) Lady Raider. *Tex. J. Agric. Natl. Resour.* **2003**, *16*, 24–28.
10. Mitchell, A.E.; Hong, Y.J.; Koh, E.; Barrett, D.M.; Bryant, D.E.; Denison, R.F.; Kaffka, S. Ten-year comparison of the influence of organic and conventional crop management practices on the content of flavonoids in tomatoes. *J. Agric. Food Chem.* **2007**, *55*, 6154–6159. [CrossRef] [PubMed]
11. Petrus, K.; Schwartz, H.; Sontag, G. Analysis of flavonoids in honey by HPLC coupled with coulometric electrode array detection and electrospray ionization mass spectrometry. *Anal. Bioanal. Chem.* **2011**, *400*, 2555–2563. [CrossRef] [PubMed]

12. Tutelian, V.A.; Lashneva, N.V. Biologically active substances of plant origin. Flavonols and flavones: Prevalence, dietary sources and consumption. *Vopr. Pitan.* **2013**, *82*, 4–22.
13. Bhagwat, S.; Haytowits, D.B.; Holden, J.M. *USDA Database for the Flavonoid Content of Selected Foods, Release 3*; U.S. Department of Agriculture: Beltsville, MD, USA, 2011.
14. Chun, O.K.; Chung, S.J.; Song, W.O. Estimated dietary flavonoid intake and major food sources of U.S. adults. *J. Nutr.* **2007**, *137*, 1244–1252. [PubMed]
15. Sun, C.; Wang, H.; Wang, D.; Chen, Y.; Zhao, Y.; Xia, W. Using an FFQ to assess intakes of dietary flavonols and flavones among female adolescents in the Suihua area of northern China. *Public Health Nutr.* **2015**, *18*, 632–639. [CrossRef] [PubMed]
16. Zhang, Y.; Li, Y.; Cao, C.; Cao, J.; Chen, W.; Zhang, Y.; Wang, C.; Wang, J.; Zhang, X.; Zhao, X. Dietary flavonol and flavone intakes and their major food sources in Chinese adults. *Nutr. Cancer* **2010**, *62*, 1120–1127. [CrossRef] [PubMed]
17. Sampson, L.; Rimm, E.; Hollman, P.C.; de Vries, J.H.; Katan, M.B. Flavonol and flavone intakes in US health professionals. *J. Am. Diet. Assoc.* **2002**, *102*, 1414–1420. [CrossRef]
18. Nishimuro, H.; Ohnishi, H.; Sato, M.; Ohnishi-Kameyama, M.; Matsunaga, I.; Naito, S.; Ippoushi, K.; Oike, H.; Nagata, T.; Akasaka, H.; *et al*. Estimated daily intake and seasonal food sources of quercetin in Japan. *Nutrients* **2015**, *7*, 2345–2358. [CrossRef] [PubMed]
19. Somerset, S.M.; Johannot, L. Dietary flavonoid sources in Australian adults. *Nutr. Cancer* **2008**, *60*, 442–449. [CrossRef] [PubMed]
20. Zamora-Ros, R.; Andres-Lacueva, C.; Lamuela-Raventos, R.M.; Berenguer, T.; Jakszyn, P.; Barricarte, A.; Ardanaz, E.; Amiano, P.; Dorronsoro, M.; Larrañaga, N.; *et al*. Estimation of dietary sources and flavonoid intake in a Spanish adult population (EPIC-Spain). *J. Am. Diet. Assoc.* **2010**, *110*, 390–398. [CrossRef] [PubMed]
21. Scholz, S.; Williamson, G. Interactions affecting the bioavailability of dietary polyphenols *in vivo*. *Int. J. Vitam. Nutr. Res.* **2007**, *77*, 224–235. [CrossRef] [PubMed]
22. Ader, P.; Wessmann, A.; Wolffram, S. Bioavailability and metabolism of the flavonol quercetin in the pig. *Free Radic. Biol. Med.* **2000**, *28*, 1056–1067. [CrossRef]
23. Guo, Y.; Mah, E.; Davis, C.G.; Jalili, T.; Ferruzzi, M.G.; Chun, O.K.; Bruno, R.S. Dietary fat increases quercetin bioavailability in overweight adults. *Mol. Nutr. Food Res.* **2013**, *57*, 896–905. [CrossRef] [PubMed]
24. Crespy, V.; Morand, C.; Manach, C. Part of quercetin absorbed in the small intestine is conjugated and further secreted in the intestinal l: Umen. *Am. J. Physiol.* **1999**, *277*, G120–G126. [PubMed]
25. Manach, C.; Morand, C.; Texier, O.; Favier, M.L.; Agullo, G.; Demigné, C.; Régérat, F.; Rémésy, C. Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *J. Nutr.* **1995**, *125*, 1911–1922. [PubMed]
26. Hollman, P.C.; Katan, M.B. Absorption, metabolism and bioavailability of flavonoids. In *Flavonoids in Health and Disease*; Rice-Evans, C.A., Packer, L., Eds.; Marcel Dekker Inc.: New York, NY, USA, 1998; pp. 483–522.
27. Day, A.J.; Bao, Y.; Morgan, M.R.; Williamson, G. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radic. Biol. Med.* **2000**, *29*, 1234–1243. [CrossRef]
28. De Boer, V.C.; Dihal, A.A.; van der Woude, H.; Arts, L.C.; Wolffram, S.; Alink, G.M.; Rietjens, I.M.; Keijer, J.; Hollman, P.C. Tissue distribution of quercetin in rats and pigs. *J. Nutr.* **2005**, *135*, 1718–1725. [PubMed]
29. Kim, D.H.; Kim, S.Y.; Park, S.Y.; Han, M.J. Metabolism of quercetin by human intestinal bacteria and its relation to some biological activities. *Biol. Pharm. Bull.* **1999**, *22*, 749–751. [CrossRef] [PubMed]
30. Manach, C.; Texier, O.; Morand, C.; Crespy, V.; Régérat, F.; Demigné, C.; Rémésy, C. Comparison of the bioavailability of quercetin and catechin in rats. *Free Radic. Biol. Med.* **1999**, *27*, 1259–1266. [CrossRef]
31. Oliveira, E.J.; Watson, D.G. *In vitro* glucuronidation of kaempferol and quercetin by human UGT-1A9 microsomes. *FEBS Lett.* **2000**, *471*, 1–6. [CrossRef]
32. Koli, R.; Erlund, I.; Jula, A.; Marniemi, J.; Mattila, P.; Alfthan, G. Bioavailability of various polyphenols from a diet containing moderate amounts of berries. *J. Agric. Food Chem.* **2010**, *58*, 3927–3932. [CrossRef] [PubMed]
33. Aziz, A.A.; Edwards, C.A.; Lean, M.E.; Crozier, A. Absorption and excretion of conjugated flavonols, including quercetin-4′-O-beta-glucoside and isorhamnetin-4′-O-beta-glucoside by human volunteers after the consumption of onions. *Free Radic. Res.* **1998**, *29*, 257–269. [CrossRef] [PubMed]
34. Morand, C.; Crespy, V.; Manach, C.; Besson, C.; Demigné, C.; Rémésy, C. Plasma metabolites of quercetin and their antioxidant properties. *Am. J. Physiol.* **1998**, *275*, R212–R219. [PubMed]
35. Boulton, D.W.; Walle, U.K.; Walle, T. Extensive binding of the bioflavonoid quercetin to human plasma proteins. *J. Pharm. Pharmacol.* **1998**, *50*, 243–249. [CrossRef] [PubMed]

36. Young, J.F.; Nielsen, S.E.; Haraldsdóttir, J.; Daneshvar, B.; Lauridsen, S.T.; Knuthsen, P.; Crozier, A.; Sandström, B.; Dragsted, L.O. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. *Am. J. Clin. Nutr.* **1999**, *69*, 87–94. [PubMed]
37. Graefe, E.U.; Derendorf, H.; Veit, M. Pharmacokinetics and bioavailability of the flavonol quercetin in humans. *Int. J. Clin. Pharmacol. Ther.* **1999**, *37*, 219–233. [PubMed]
38. Manach, C.; Mazur, A.; Scalbert, A. Polyphenols and prevention of cardiovascular diseases. *Curr. Opin. Lipidol.* **2005**, *16*, 77–84. [CrossRef] [PubMed]
39. Konrad, M.; Nieman, D.C. Evaluation of quercetin as a countermeasure to exercise-induced physiological stress. In *Source Antioxidants in Sport Nutrition*; Lamprecht, M., Ed.; CRC Press: Boca Raton, FL, USA, 2015; Chapter 10.
40. Moon, Y.J.; Wang, L.; DiCenzo, R.; Morris, M.E. Quercetin pharmacokinetics in humans. *Biopharm. Drug Dispos.* **2008**, *29*, 205–217. [CrossRef] [PubMed]
41. Walle, T.; Walle, U.K.; Halushka, P.V. Carbon dioxide is the major metabolite of quercetin in humans. *J. Nutr.* **2001**, *131*, 2648–2652. [PubMed]
42. Harwood, M.; Danielewska-Nikiel, B.; Borzelleca, J.F.; Flamm, G.W.; Williams, G.M.; Lines, T.C. A critical review of the data related to the safety of quercetin and lack of evidence of *in vivo* toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem. Toxicol.* **2007**, *45*, 2179–2205. [CrossRef] [PubMed]
43. Read, M.A. Flavonoids: Naturally occurring anti-inflammatory agents. *Am. J. Pathol.* **1995**, *147*, 235–237. [PubMed]
44. Orsolic, N.; Knezevic, A.H.; Sver, L.; Terzic, S.; Basic, I. Immunomodulatory and antimetastatic action of propolis and related polyphenolic compounds. *J. Ethnopharmacol.* **2004**, *94*, 307–315. [CrossRef] [PubMed]
45. Manjeet, K.R.; Ghosh, B. Quercetin inhibits LPS-induced nitric oxide and tumor necrosis factor-alpha production in murine macrophages. *Int. J. Immunopharmocol.* **1999**, *21*, 435–443.
46. Gerates, L.; Moonen, H.J.J.; Brauers, K.; Wouters, E.F.M.; Bast, A.; Hageman, G.J. Dietary flavones and flavonols are inhibitor of poly (ADP-ribose) polymerase-1 in pulmonary epithelial cells. *J. Nutr.* **2007**, *137*, 2190–2195.
47. Bureau, G.; Longpre, F.; Martinoli, M.G. Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation. *J. Neurosci. Res.* **2008**, *86*, 403–410. [CrossRef] [PubMed]
48. Kim, H.P.; Mani, I.; Iversen, L.; Ziboh, V.A. Effects of naturally-occurring flavonoids and bioflavonoids on epidermal cyclooxygenase and lipoxygenase from guinea-pigs. *Prostaglandins Leukot. Essent. Fat. Acids* **1998**, *58*, 17–24. [CrossRef]
49. Lee, K.M.; Hwang, M.K.; Lee, D.E.; Lee, K.W.; Lee, H.J. Protective effect of quercetin against arsenite-induced COX-2 expression by targeting PI3K in rat liver epithelial cells. *J. Agric. Food Chem.* **2010**, *58*, 5815–5820. [CrossRef] [PubMed]
50. Endale, M.; Park, S.C.; Kim, S.; Kim, S.H.; Yang, Y.; Cho, J.Y.; Rhee, M.H. Quercetin disrupts tyrosine-phosphorylated phosphatidylinositol 3-kinase and myeloid differentiation factor-88 association, and inhibits MAPK/AP-1 and IKK/NF-κB-induced inflammatory mediators production in RAW 264.7 cells. *Immunobiology* **2013**, *218*, 1452–1467. [CrossRef] [PubMed]
51. Kempuraj, D.; Madhappan, B.; Christodoulou, S.; Boucher, W.; Cao, J.; Papadopoulou, N.; Cetrulo, C.L.; Theoharides, T.C. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. *Br. J. Pharmacol.* **2005**, *145*, 934–944. [CrossRef] [PubMed]
52. Yang, D.; Liu, X.; Liu, M.; Chi, H.; Liu, J.; Han, H. Protective effects of quercetin and taraxasterol against H_2O_2-induced human umbilical vein endothelial cell injury *in vitro*. *Exp. Ther. Med.* **2015**, *10*, 1253–1260. [CrossRef] [PubMed]
53. Chirumbolo, S. The role of quercetin, flavonols and flavones in modulating inflammatory cell function. *Inflamm. Allergy Drug Targets* **2010**, *9*, 263–285.
54. Penissi, A.B.; Rudolph, M.I.; Piezzi, R.S. Role of mast cells in gastrointestinal mucosal defense. *Biocell* **2003**, *27*, 163–172. [PubMed]
55. Huang, R.Y.; Yu, Y.L.; Cheng, W.C.; OuYang, C.N.; Fu, E.; Chu, C.L. Immunosuppressive effect of quercetin on dendritic cell activiation and function. *J. Immunol.* **2010**, *184*, 6815–6821. [CrossRef] [PubMed]
56. Nair, M.P.N.; Kandaswami, C.; Mahajan, S.; Chadha, K.C.; Chawda, R.; Nair, H.; Kumar, N.; Nair, R.E.; Schwartz, S.A. The flavonoid, quercetin, differentially regulates Th-1 (IFNg) and Th-2 (IL4) cytokine gene expression by normal peripheral blood mononuclear cells. *Biochim. Biophys. Acta* **2002**, *1593*, 29–36. [CrossRef]

57. Lim, H.; Kim, H.P. Inhibition of mammalian collagenase, matrix metalloproteinase-1, by naturally-occurring flavonoids. *Planta Med.* **2007**, *73*, 1267–1274. [CrossRef] [PubMed]
58. Kandere-Grzybowska, K.; Kempuraj, D.; Cao, J.; Cetrulo, C.L.; Theoharides, T.C. Regulation of IL-1-induced selective IL-6 release from human mast cells and inhibition by quercetin. *Br. J. Pharmacol.* **2006**, *148*, 208–215. [CrossRef] [PubMed]
59. Muthian, G.; Bright, J.J. Quercetin, a flavonoid phytoestrogen, ameliorates experimental allergic encephalomyelitis by blocking IL-12 signaling through JAK-STAT pathway in T lymphocyte. *J. Clin. Immunol.* **2004**, *24*, 542–552. [CrossRef] [PubMed]
60. Morikawa, K.; Nonaka, M.; Narahara, M.; Torii, I.; Kawaguchi, K.; Yoshikawa, T.; Kumazawa, Y.; Morikawa, S. Inhibitory effect of quercetin on carrageenan-induced inflammation in rats. *Life Sci.* **2003**, *74*, 709–721. [CrossRef] [PubMed]
61. Stewart, L.K.; Soileau, J.L.; Ribnicky, D.; Wang, Z.Q.; Raskin, I.; Poulev, A.; Majewski, M.; Cefalu, W.T.; Gettys, T.W. Quercetin transiently increases energy expenditure but persistently decreases circulating markers of inflammation in C57BL/6J mice fed a high-fat diet. *Metabolism* **2008**, *57*, S39–S46. [CrossRef] [PubMed]
62. Rivera, L.; Morón, R.; Sánchez, M.; Zarzuelo, A.; Galisteo, M. Quercetin ameliorates metabolic syndrome and improves the inflammatory status in obese Zucker rats. *Obesity (Silver Spring)* **2008**, *16*, 2081–2087. [CrossRef] [PubMed]
63. Mamani-Matsuda, M.; Kauss, T.; Al-Kharrat, A.; Rambert, J.; Fawaz, F.; Thiolat, D.; Moynet, D.; Coves, S.; Malvy, D.; Mossalayi, M.D. Therapeutic and preventive properties of quercetin in experimental arthritis correlate with decreased macrophage inflammatory mediators. *Biochem. Pharmacol.* **2006**, *72*, 1304–1310. [CrossRef] [PubMed]
64. Schültke, E.; Kendall, E.; Kamencic, H.; Ghong, Z.; Griebel, R.W.; Juurlink, B.H. Quercetin promotes functional recovery following acute spinal cord injury. *J. Neurotrauma* **2003**, *20*, 583–591. [CrossRef] [PubMed]
65. Schültke, E.; Kamencic, H.; Skihar, V.M.; Griebel, R.; Juurlink, B. Quercetin in an animal model of spinal cord compression injury: Correlation of treatment duration with recovery of motor function. *Spinal Cord* **2010**, *48*, 112–117. [CrossRef] [PubMed]
66. Jung, J.H.; Kang, J.I.; Kim, H.S. Effect of quercetin on impaired immune function in mice exposed to irradiation. *Nutr. Res. Pract.* **2012**, *6*, 301–307. [CrossRef] [PubMed]
67. Liu, H.; Zhang, L.; Lu, S.P. Evaluation of antioxidant and immunity activities of quercetin in isoproterenol-treated rats. *Molecules* **2012**, *17*, 4281–4291. [CrossRef] [PubMed]
68. Dong, Y.S.; Wang, J.L.; Feng, D.Y.; Qin, H.Z.; Wen, H.; Yin, Z.M.; Gao, G.D.; Li, C. Protective effect of quercetin against oxidative stress and brain edema in an experimental rat model of subarachnoid hemorrhage. *Int. J. Med. Sci.* **2014**, *11*, 282–290. [CrossRef] [PubMed]
69. Milenković, M.; Arsenović-Ranin, N.; Stojić-Vukanić, Z.; Bufan, B.; Vučićević, D.; Jančić, I. Quercetin ameliorates experimental autoimmune myocarditis in rats. *J. Pharm. Pharm. Sci.* **2010**, *13*, 311–319. [PubMed]
70. Kobori, M.; Takahashi, Y.; Sakurai, M.; Akimoto, Y.; Tsushida, T.; Oike, H.; Ippoushi, K. Quercetin suppresses immune cell accumulation and improves mitochondrial gene expression in adipose tissue of diet-induced obese mice. *Mol. Nutr. Food Res.* **2016**, *60*, 300–312. [CrossRef] [PubMed]
71. Qureshi, A.A.; Khan, D.A.; Mahjabeen, W.; Papasian, C.J.; Qureshi, N. Suppression of nitric oxide production and cardiovascular risk factors in healthy seniors and hypercholesterolemic subjects by a combination of polyphenols and vitamins. *J. Clin. Exp. Cardiol.* **2012**, *S5*, 8.
72. Heinz, S.A.; Henson, D.A.; Austin, M.D.; Jin, F.; Nieman, D.C. Quercetin supplementation and upper respiratory tract infection: A randomized community clinical trial. *Pharmacol. Res.* **2010**, *62*, 237–242. [CrossRef]
73. Heinz, S.A.; Henson, D.A.; Nieman, D.C.; Austin, M.D.; Jin, F. A 12-week supplementation with quercetin does not affect natural killer cell activity, granulocyte oxidative burst activity or granulocyte phagocytosis in female human subjects. *Br. J. Nutr.* **2010**, *104*, 849–857. [CrossRef] [PubMed]
74. Nieman, D.C.; Henson, D.A.; Gross, S.J.; Jenkins, D.P.; Davis, J.M.; Murphy, E.A.; Carmichael, M.D.; Dumke, C.L.; Utter, A.C.; McAnulty, S.R.; *et al.* Quercetin reduces illness but not immune perturbations after intensive exercise. *Med. Sci. Sports Exerc.* **2007**, *39*, 1561–1569. [CrossRef] [PubMed]
75. Henson, D.; Nieman, D.; Davis, J.M.; Dumke, C.; Gross, S.; Murphy, A.; Carmichael, M.; Jenkins, D.P.; Quindry, J.; McAnulty, S.; *et al.* Post-160-km race illness rates and decreases in granulocyte respiratory burst and salivary IgA output are not countered by quercetin ingestion. *Int. J. Sports Med.* **2008**, *29*, 856–863. [CrossRef] [PubMed]

76. Nieman, D.C.; Henson, D.A.; Maxwell, K.R.; Williams, A.S.; McAnulty, S.R.; Jin, F.; Shanely, R.A.; Lines, T.C. Effects of quercetin and EGCG on mitochondrial biogenesis and immunity. *Med. Sci. Sports Exerc.* **2009**, *41*, 1467–1475. [CrossRef] [PubMed]
77. Nieman, D.C.; Williams, A.S.; Shanely, R.A.; Jin, F.; McAnulty, S.R.; Triplett, N.T.; Austin, M.D.; Henson, D.A. Quercetin's influence on exercise performance and muscle mitochondrial biogenesis. *Med. Sci. Sports Exerc.* **2010**, *42*, 338–345. [CrossRef] [PubMed]

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Review

The Role of Vitamin E in Immunity

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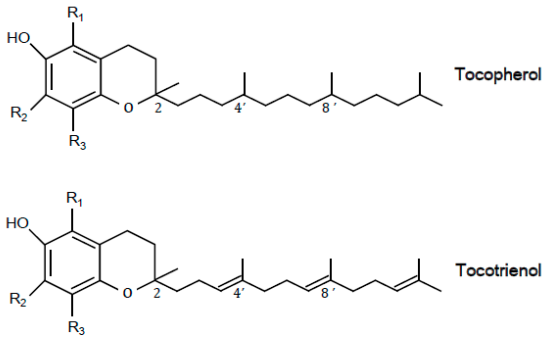
Abstract: Vitamin E is a fat-soluble antioxidant that can protect the polyunsaturated fatty acids (PUFAs) in the membrane from oxidation, regulate the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and modulate signal transduction. Immunomodulatory effects of vitamin E have been observed in animal and human models under normal and disease conditions. With advances in understating of the development, function, and regulation of dendritic cells (DCs), macrophages, natural killer (NK) cells, T cells, and B cells, recent studies have focused on vitamin E's effects on specific immune cells. This review will summarize the immunological changes observed with vitamin E intervention in animals and humans, and then describe the cell-specific effects of vitamin E in order to understand the mechanisms of immunomodulation and implications of vitamin E for immunological diseases.

Keywords: vitamin E; macrophages; T cells; dendritic cells; immunomodulation; infection

1. Vitamin E: Definition, Structure, Sources, and Functions

1.1. Definition and Structure

Vitamin E is the collective term for four tocopherols (α-, β-, γ-, and δ-tocopherols) and four tocotrienols (α-, β-, γ-, and δ-tocotrienols) found in food. These forms have antioxidant activities, but cannot be interconverted, and only α-tocopherol meets the human vitamin E requirement [1]. Tocopherols have a chromanol ring and a phytyl tail, while tocotrienols have a chromanol ring and an unsaturated tail. The α-, β-, γ-, and δ- forms differ in the number and position of methyl groups on the chromanol structure. Natural tocopherols have only *RRR* stereochemistry, but synthetic tocopherols are mixtures of eight stereoisomers (*RRR*-, *RSR*-, *RRS*-, *RSS*-, *SRR*-, *SSR*-, *SRS*-, *SSS*-), because there are three asymmetric carbon atoms (2*R*, 4′*R*, 8′*R*) present in the phytyl tail. The structures of tocopherols and tocotrienols are shown in Figure 1.



R_1	R_2	R_3	
CH_3	CH_3	CH_3	α-
CH_3	H	CH_3	β-
H	CH_3	CH_3	γ-
H	H	CH_3	δ-

Figure 1. The structures of tocopherol and tocotrienols.

1.2. Sources

The major dietary sources of vitamin E are vegetable oils. Nuts are good sources of vitamin E as well [2]. Soybean, sunflower, corn, walnut, cottonseed, palm, and wheat germ oils contain relatively higher amounts (more than approximately 50 mg vitamin E/100 g oil) of vitamin E than other oils. The proportions of α-, β-, γ-, and δ-tocopherols vary depending on the oil type. Safflower and sunflower oils are high in α-tocopherol, soybean and corn oils contain mainly γ-tocopherol, and cottonseed oil contains similar proportions of α- and γ-tocopherols. Therefore, the types of oils consumed through the diet affect the dietary intake levels of α-tocopherol. Vitamin E supplements are quite popular and contribute considerably to vitamin E intake among some populations. Either natural or synthetic forms of α-tocopherol are used as supplements.

Despite the relatively higher intake of γ-tocopherol from the diet than α-tocopherol, α-tocopherol is the major form of vitamin E in the circulation because α-tocopherol transfer protein (α-TTP) has the preferential binding affinity for α-tocopherol. α-TTP is involved in the transfer of α-tocopherol to the plasma membrane [1].

1.3. Functions

Vitamin E is a major fat-soluble antioxidant that scavenges peroxyl radicals and terminates the oxidation of polyunsaturated fatty acids (PUFAs). In the presence of vitamin E, peroxyl radicals react with α-tocopherol instead of lipid hydroperoxide, the chain reaction of peroxyl radical production is stopped, and further oxidation of PUFAs in the membrane is prevented [1]. Tocopheroxyl radicals—produced from α-tocopherol and peroxyl radicals—are reduced by vitamin C or glutathione, form tocopherol dimers, undergo further oxidation, or act as prooxidants. The antioxidant activity of vitamin E may be responsible for the regulation of several enzymes involved in signal transduction because the activity of signaling enzymes is regulated by the redox state.

Vitamin E inhibits protein kinase C (PKC) activity by increasing PKC-α dephosphorylation through the activation of protein phosphatase 2A. The inhibition of PKC by vitamin E has been reported in various cells, and consequently, the inhibition of platelet aggregation; reduced proliferation of monocytes, macrophages, neutrophils, and vascular smooth muscle cells; and decreased superoxide production in neutrophils and macrophages have been observed [3,4].

Vitamin E may directly bind to the enzymes involved in the generation of lipid mediators or to the transport proteins involved in signal transduction. Vitamin E may affect the membrane protein

interaction and translocation of the enzymes to the plasma membrane and therefore change the activity of signal transduction enzymes [4].

2. Modulation of Immune Responses and Infectious Diseases by Vitamin E Supplementation

2.1. Immune Responses in Animals

Dietary interventions of vitamin E at supplemental levels have been shown to enhance cell-mediated and humoral immune responses in various species of animals. Increased lymphocyte proliferation, immunoglobulin levels, antibody responses, natural killer (NK) cell activity, and interleukin (IL)-2 production have been reported with vitamin E supplementation (Table 1).

2.2. Immune Responses in Humans

In humans, many intervention studies have reported increased lymphocyte proliferation in response to mitogenic stimulation, enhanced delayed type hypersensitivity (DTH) response, increased IL-2 production, and decreased IL-6 production with vitamin E supplementation above the recommended levels. However, some studies showed no difference or decreased lymphocyte proliferation responses and decreased chemiluminescence. (Table 2). Differences in dose of vitamin E supplementation used, magnitude of vitamin E level changes with supplementation, age of subjects, and methodology (determination of antibody levels with or without specific vaccination) might have contributed to the different results observed.

2.3. Infectious Diseases in Animals

The immunostimulatory effect of vitamin E has resulted in enhanced resistance against several pathogens. Animal studies in which infectious disease models were used to test the effects of vitamin E supplementation are listed in Table 3.

The mechanisms involved with protection against infectious agents were increased macrophage activity and antibody (Ab) production for *D. pneumoniae* type 1 [5], and higher NK activity and Th1 response for influenza virus [6,7].

2.4. Infectious Diseases in Humans

In humans, the effects of vitamin E on the natural incidence of infectious diseases have been determined in several studies (Table 4). Many studies provided evidence that the immunostimulatory effects of vitamin E confer improved resistance to infections. However, the magnitudes of the effects were rather small, and in some studies, positive effects were only observed in subgroups of subjects.

Table 1. Modulation of immune responses by vitamin E in animal models.

Species	Dosage and Duration	Form of Vitamin E Used	Results	References
Chicks, female broiler (n = 6/group, 6 replicate)	100 mg/kg diet for 21 days	DL-α-tocopheryl acetate	↑Plasma IgM levels at day 21 ↔Splenic expressions of TNF-α, IFN-γ, IL-2, IL-10	Dalia et al. 2018 [8]
Pregnant cows (n = 24/group)	250 IU/day from day 107 of gestation to day 21 of lactation	NA	↑IgG and IgA concentration in sow plasma	Wang et al. 2017 [9]
Domestic cats (39 castrated male and 33 intact female) (n = 8/group)	225, 450 mg/kg diet for 28 days	α-tocopherol	↑Lymphocyte proliferation (ConA, PHA)	O' Brien et al. 2015 [10]
Young and old mice (n = 11–13/group)	500 mg/kg diet for 6 weeks	DL-α-tocotrienol	↑Lymphocyte proliferation in old (ConA, PHA) ↑IL-1β production in young	Ren et al. 2010 [11]
Young rats (n = 6/group)	50, 200 mg/kg diet for 8–10 weeks		↑Lymphocyte proliferation (ConA, LPS)	Bendich et al. 1986 [12]
Old mice (n = 10/group)	500 mg/kg diet for 6 weeks	DL-α-tocopheryl acetate	↑Lymphocyte proliferation (ConA, LPS) ↑DTH response ↑IL-2 production ↓PGE_2 production	Meydani et al. 1986 [13]
Young and old mice (n = 5/group)	500 IU (500 mg) for 9 weeks	DL-α-tocopherol acetate	↑Lymphocyte proliferation (ConA) in young ↔Lymphocyte proliferation (ConA) in old ↑IFN-γ in young under restraint stress	Wakikawa et al. 1999 [14]
Young rats (n = 10/group)	50, 100, 250, 500, 2500 mg/kg diet for 7 days	DL-α-tocopheryl acetate	↑Lymphocyte proliferation (>100 mg/kg diet, ConA) (>250 mg/kg diet, LPS) ↑NK activity (>250 mg/kg diet)	Moriguchi et al. 1990 [15]
Old rats (n = 5/group)	585 mg/kg diet for 12 months	DL-α-tocopheryl nicotinate	↑Lymphocyte proliferation (ConA, PHA) ↑IL-2 production	Sakai S & Moriguchi 1997 [16]
Young calves (n = 8/group)	125, 250, 500 IU (125, 250, 500 mg)/day for 24 weeks	DL-α-tocopheryl acetate	↑Lymphocyte proliferation (PHA, ConA, pokeweed mitogen) ↑Antibovine herpesvirus Ab titer to booster in 125 IU/day group	Reddy et al. 1987 [17]
Young mice (n = 8/group)	200 mg/kg diet for 6–12 weeks	α-tocopheryl acetate	↑Ab response ↑Helper T cell activity	Tanake et al. 1979 [18]
Mice (n = 10/group)	500 mg/kg diet for 6 months	α-tocopherol acetate (Tekland, Madison, WI)	↓IL-6 and PGEs (unstimulated) production by macrophages ↓Nitric oxide production (LPS) by macrophages	Beharka et al. 2000 [19]

Ab, antibody; ConA, concanavalin A; IFN-γ, interferon-γ; LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin; TNF, Tumor necrosis factor.

Table 2. Modulation of immune responses by vitamin E in humans.

Subjects	Age	Amount and Duration of Supplementation	Form of Vitamin E Used	Effects on Immune Function	References
Young (n = 5) and senior athletes (n = 5)	18–25, 35–57	4.6 ± 0.3 mg/100 mL of vitamin E-enriched beverage 5 days/week for 5 weeks	α-tocopherol acetate	↑15LOX2, TNF-α expression	Capo et al. 2016 [20]
Healthy women (n = 108)	18–25	400 mg TRF/day for 56 days	D-α-tocotrienol D-γ-tocotrienol	↑IL-4 (TT vaccine), IFN-γ (ConA)	Mahalingam et al. 2011 [21]
			D-δ-tocotrienol D-α-tocopherol	↓IL-6 (LPS)	
Healthy men and women (n = 19, 34)	20–50	200 mg/day for 56 days	α-tocopherol	↔IL-4, IFN-γ production (ConA)	Radhakrishnan et al. 2009 [22]
Adult males and young boys (n = 18)	25–30, 13–18	300 mg/day for 3 weeks	DL-α-tocopheryl acetate	↓Lymphocyte proliferation (PHA) ↔DTH ↓Bactericidal activity	Prasad 1980 [23]
Institutionalized adult males and females (n = 103)	24–104	200, 400 mg/day for 6 months	α-tocopherol acetate	↔Ab development to influenza virus	Harman and Miller 1986 [24]
Healthy elderly males and females (n = 32)	≥60	800 mg/day for 30 days	DL-α-tocopheryl acetate	↑Lymphocyte proliferation (ConA) ↑DTH ↑IL-2 production (ConA) ↓PGE_2 production (PHA)	Meydani et al. 1990 [25]
Eldery males and females (n = 74)	≥65	100 mg/day for 3 months	DL-α-tocopheryl acetate	↔Lymphocyte proliferation (ConA, PHA) ↔IgG, IgA levels	De Waart et al. 1997 [26]
Healthy elderly males and females (n = 88)	≥65	60, 200, 800 mg/day for 235 days	DL-α-tocopherol	↑DTH and antibody titer to hepatitis B with 200, 800 mg	Meydani et al. 1997 [27]
Healthy elderly males and females (n = 161)	65–80	50, 100 mg/day for 6 months	DL-α-tocopheryl acetate	↑No. of positive DTH reaction with 100 mg ↑dDiameter of induration of DTH reaction in a subgroup supplemented with 100 mg ↔IL-2 production ↓IFN-γ production	Pallast et al. 1999 [28]
Healthy young adults (n = 31) and premature infants (n = 10)	24–31	600 mg/day for 3 months40 mg/kg body weight for 8–14 days		↓Chemiluminescence	Okano et al. 1990 [29]
Cigarette smoker (n = 60)	33 ± 4	900 IU/day for 6 weeks		↓Chemiluminescence	Richards et al. 1990 [30]
Healthy males (n = 40)	24–57	200 mg/day for 4 months	*all-rac*-α-tocopherol	Prevented fish-oil-induced suppression of ConA mitogenesis	Kramer et al. 1991 [31]
Healthy elderly (n = 40)	>65	100, 200, or 400 mg/day for 3 months	DL-α-tocopherol	↑DTH (maximal diameter) in 100, 200, 400 mg groups ↑Lymphocyte proliferation (ConA) in 200 mg group	Wu et al. 2006 [32]
Sedentary young and elderly males (n = 21)	22–29, 55–74	800 IU (727 mg)/day for 48 days	DL-α-tocopherol	↓IL-6 secretion ↓Exercise-enhanced IL-1β secretion	Cannon et al. 1991 [33]

ConA, concanavalin A; DTH, delayed type hypersensitivity; IFN-γ, interferon-γ; 15LOX2, 15-lipoxygenase-2; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin; TRF, tocotrienol-rich fraction; TT vaccine, tetanous toxoid vaccine.

Table 3. Effects of vitamin E supplementation on infectious diseases in animal models.

Subjects	Age	Dose and Duration of Supplementation	Form of Vitamin E Used	Infection Organism and Route of Infection	Results: Effects of Vitamin E Supplementation	References
Mice BALB/c (n = 3–6/group)	6 months	100 mg/kg for 8 days before MRSA-challenge	δ-, γ-Tocotrienol	MRSA, inoculated onto superficial surgical wounds	Higher NK cytotoxicity Higher IL-24 mRNA expression levels	Pierpaoli et al. 2017 [34]
Young and aged male mice C57BL/6 (n = 6/group)	2, 22–26 months	500 mg/kg for 4 weeks prior to infection	D-α-tocopheryl acetate	*Streptococcus pneumoniae*, intra-tracheally injected	1000-fold fewer bacteria in their lung Age-associated higher production of proinflammatory cytokines (TNF-, IL-6) were reduced 3-fold reduction in the number of PMNs	Bou Ghanem et al. 2015 [35]
Worm-free lambs (n =10/group)	28–32 weeks	5.3 IU (3.56 mg)/kg BW for 12 weeks	D-α-tocopherol	*H. contortus* L3 larvae, route NA	No difference in serum IgG or peripheral mRNA expression of IL-4 or IFN-γ Lower PCV, FEC, and worm burden	De Wolf et al. 2014 [36]
Male mice BALB/c (n = 6–7/group)	At weaning	Deficient, Adequate (38.4 mg/kg diet), or Supplemented (384 mg/kg diet) for 4 weeks	DL-α-tocopheryl acetate	HSV-1, intranasally	Higher viral titre and ILβ, TNF-α, RANTES in the brain with E deficiency No difference in expressions of IL-6, TNFα, IL-1β, and IL-10 between adequate and supplemented	Sheridan & Beck. 2008 [37]
Mice C57BL (n = 6–9/group)	22 months	500 mg/kg diet for 8 weeks	DL-α-tocopherol acetate	Influenza by nasal inoculation	Lower viral titer Higher IL-2 and IFN-γ production	Han et al. 2000 [6]
Mice, C57BL/6 (n = 4–9)	22 months	500mg/kg diet for 6 weeks	DL-α-tocopherol acetate	Influenza A/PC/1/73 (H3N2) by nasal inoculation	Lower viral titre	Hayek et al. 1997 [7]
Mice, C57BL/6 (n = 6)	5 weeks	160 IU/L liquid diet for 4, 8, 12, 16 weeks	*all-rac*-α-tocopheryl acetate	Murine LP-BM5 leukaemia retrovirus by IP injection	Restored IL-2 and IFN-γ production by splenocytes following infection	Wang et al. 1994 [38]
Calves, Holstein (n = 7)	1d	1400 or 2800 mg orally once per week, 1400 mg injection once per week for 12 weeks	DL-α-tocopheryl acetate	Bovine rhinotracheitis virus, in vitro	Serum from vitamin E-supplemented calves inhibited the replication of bovine rhinotracheitis virus in vitro	Reddy et al. 1986 [39]
Mice, Swiss Webster (n = 10)	4 weeks	180 mg/kg diet for 4 weeks	DL-α-tocopheryl acetate	*Diplococcus pneumoniae* type I by IP injection	Higher survival	Heinzerling et al. 1974a [5]
Mice, BALB/C (n = 25)	NA	25 or 250 mg/kg bw orally for 4 days, starting 2 days before burn injury	DL-α-tocopheryl acetate	*Pseudomonas aeruginosa*, subeschar injection to burned mice	Lower mortality rate	Fang et al. 1990 [40]
Mice, BALB/C (NA)	3 weeks	4000mg/kg diet for 2, 4, or 14 weeks	Vitamin E injectable (aqueous)	*Listeria monocytogenes* by IP injection	No difference in resistance	Watson & Petro 1982 [41]
Rats, Sprague-Dawley (n = 6)	3 weeks	180 mg/kg diet + 6000 IU vitamin A/kg diet for 6 weeks	DL-α-tocopheryl acetate	*Mycoplasma pulmonis* by aerosol	Higher resistance to infection	Tvedten et al. 1973 [42]
Lambs (n = 10)	NA	1000 IU orally, 300 mg/kg diet for 23 days	DL-α-tocopheryl acetate	Chlamydia by intratracheal inoculation	Faster recovery (higher food intake and weight gains)	Stephens et al. 1979 [43]

Table 3. *Cont.*

Subjects	Age	Dose and Duration of Supplementation	Form of Vitamin E Used	Infection Organism and Route of Infection	Results: Effects of Vitamin E Supplementation	References
Turkey, broadbreasted white poults (*n* = 6)	1 day	500 mg/kg diet for 14 days before infection and 18–21 days after infection	DL-α-tocopheryl acetate	*Histomonas meleagridis*, oral	No effect on mortality by vitamin E supplementation alone Lower mortality and lesion score in combination with ipronidazole	Schildknecht & Squibb 1979 [44]
Pigs (*n* = 6)	NA	200 mg/pig per day for 59 days before infection and 22 days after infection	DL-α-tocopheryl acetate	*Treponema hyodysenteriae*, oral	Improved weight gain and recovery rate No beneficial effect on appetite and diarrhoea	Teige et al. 1982 [45]
Sheep (*n* = 12)	3–6 months	300 mg/kg diet starting 2 weeks before first vaccination	DL-α-tocopheryl acetate	*Clostridium perfringens* type D by IV injection after two IM vaccinations	Higher Ab titre Fail to prove beneficial effect of vitamin E on protection (none of the vaccinated lambs died)	Tengerdy et al. 1983 [46]
Cows (*n* = 20)	NA	740 mg/cow per day, duration NA	DL-α-tocopheryl acetate	Natural occurrence of clinical mastitis due to *Streptococci, Coliform, Staphylococci, Clostridium bovis*	Lower clinical cases of mastitis	Smith et al. 1984 [47]
Chicks, broiler (*n* = 12–14)	1day	150 mg or 300mg/kg diet for 2 weeks before infection	DL-α-tocopheryl acetate	*Escherichia coli*, orally and post-thoracic air sac	Lower mortality Higher Ab titre	Heinzerling et al. 1974b [48]
Chicks, broiler (*n* = 10)	1 day	300 mg/kg diet for 6 weeks, starting 3 weeks before first infection	DL-α-tocopheryl acetate	*E. coli*, post-thoracic air sac	Lower mortality	Tengerdy & Nockels 1975 [49]
Chicks, Leghorn (*n* = 22)	1 day	300 mg/kg diet for 4 weeks before infection	DL-α-tocopheryl acetate	*E. coli* by IV injection	Lower mortality	Likoff et al. 1981 [50]
Pigs (*n* = 10)	6–8 weeks	100, 000 mg/t diet for 10 weeks, starting 2 weeks before infection	Vitamin E; Tompson-Hayward, Minneapolis, MN, USA	*E. coli* by IM injection	Higher serum Ab titre	Ellis & Vorhies 1976 [51]

Ab, antibody; FEC, fecal egg count; HSV, Herpes simplex virus; MRSA, IFN-γ, interferon-γ; IM, intramuscular; IV, intravenous; Methicillin-resistant *Staphylococcus aureus*; NK, natural killer; PCV, packed cell volume; PMN, polymorphonuclear leukocyte, RANTES, regulated on activation, normal T cell expressed and secreted; TNF-α, tumor necrosis factor-α.

Table 4. Effects of vitamin E supplementation on infectious diseases in humans.

Subjects	Age	Dose and Duration of Supplementation	Form of Vitamin E Used	Infection Organism and Route of Infection	Results: Effects of Vitamin E Supplementation	References
Male smoker	50–69	50 mg/d for median of 6 years	DL-α-tocopheryl acetate	Natural incidence of pneumonia	69% Lower incidence of pneumonia among subgroups including participants who smoked 5–19 cigarettes per day at baseline and exercised at leisure time 14% Lower incidence of pneumonia among subgroups including participants who smoked ≥20 cigarettes per day at baseline and did not exercise	Hemila et al. 2016 [52]
HIV-infected pregnant Tanzanian women	25.4	30 mg during pregnancy (multivitamin form with 20 mg vitamins B1, 20 mg B2, 25 mg B6, 100 mg niacin, 50 μg B12, 500 mg C, and 800 μg folic acid)	NA	Natural incidence of malaria after having received malaria prophylaxis during pregnancy	Lower incidence of presumptive clinical malaria, but higher risk of any malaria parasitemia	Olofin et al. 2014 [53]
Patients with HCV-related cirrhosis	54–75	900 IU (604.03 mg for D- or 818.18 mg for DL-)/day for 6 months	α-tocopherol	Natural incidence of cirrhosis	Reduced glutathione (GSH) and glutathione peroxidase, which are significantly lower in cirrhotic patients ($p < 0.05$), were comparably improved by vitamin E regimens	Marotta et al. 2007 [54]
Patients with chronic HCV	18–75	945 IU (634.23 mg)/day for 6 months with 500 mg ascorbic acid and 200 μg of selenium	D-α-tocopherol	Natural incidence of HCV	No difference in median log plasma HCV-RNA	Groenbak et al. 2006 [55]
Nursing home residents	>65	200 IU/day for 1 year	DL-α-tocopherol	Natural incidence of respiratory infections	Fewer numbers of subjects with all and upper respiratory infections Lower incidence of common cold No effect on lower respiratory infection	Meydani et al. 2004 [56]
Male smokers	50–69	50 mg/day during 4-year follow-up	α-tocopherol	Natural incidence of common cold episodes	Lower incidence of common cold Reduction was greatest among older city dwellers who smoked fewer than 15 cigarettes per day	Hemila et al. 2002 [57]
Male smokers	50–69 years	50 mg/day for median of 6.1 years	DL-α-tocopheryl acetate	Natural incidence of pneumonia	No overall effect on the incidence of pneumonia. Lower incidence of pneumonia among the subjects who had initiated smoking at a later age (>21)	Hemila et al. 2004 [58]
Non-institutionalized individuals	>60 years	200 mg/day for median of 441 days	α-tocopherol acetate	Natural incidence and severity of self-reported acute respiratory tract infections	No effect on incidence and severity of acute respiratory tract infections	Graat et al. 2002 [59]

HCV, hepatitis C virus.

3. Vitamin E and Immune Cells

The immunomodulatory mechanisms of α-tocopherol in immune cells are depicted in Figure 2.

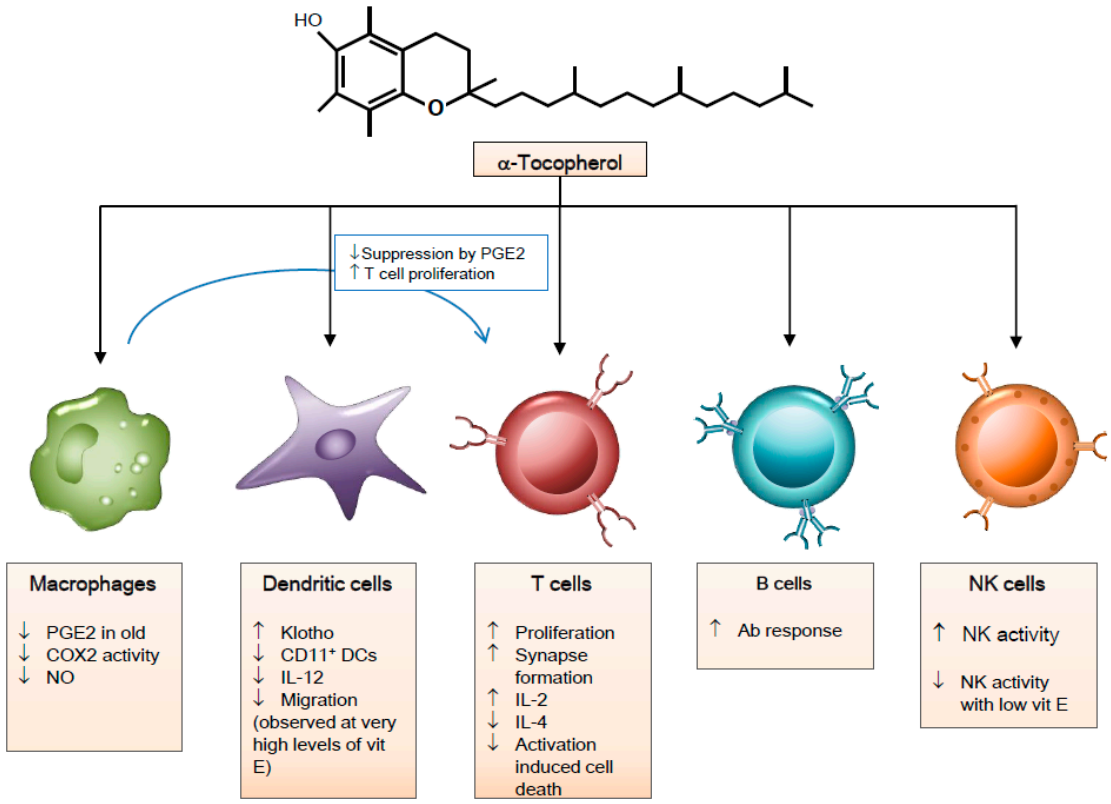


Figure 2. Immunomodulatory effects of vitamin E on immune cells. Abbreviations: PGE_2, prostaglandin E_2; COX2, Cyclooxygenase 2; NO, Nitric oxide; CD, Clusters of Differentiation; DCs, Dendritic cells; IL-12, Interleukin-12; Ab, antibody; NK, Natural killer.

3.1. Macrophages

Macrophages, important effector cells in the innate immune response, serve as antigen presenting cells (APC) and regulate NK cells and T cells by producing cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), and prostaglandins. Cytokines produced by T cells and other immune cells can shift the macrophages into different populations with distinct physiologies [60].

The effects of vitamin E on prostaglandin (PG)E_2 production by macrophages from the aged have been suggested as one of the mechanisms by which vitamin E improves the age-associated decrease in the T cell-mediated immune response [61]. In a co-culture experiment in which purified T cells and macrophages from young and old mice were cultured together, T cells from young mice showed suppressed proliferation and IL-2 secretion when cultured with macrophages from old mice. When macrophages from old mice were pre-incubated with 10 μg/mL vitamin E for 4 h, co-cultures of old macrophages and young T cells showed significant improvement in proliferation. Vitamin E pre-incubation of old macrophages improved proliferation and IL-2 production in co-cultures of old macrophages and old T cells [62]. Macrophages from old mice produced significantly higher levels of PGE_2, which was due to higher cyclooxygenase (COX) activity. Macrophages from old mice expressed higher levels of inducible COX2 protein and mRNA [63]. These increases in PGE_2 synthesis and COX activity were lowered by in vivo vitamin E supplementation [64]. Macrophages isolated from old mice fed a diet containing 500 ppm vitamin E for 30 days produced lower amounts of PGE_2 and had lower COX activity than those from old mice fed a control diet containing 30 ppm vitamin E, but the COX2 mRNA levels and protein expression of the control and supplemented groups did not

differ. Thus, vitamin E's effect on COX activity seemed to be through post-translational mechanisms rather than through its effect at transcriptome or translational levels. In a subsequent study, it was shown that vitamin E reduced COX activity in macrophages from old mice by decreasing peroxynitrite production [65]. The inhibition of COX activity by vitamin E in old mice disappeared specifically with the addition of a nitric oxide (NO) donor in the presence of a superoxide to elevate peroxynitrite levels in the macrophage culture. There is a complex interplay between the nitric oxide synthase (NOS) and COX pathways and NO increases COX2 activity, which seems to be due to the NO preventing self-deactivation of COX by the superoxide as NO interacts with the superoxide [66].

In vivo supplementation of vitamin E (1500 IU D-α-tocopheryl acetate/day for 16 weeks) in allergic asthmatic patients prevented the suppression of alveolar macrophage nuclear factor (erythroid-derived 2)-like 2 (NRF2) activity after allergen challenge [67]. This study presented the possibility of vitamin E's protective role in allergies and asthmas through regulation of macrophage NRF2 activity, but, further studies are needed to confirm the findings because of the small number of patients (nine mild non-smoking allergic asthmatics) and the lack of appropriate controls.

3.2. Natural Killer Cells

NK activity seems to be related with vitamin E status. The NK activity of a boy with Shwachman syndrome who had a severe vitamin E deficiency was low, but improved after eight weeks of 100 mg/d α-tocopherol supplementation. When α-tocopherol supplementation was stopped, NK activity and $CD16^+$ $CD56^+$ cells decreased. NK activity and $CD16^+$ $CD56^+$ cells were restored upon resuming eight weeks of 100 mg/d α-tocopherol supplementation [68]. In 37 women aged 90–106 years old, NK cell cytotoxicity was positively associated with plasma vitamin E concentration [69]. A two-week supplementation of 750 mg vitamin E in colorectal cancer patients resulted in increased NK activity in six out of seven patients. Vitamin E treatment did not result in changes in perforin expression or IFN-γ production; therefore, mechanisms of improved NK activity by vitamin E could not be determined from the study [70].

NO appears to be involved in the impairment of NK cell function. Co-culture of NK cells and myeloid-derived suppressor cells (MDSCs) showed that NK cell cytotoxicity and IFN-γ were impaired by MDSCs and that the inhibition of inducible nitric oxide synthase (iNOS) rescued the impairment by MDSCs. Exposure of NK cells to NO by treatment with an NO producer caused the nitration of tyrosine residues on $CD16^+$ NK cells. These results suggested that MDSCs impair NK cell function via the production of NO and the nitration of protein tyrosine residues [71]. Vitamin E might exert its effects on NK cell function by modulating NO levels.

3.3. Dendritic Cells

Dendritic cells (DCs) are effective antigen-presenting cells that recognize pathogens and present pathogen-derived antigens to T cells. The interaction of DCs with pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) elicits the activation and maturation of DCs. The increased expression of surface major histocompatibility complex (MHC) molecules and co-stimulatory molecules and the increased production of cytokines occur with the activation of DCs, which allows the effective induction of the T cell response [72–74]. DCs are also involved in tolerance and autoimmunity. DCs might promote tolerance by the generation of Treg cells and/or by the induction of T cell unresponsiveness. DCs might be involved in the pathogenesis of autoimmune disease by promoting the priming or differentiation of self-reactive T cells [72]. Therefore, understanding the regulation of DCs by vitamin E will provide insight into the mechanisms of vitamin E's immune response modulation and implications of vitamin E in immunological diseases.

Several studies have shown that vitamin E could regulate the maturation and functions of DCs. Tan et al. [75] investigated the effects of α-tocopherol and vitamin C, alone or in combination, on the phenotype and functions of human DCs generated from peripheral blood mononuclear cells (PBMCs). During the differentiation of human PBMCs into DCs, various concentrations of α-tocopherol

were treated in culture starting from day 2, cells were stimulated on day 5, and then the surface phenotype was determined on day 6. The expression of human leukocyte antigen(HLA)-DR, CD40 CD80, and CD86 appeared to be increased with lower concentrations of α-tocopherol (<0.05 mM), but the combination of vitamin E and C prevented DC activation, as the upregulation of surface markers was not observed. DCs treated with 0.5 mM vitamin E and 10 mM vitamin C showed lower levels of intracellular ROS and inhibition of the nuclear factor (NF)-κB, PKC, and p38 mitogen-activated protein kinase (MAPK) pathways. When bone marrow-derived dendritic cells (BMDCs) from Balb/c mice were treated with 500 μM of α-tocopherol for 2 h, upregulation of phosphorylated inhibitor of κB (IκB) by lipopolysaccharide (LPS)-stimulation was suppressed. Vitamin E treatment for 24 h resulted in a reduced number of $CD11^+CD86^+$ cells and ROS-positive cells, lower production of IL-12p70 and TNF-α, and decreased transwell migration of BMDCs. These effects of vitamin E on BMDCs were partly dependent on Klotho expression. Vitamin E treatment on BMDCs resulted in higher Klotho transcript and protein levels, and silencing of Klotho by transfection of *Klotho* siRNA abolished the inhibitory effects of vitamin E on IL-12p70 production, number of ROS-positive cells, and DC migration [76]. Klotho is a membrane protein that has been shown to mediate calcium transport into the cells; regulate intracellular signaling pathways such as p53/p21, cyclin adenosine monophosphate (cAMP), PKC, and Wnt; and inhibit the NF-κB pathway [77]. Therefore, the upregulation of Klotho by vitamin E could be one of the mechanisms by which vitamin E modulates NF-κB mediated DC function and maturation. However, the level of α-tocopherol used for in vitro treatment (500 μM) was high and, therefore, further research is needed to elucidate the physiological relevance of vitamin E treatment on the expression of Klotho and its involvement in the modulation of DC function.

In vivo supplementation of α-tocopherol at 150, 250, and 500 mg/kg diet in allergic female mice reduced the lung $CD11b^+$ DCs and mRNA levels of IL-4, IL-33, thymic stromal lymphopoietin (TSLP), eotaxin 1 (CCL11), and eotaxin 2 (CCL24) in allergen challenged pups. Furthermore, when BMDCs from 10-day-old neonates born to a control female were treated with 80 μM α-tocopherol for 24 h, the number of $CD45^+$ $CD11b^+$ $CD11^+$ DCs and the number of $CD45^+$ $CD11b^+$ $CD11c^+$ $Ly6c^-$ $MHCII^-$ DCs were reduced. Maternal supplementation with α-tocopherol was effective in decreasing allergic responses in offspring from allergic mothers by affecting the development of subsets of DCs that are critical for allergic responses [78]. On the other hand, γ-tocopherol supplementation exerted an opposite response in the same model. In vivo supplementation of γ-tocopherol at 250 mg/kg diet in allergic female mice resulted in a higher number of lung eosinophils, a higher number of lung $CD11c^+$ $CD11b^+$ DCs, and higher levels of lung lavage CCL11 in the offspring [79].

Modulation of the immune response by vitamin E has been observed in animal and human studies, and DCs play a critical role in bridging innate and adaptive immune systems and initiating adaptive immune responses. Despite the importance of DCs' role in adaptive immune responses and in diseases such as autoimmune diseases, few studies have investigated the DC-specific effect of vitamin E.

3.4. T Cells

The effects of vitamin E on immune cells have been studied the most with T cells. The dysregulation of immune function occurs with aging and the most significant changes are observed in T cells. Age-associated changes in T cells include, but are not limited to, (1) defects in T cell receptor (TCR) signal transduction such as a decrease in linker for the activation of T cells (LAT) phosphorylation by zeta chain of T cell receptor associated protein kinase 70 (ZAP-70), (2) decreased intracellular influx of calcium following stimulation, (3) diminished synapse formation, (4) diminished activation of the mitogen activated protein kinase (MAP kinase) pathway, (5) decreased nuclear factor of activated T-cells (NFAT) binding activity, and (6) a shift of the T cell population toward memory T cells [80]. As a result, diminished production of IL-2 and reduced proliferative capacity of naive T cells are observed and impaired T cell functions contribute to increased susceptibility to infectious diseases and poor response to immunization.

Vitamin E has been shown to increase the cell division and IL-2 producing capacity of naïve T cells, increase the percentage of T cells capable of forming an effective immune synapse, and reverse the age-associated defect in the phosphorylation of LAT in T cells from old animals [81–83].

In vitro pre-incubation with 46 μM vitamin E for 4 h increased proliferation and IL-2 production in T cells purified from old mice stimulated with anti-CD3 and anti-CD28. Increased IL-2 production was due to both an increase in the number of activation-induced IL-2^+ cells and an increase in the level of IL-2 accumulated per cell. Vitamin E specifically increased the naive T cells' ability to progress through the cell division cycle in old mice [81]. The gene expression profile of T cells isolated from young and old mice fed a diet supplemented with 500 ppm vitamin E for four weeks provided evidence that vitamin E influences cell cycle-related molecules at the gene expression level. Higher expression of cell cycle-related genes *Ccnb2*, *Cdc2*, and *Cdc6* was observed in stimulated T cells from old mice fed the vitamin E-supplemented diet compared with those fed the control diet, which was not observed in young mice [84]. Cyclin B2, encoded by *Ccnb2*, binds to cyclin-dependent kinase 1 (also known as Cdc2) and regulates the events during both the G_2/M transition and progression through mitosis. Cdc6 is a key regulator in the early steps of DNA replication, as the binding of Cdc6 to chromatin is a necessary and universal step in the acquisition of replication competences [85]. These alterations in the expression of cell cycle-related genes observed with vitamin E might contribute to vitamin E improving the proliferative ability of old T cells.

Marko et al. [82] showed that pre-incubation of CD4+ T cells isolated from old T cells with 46 μM vitamin E for 4 h increased the percentage of $CD4^+$ T cells displaying effective immune synapses. Redistribution of Zap70, LAT, Vav, and phospholipase Cγ (PLCγ) into immune synapse increased significantly with vitamin E treatment. This change was confirmed with in vivo supplementation of vitamin E. In old mice fed a diet containing 500 ppm vitamin E for eight weeks, LAT and Vav showed significantly higher redistribution into the T cell/APC contact area when purified $CD4^+$ T cells were stimulated with murine CD3ε hybridoma. In a subsequent study, it was shown that vitamin E could reverse the age-associated defect in the phosphorylation of LAT on tyrosine 191 [83]. The phosphorylation of LAT is required for the recruitment of adaptor and effector proteins. Therefore, it plays a pivotal role in the assembly of microcluster structures in the initiation of T cell activation signals. This evidence suggests that vitamin E can modulate the early stages of T cell activation.

Vitamin E seems to modulate Th1 and Th2 responses. The polarization of CD4 T cells to T helper (Th)1 or Th2 cells has implications for the protection against different pathogens (intracellular vs. extracellular pathogens) and the development of different types of chronic diseases (inflammatory vs. allergic diseases). PBMCs isolated from allergic donors treated with vitamin E (12.5–50 μM) showed dose-dependent decreases in IL-4 production [86]. IL-4 mRNA levels in activated PBMCs were downregulated by 25 μM vitamin E treatment. Jurkat T cells treated with 50 μM vitamin E exhibited downregulation of IL-4 promoter activity, which might be related to vitamin E blocking the interaction of transcription factors with PRE-1 and P1. In vivo supplementation of vitamin E enhancing the Th1 response has been observed in mice infected with influenza virus and in colorectal cancer patients [6,87]. In colorectal cancer patients, two weeks of supplementation with 750 mg vitamin E led to an increased frequency of IL-2 producing CD4+ T cells and increased IFN-γ production [87]. In old mice infected with influenza virus, 500 ppm vitamin E supplementation for eight weeks prior to infection lowered the viral titer in the lung, and this protective effect of vitamin E was associated with the enhancement of Th1 response. IFN-γ production levels correlated negatively with viral titer, and old mice fed a vitamin E-supplemented diet produced significantly higher levels of IFN-γ and IL-2 [6]. The gene expression profile of T cells isolated from young and old mice fed a diet supplemented with 500 ppm vitamin E for four weeks provided evidence that vitamin E influences the Th1/Th2 balance at the gene expression level. The increase in IL-4 expression following stimulation was lower in T cells from old mice fed the vitamin E-supplemented diet compared with those fed the control diet, and the ratio of IFN-γ and IL-4 expression levels was significantly higher in the vitamin E group than in the control group [84].

Vitamin E can affect activation-induced cell death in T cells. In vitro treatment of primary human T cells with 25 μM vitamin E suppressed CD95L expression and activation-induced cell death [88]. The reduction of CD95L mRNA levels and the proportion of CD95L-positive cells were related to the suppression of NF-κB and AP-1 binding to the CD95L promoter target site by vitamin E. On the other hand, α-tocopheryl succinate was shown to trigger apoptosis in Jurkat cells with caspase-activation involved [89].

3.5. B Cells

Vitamin E supplementation has been reported to enhance humoral responses. Higher antibody responses have been observed in animals and humans [19,27]. However, it is hard to differentiate whether vitamin E's direct effect on B cells or indirect effect through T cells contributes to higher antibody responses.

4. Conclusions

Vitamin E has been shown to enhance immune responses in animal and human models and to confer protection against several infectious diseases. Suggested mechanisms involved with these changes are (1) the reduction of PGE_2 production by the inhibition of COX2 activity mediated through decreasing NO production, (2) the improvement of effective immune synapse formation in naive T cells and the initiation of T cell activation signals, and (3) the modulation of Th1/Th2 balance. Higher NK activity and changes in dendritic function such as lower IL-12 production and migration were observed with vitamin E, but underlying mechanisms need to be further elucidated

Several considerations are warranted for the advancement in our understanding of vitamin E's role in immunity. For in vitro studies to support implications for the regulation of immunological diseases, the physiological relevance of vitamin E levels used for treatment should be considered. Different forms of vitamin E exert differential effects on immune cells. Cell-specific effects of vitamin E provide valuable evidence regarding the immunomodulatory mechanisms of vitamin E, but the interplay between immune cells should not be ignored, because interactions between immune cells are critical in the regulation of immune function.

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References

1. Traber, M.G. Vitamin E regulatory mechanisms. *Annu. Rev. Nutr.* **2007**, *27*, 347–362. [CrossRef] [PubMed]
2. Sheppard, A.J.; Pennington, J.A.T.; Weihrauch, J.L. Analysis and distribution of vitamin E in vegetable oils and foods. In *Vitamin E in Health and Disease*; Packer, L., Fuchs, J., Eds.; Marcel Dekker: New York, NY, USA, 1980; pp. 7–65.
3. Traber, M.G.; Atkinson, J. Vitamin E, antioxidant and nothing m more. *Free Rad. Biol. Med.* **2007**, *43*, 4–15. [CrossRef] [PubMed]
4. Zingg, J.M. Vitamin E: A role in signal transduction. *Annu. Rev. Nutr.* **2015**, *35*, 135–173. [CrossRef] [PubMed]
5. Heinzerling, R.H.; Tengerdy, R.P.; Wick, L.L.; Lueker, D.C. Vitamin E protects mice against Diplococcus pneumoniae type I infection. *Infect. Immun.* **1974**, *10*, 1292–1295. [PubMed]
6. Han, S.N.; Wu, D.; Ha, W.K.; Beharka, A.; Smith, D.E.; Bender, B.S.; Meydani, S.N. Vitamin E supplementation increases T helper 1 cytokine production in old mice infected with influenza virus. *Immunology* **2000**, *100*, 487–493. [CrossRef] [PubMed]
7. Hayek, M.G.; Taylor, S.F.; Bender, B.S.; Han, S.N.; Meydani, M.; Smith, D.E.; Eghtesada, S.; Meydani, S.N. Vitamin E supplementation decreases lung virus titers in mice infected with influenza. *J. Infect. Dis.* **1997**, *176*, 273–276. [CrossRef] [PubMed]

8. Dalia, A.M.; Loh, T.C.; Sazili, A.Q.; Jahromi, M.F.; Samsudin, A.A. Effects of vitamin E, inorganic selenium, bacterial organic selenium, and their combinations on immunity response in broiler chickens. *BMC Vet. Res.* **2018**, *14*, 249. [CrossRef] [PubMed]
9. Wang, L.; Xu, X.; Su, G.; Shi, B.; Shan, A. High concentration of vitamin E supplementation in sow diet during the last week of gestation and lactation affects the immunological variables and antioxidative parameters in piglets. *J. Dairy Res.* **2017**, *84*, 8–13. [CrossRef] [PubMed]
10. O'Brien, T.; Thomas, D.G.; Morel, P.C.; Rutherfurd-Markwick, K.J. Moderate dietary supplementation with vitamin E enhances lymphocyte functionality in the adult cat. *Res. Vet. Sci.* **2015**, *99*, 63–69. [CrossRef] [PubMed]
11. Ren, Z.; Pae, M.; Dao, M.C.; Smith, D.; Meydani, S.N.; Wu, D. Dietary supplementation with tocotrienols enhances immune function in C57BL/6 mice. *J. Nutr.* **2010**, *140*, 1335–1341. [CrossRef] [PubMed]
12. Bendich, A.; Gabriel, E.; Machlin, L.J. Dietary vitamin E requirement for optimum immune responses in the rat. *J. Nutr.* **1986**, *116*, 675–681. [CrossRef] [PubMed]
13. Meydani, S.N.; Meydani, M.; Verdon, C.P.; Shapiro, A.A.; Blumberg, J.B.; Hayes, K.C. Vitamin E supplementation suppresses prostaglandin E1(2) synthesis and enhances the immune response of aged mice. *Mech. Ageing Dev.* **1986**, *34*, 191–201. [CrossRef]
14. Wakikawa, A.; Utsuyama, M.; Wakabayashi, A.; Kitagawa, M.; Hirokawa, K. Vitamin E enhances the immune functions of young but not old mice under restraint stress. *Exp. Gerontol.* **1999**, *34*, 853–862. [CrossRef]
15. Moriguchi, S.; Kobayashi, N.; Kishino, Y. High dietary intakes of vitamin E and cellular immune functions in rats. *J. Nutr.* **1990**, *120*, 1096–1102. [CrossRef] [PubMed]
16. Sakai, S.; Moriguchi, S. Long-term feeding of high vitamin E diet improves the decreased mitogen response of rat splenic lymphocytes with aging. *J. Nutr. Sci. Vitaminol.* **1997**, *43*, 113–122. [CrossRef] [PubMed]
17. Reddy, P.G.; Morrill, J.L.; Minocha, H.C.; Stevenson, J.S. Vitamin E is Immunostimulatory in calves. *J. Dairy Sci.* **1987**, *70*, 993–999. [CrossRef]
18. Tanaka, J.; Fujiwara, H.; Torisu, M. Vitamin E and immune response. I. Enhancement of helper T cell activity by dietary supplementation of vitamin E in mice. *Immunology* **1979**, *38*, 727–734. [PubMed]
19. Beharka, A.A.; Han, S.N.; Adolfsson, O.; Wu, D.; Lipman, R.; Smith, D.; Cao, G.; Meydani, M.; Meydani, S.N. Long-term dietary antioxidant supplementation reduces production of selected inflammatory mediators by murine macrophages. *Nutr. Res.* **2000**, *20*, 281–296. [CrossRef]
20. Capó, X.; Martorell, M.; Sureda, A.; Riera, J.; Drobnic, F.; Tur, J.A.; Pons, A. Effects of Almond- and Olive Oil-Based Docosahexaenoic- and Vitamin E-Enriched Beverage Dietary Supplementation on Inflammation Associated to Exercise and Age. *Nutrients.* **2016**, *8*, 619. [CrossRef] [PubMed]
21. Mahalingam, D.; Radhakrishnan, A.K.; Amom, Z.; Ibrahim, N.; Nesaretnam, K. Effects of supplementation with tocotrienol-rich fraction on immune response to tetanus toxoid immunization in normal healthy volunteers. *Eur. J. Clin. Nutr.* **2011**, *65*, 63–69. [CrossRef] [PubMed]
22. Radhakrishnan, A.K.; Lee, A.L.; Wong, P.F.; Kaur, J.; Aung, H.; Nesaretnam, K. Daily supplementation of tocotrienol-rich fraction or alpha-tocopherol did not induce immunomodulatory changes in healthy human volunteers. *Br. J. Nutr.* **2009**, *101*, 810–815. [CrossRef] [PubMed]
23. Prasad, J.S. Effect of vitamin E supplementation on leukocyte function. *Am. J. Clin. Nutr.* **1980**, *33*, 606–608. [CrossRef] [PubMed]
24. Harman, D.; Miller, R.W. Effect of vitamin E on the immune response to influenza virus vaccine and the incidence of infectious disease in man. *Age* **1986**, *9*, 21–23. [CrossRef]
25. Meydani, S.N.; Barklund, M.P.; Liu, S.; Meydani, M.; Miller, R.A.; Cannon, J.G.; Morrow, F.D.; Rocklin, R.; Blumberg, J.B. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. *Am. J. Clin. Nutr.* **1990**, *52*, 557–563. [CrossRef] [PubMed]
26. De Waart, F.G.; Portengen, L.; Doekes, G.; Verwaal, C.J.; Kok, F.J. Effect of 3 months vitamin E supplementation on indices of the cellular and humoral immune response in elderly subjects. *Br. J. Nutr.* **1997**, *78*, 761–774. [CrossRef] [PubMed]
27. Meydani, S.N.; Meydani, M.; Blumberg, J.B.; Leka, L.S.; Siber, G.; Loszewski, R.; Thompson, C.; Pedrosa, M.C.; Diamond, R.D.; Stollar, B.D. Vitamin E supplementation and in vivo immune response in healthy elderly subjects. A randomized controlled trial. *JAMA* **1997**, *277*, 1380–1386. [CrossRef] [PubMed]

28. Pallast, E.G.; Schouten, E.G.; de Waart, F.G.; Fonk, H.C.; Doekes, G.; von Blomberg, B.M.; Kok, F.J. Effect of 50- and 100-mg vitamin E supplements on cellular immune function in noninstitutionalized elderly persons. *Am. J. Clin. Nutr.* **1999**, *69*, 1273–1281. [CrossRef] [PubMed]
29. Okano, T.; Tamai, H.; Mino, M. Superoxide generation in leukocytes and vitamin E. *Int. J. Vitam. Nutr. Res.* **1991**, *61*, 20–26. [PubMed]
30. Richards, G.A.; Theron, A.J.; van Rensburg, C.E.; van Rensburg, A.J.; van der Merwe, C.A.; Kuyl, J.M.; Anderson, R. Investigation of the effects of oral administration of vitamin E and beta-carotene on the chemiluminescence responses and the frequency of sister chromatid exchanges in circulating leukocytes from cigarette smokers. *Am. Rev. Respir. Dis.* **1990**, *142*, 648–654. [CrossRef] [PubMed]
31. Kramer, T.R.; Schoene, N.; Douglass, L.W.; Judd, J.T.; Ballard-Barbash, R.; Taylor, P.R.; Bhagavan, H.N.; Nair, P.P. Increased vitamin E intake restores fish-oil-induced suppressed blastogenesis of mitogen-stimulated T lymphocytes. *Am. J. Clin. Nutr.* **1991**, *54*, 896–902. [CrossRef] [PubMed]
32. Wu, D.; Han, S.N.; Meydani, M.; Meydani, S.N. Effect of concomitant consumption of fish oil and vitamin E on T cell mediated function in the elderly: A randomized double-blind trial. *J. Am. Coll. Nutr.* **2006**, *25*, 300–306. [CrossRef] [PubMed]
33. Cannon, J.G.; Meydani, S.N.; Fielding, R.A.; Fiatarone, M.A.; Meydani, M.; Farhangmehr, M.; Orencole, S.F.; Blumberg, J.B.; Evans, W.J. Acute phase response in exercise. II. Associations between vitamin E, cytokines, and muscle proteolysis. *Am. J. Physiol.* **1991**, *260*, 1235–1240. [CrossRef] [PubMed]
34. Pierpaoli, E.; Orlando, F.; Cirioni, O.; Simonetti, O.; Giacometti, A.; Provinciali, M. Supplementation with tocotrienols from Bixa orellana improves the in vivo efficacy of daptomycin against methicillin-resistant Staphylococcus aureus in a mouse model of infected wound. *Phytomedicine* **2017**, *36*, 50–53. [CrossRef] [PubMed]
35. Bou Ghanem, E.N.; Clark, S.; Du, X.; Wu, D.; Camilli, A.; Leong, J.M.; Meydani, S.N. The α-tocopherol form of vitamin E reverses age-associated susceptibility to streptococcus pneumoniae lung infection by modulating pulmonary neutrophil recruitment. *J. Immunol.* **2015**, *194*, 1090–1099. [CrossRef] [PubMed]
36. De Wolf, B.M.; Zajac, A.M.; Hoffer, K.A.; Sartini, B.L.; Bowdridge, S.; LaRoith, T.; Petersson, K.H. The effect of vitamin E supplementation on an experimental Haemonchus contortus infection in lambs. *Vet. Parasitol.* **2014**, *205*, 140–149. [CrossRef] [PubMed]
37. Sheridan, P.A.; Beck, M.A. The immune response to herpes simplex virus encephalitis in mice is modulated by dietary vitamin E. *J. Nutr.* **2008**, *138*, 130–137. [CrossRef] [PubMed]
38. Wang, Y.; Huang, D.S.; Eskelson, C.D.; Watson, R.R. Long-Term Dietary Vitamin E Retards Development of Retrovirus-Induced Disregulation in Cytokine Production. *Clin. Immunol. Immunopathol.* **1994**, *72*, 70–75. [CrossRef] [PubMed]
39. Reddy, P.G.; Morrill, J.L.; Minocha, H.C.; Morrill, M.B.; Dayton, A.D.; Frey, R.A. Effect of supplemental vitamin E on the immune system of calves. *J. Dairy Sci.* **1986**, *69*, 164–171. [CrossRef]
40. Fang, C.H.; Peck, M.D.; Alexander, J.W.; Babcock, G.F.; Warden, G.D. The effect of free radical scavengers on outcome after infection in burned mice. *J. Trauma.* **1990**, *30*, 453–456. [CrossRef] [PubMed]
41. Watson, R.; Petro, T.M. Cellular immune response, corticosteroid levels and resistance to Listeria monocytogenes and murine leukemia in mice fed a high vitamin E diet. *N.Y. Acad. Sci.* **1982**, *393*, 205–210. [CrossRef]
42. Tvedten, H.W.; Whitehair, C.K.; Langham, R.F. Influence of vitamins A and E on gnotobiotic and conventionally maintained rats exposed to Mycoplasma pulmonis. *J. Am. Vet. Med. Assoc.* **1973**, *163*, 605–612. [PubMed]
43. Stephens, L.C.; McChesney, A.E.; Nockels, C.F. Improved recovery of vitamin E-treated lambs that have been experimentally infected with intratracheal Chlamydia. *Br. Vet. J.* **1979**, *135*, 291–293. [CrossRef]
44. Schildknecht, E.G.; Squibb, R.L. The effect of vitamins A, E and K on experimentally induced histomoniasis in turkeys. *Parasitology* **1979**, *78*, 19–31. [CrossRef] [PubMed]
45. Teige, J.; Tollersrud, S.; Lund, A.; Larsen, H.J. Swine dysentery: The influence of dietary vitamin E and selenium on the clinical and pathological effects of Treponema hyodysenteriae infection in pigs. *Res. Vet. Sci.* **1982**, *32*, 95–100. [PubMed]
46. Tengerdy, R.P.; Meyer, D.L.; Lauerman, L.H.; Lueker, D.C.; Nockels, C.F. Vitamin E-enhanced humoral antibody response to Clostridium perfringens type D in sheep. *Br. Vet. J.* **1983**, *139*, 147–152. [CrossRef]

47. Smith, K.L.; Harrison, J.H.; Hancock, D.D.; Todhunter, D.A.; Conrad, H.R. Effect of vitamin E and selenium supplementation on incidence of clinical mastitis and duration of clinical symptoms. *J. Dairy Sci.* **1984**, *67*, 1293–1300. [CrossRef]
48. Heinzerling, R.H.; Nockels, C.F.; Quarles, C.L.; Tengerdy, R.P. Protection of chicks against *E.coli* infection by dietary supplementation with vitamin E. *Proc. Soc. Exp. Biol. Med.* **1974**, *146*, 279–283. [CrossRef] [PubMed]
49. Tengerdy, R.P.; Nockels, C.F. Vitamin E or vitamin A protects chickens against E. coli infection. *Poult. Sci.* **1975**, *54*, 1292–1296. [CrossRef] [PubMed]
50. Likoff, R.O.; Guptill, D.R.; Lawrence, L.M.; McKay, C.C.; Mathias, M.M.; Nockels, C.F.; Tengerdy, R.P. Vitamin E and aspirin depress prostaglandins in protection of chickens against Escherichia coli infection. *Am. J. Clin. Nutr.* **1981**, *34*, 245–251. [CrossRef] [PubMed]
51. Ellis, R.P.; Vorhies, M.W. Effect of supplemental dietary vitamin E on the serologic response of swine to an Escherichia coli bacterin. *J. Am. Vet. Med. Assoc.* **1976**, *168*, 231–232. [PubMed]
52. Hemilä, H. Vitamin E administration may decrease the incidence of pneumonia in elderly males. *Clin. Interv. Aging* **2016**, *11*, 1379–1385. [CrossRef] [PubMed]
53. Olofin, I.O.; Spiegelman, D.; Aboud, S.; Duggan, C.; Danaei, G.; Fawzi, W.W. Supplementation with multivitamins and vitamin A and incidence of malaria among HIV-infected Tanzanian women. *J. Acquir. Immune Defic. Syndr.* **2014**, *67* (Suppl. S4), S173–S178. [CrossRef] [PubMed]
54. Marotta, F.; Yoshida, C.; Barreto, R.; Naito, Y.; Packer, L. Oxidative-inflammatory damage in cirrhosis: Effect of vitamin E and a fermented papaya preparation. *J. Gastroenterol. Hepatol.* **2007**, *22*, 697–703. [CrossRef] [PubMed]
55. Groenbaek, K.; Friis, H.; Hansen, M.; Ring-Larsen, H.; Krarup, H.B. The effect of antioxidant supplementation on hepatitis C viral load, transaminases and oxidative status: A randomized trial among chronic hepatitis C virus-infected patients. *Eur. J. Gastroenterol. Hepatol.* **2006**, *18*, 985–989. [CrossRef] [PubMed]
56. Meydani, S.N.; Leka, L.S.; Fine, B.C.; Dallal, G.E.; Keusch, G.T.; Singh, M.F.; Hamer, D.H. Vitamin E and respiratory tract infections in elderly nursing home residents: A randomized controlled trial. *JAMA* **2004**, *292*, 828–836. [CrossRef] [PubMed]
57. Hemilä, H.; Kaprio, J.; Albanes, D.; Heinonen, O.P.; Virtamo, J. Vitamin C, vitamin E, and beta-carotene in relation to common cold incidence in male smokers. *Epidemiology* **2002**, *13*, 32–37. [CrossRef] [PubMed]
58. Hemilä, H.; Virtamo, J.; Albanes, D.; Kaprio, J. Vitamin E and beta-carotene supplementation and hospital-treated pneumonia incidence in male smokers. *Chest* **2004**, *125*, 557–565. [CrossRef] [PubMed]
59. Graat, J.M.; Schouten, E.G.; Kok, F.J. Effect of daily vitamin E and multivitamin-mineral supplementation on acute respiratory tract infections in elderly persons: A randomized controlled trial. *JAMA* **2002**, *288*, 715–721. [CrossRef] [PubMed]
60. Mosser, D.M.; Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **2008**, *8*, 958–969. [CrossRef] [PubMed]
61. Meydani, S.N.; Han, S.N.; Wu, D. Vitamin E and immune response in the aged: Molecular mechanisms and clinical implications. *Immunol. Rev.* **2005**, *205*, 269–284. [CrossRef] [PubMed]
62. Beharka, A.A.; We, D.; Han, S.N.; Meydani, S.N. Macrophage prostaglandin production contributes to the age-associated decrease in T cell function which is reversed by the dietary antioxidant vitamin E. *Mech. Age. Dev.* **1997**, *93*, 59–77. [CrossRef]
63. Hayek, M.G.; Mura, C.; Wu, D.; Beharka, A.A.; Han, S.N.; Paulson, K.E.; Hwang, D.; Meydani, S.N. Enhanced expression of inducible cyclooxygenase with age in murine macrophages. *J. Immunol.* **1997**, *159*, 2445–2451. [PubMed]
64. Wu, D.; Mura, C.; Beharka, A.A.; Han, S.N.; Paulson, K.E.; Hwang, D.; Meydani, S.N. Age-associated increase in PGE2 synthesis and COX activity in murine macrophages is reversed by vitamin E. *Am. J. Physiol.* **1998**, *275*, C661–C668. [CrossRef] [PubMed]
65. Beharka, A.A.; Wu, D.; Serafini, M.; Meydani, S.N. Mechanism of vitamin E inhibition of cyclooxygenase activity in macrophages from old mice: Role of peroxynitrite. *Free. Radic. Biol. Med.* **2002**, *32*, 503–511. [CrossRef]
66. Sorokin, A. Nitric Oxide Synthase and Cyclooxygenase Pathways: A Complex Interplay in Cellular Signaling. *Curr. Med. Chem.* **2016**, *23*, 2559–2578. [CrossRef] [PubMed]

67. Dworski, R.; Han, W.; Blackwell, T.S.; Hoskins, A.; Freeman, M.L. Vitamin E prevents NRF2 suppression by allergens in asthmatic alveolar macrophages in vivo. *Free Radic. Biol. Med.* **2011**, *51*, 516–521. [CrossRef] [PubMed]
68. Adachi, N.; Migita, M.; Ohta, T.; Higashi, A.; Matsuda, I. Depressed natural killer cell activity due to decreased natural killer cell population in a vitamin E-deficient patient with Shwachman syndrome: Reversible natural killer cell abnormality by alpha-tocopherol supplementation. *Eur. J. Pediatr.* **1997**, *156*, 444–448. [CrossRef] [PubMed]
69. Ravaglia, G.; Forti, P.; Maioli, F.; Bastagli, L.; Facchini, A.; Mariani, E.; Savarino, L.; Sassi, S.; Cucinotta, D.; Lenaz, G. Effect of micronutrient status on natural killer cell immune function in healthy free-living subjects aged ≥90 y. *Am. J. Clin. Nutr.* **2000**, *71*, 590–598. [CrossRef] [PubMed]
70. Hanson, M.G.; Ozenci, V.; Carlsten, M.C.; Glimelius, B.L.; Frödin, J.E.; Masucci, G.; Malmberg, K.J.; Kiessling, R.V. A short-term dietary supplementation with high doses of vitamin E increases NK cell cytolytic activity in advanced colorectal cancer patients. *Cancer Immunol. Immunother.* **2007**, *56*, 973–984. [CrossRef] [PubMed]
71. Stiff, A.; Trikha, P.; Mundy-Bosse, B.; McMichael, E.; Mace, T.A.; Benner, B.; Kendra, K.; Campbell, A.; Gautam, S.; Abood, D.; et al. Nitric Oxide Production by Myeloid-Derived Suppressor Cells Plays a Role in Impairing Fc Receptor-Mediated Natural Killer Cell Function. *Clin. Cancer Res.* **2018**, *24*, 1891–1904. [CrossRef] [PubMed]
72. Ganguly, D.; Haak, S.; Sisirak, V.; Reizis, B. The role of dendritic cells in autoimmunity. *Nat. Rev. Immunol.* **2013**, *13*, 566–577. [CrossRef] [PubMed]
73. Pearce, E.J.; Everts, B. Dendritic cell metabolism. *Nat. Rev. Immunol.* **2015**, *15*, 18–29. [CrossRef] [PubMed]
74. Alloatti, A.; Kotsias, F.; Magalhaes, J.G.; Amigorena, S. Dendritic cell maturation and cross-presentation: Timing matters! *Immunol. Rev.* **2016**, *272*, 97–108. [CrossRef] [PubMed]
75. Tan, P.H.; Sagoo, P.; Chan, C.; Yates, J.B.; Campbell, J.; Beutelspacher, S.C.; Foxwell, B.M.; Lombardi, G.; George, A.J. Inhibition of NF-kappa B and oxidative pathways in human dendritic cells by antioxidative vitamins generates regulatory T cells. *J. Immunol.* **2005**, *174*, 7633–7644. [CrossRef] [PubMed]
76. Xuan, N.T.; Trang, P.T.; Van Phong, N.; Toan, N.L.; Trung, D.M.; Bac, N.D.; Nguyen, V.L.; Hoang, N.H.; van Hai, N. Klotho sensitive regulation of dendritic cell functions by vitamin E. *Biol. Res.* **2016**, *49*, 45. [CrossRef] [PubMed]
77. Buendía, P.; Ramírez, R.; Aljama, P.; Carracedo, J. Klotho Prevents Translocation of NFκB. *Vitam. Horm.* **2016**, *101*, 119–150. [PubMed]
78. Abdala-Valencia, H.; Berdnikovs, S.; Soveg, F.W.; Cook-Mills, J.M. α-Tocopherol supplementation of allergic female mice inhibits development of CD11c+CD11b+ dendritic cells in utero and allergic inflammation in neonates. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2014**, *307*, L482–L496. [CrossRef] [PubMed]
79. Abdala-Valencia, H.; Soveg, F.; Cook-Mills, J.M. γ-Tocopherol supplementation of allergic female mice augments development of CD11c+CD11b+ dendritic cells in utero and allergic inflammation in neonates. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2016**, *310*, L759–L771. [CrossRef] [PubMed]
80. Molano, A.; Meydani, S.N. Vitamin E, signalosomes and gene expression in T cells. *Mol. Aspects. Med.* **2012**, *33*, 55–62. [CrossRef] [PubMed]
81. Adolfsson, O.; Huber, B.T.; Meydani, S.N. Vitamin E-enhanced IL-2 production in old mice: Naive but not memory T cells show increased cell division cycling and IL-2-producing capacity. *J. Immunol.* **2001**, *167*, 3809–3817. [CrossRef] [PubMed]
82. Marko, M.G.; Ahmed, T.; Bunnell, S.C.; Wu, D.; Chung, H.; Huber, B.T.; Meydani, S.N. Age-associated decline in effective immune synapse formation of CD4(+) T cells is reversed by vitamin E supplementation. *J. Immunol.* **2007**, *178*, 1443–1449. [CrossRef] [PubMed]
83. Marko, M.G.; Pang, H.J.; Ren, Z.; Azzi, A.; Huber, B.T.; Bunnell, S.C.; Meydani, S.N. Vitamin E reverses impaired linker for activation of T cells activation in T cells from aged C57BL/6 mice. *J. Nutr.* **2009**, *139*, 1192–1197. [CrossRef] [PubMed]
84. Han, S.N.; Adolfsson, O.; Lee, C.K.; Prolla, T.A.; Ordovas, J.; Meydani, S.N. Age and vitamin E-induced changes in gene expression profiles of T cells. *J. Immunol.* **2006**, *177*, 6052–6061. [CrossRef] [PubMed]
85. Pelizon, C. Down to the origin: Cdc6 protein and the competence to replicate. *Trends Cell Biol.* **2003**, *13*, 110–113. [CrossRef]

86. Li-Weber, M.; Giaisi, M.; Treiber, M.K.; Krammer, P.H. Vitamin E inhibits IL-4 gene expression in peripheral blood T cells. *Eur. J. Immunol.* **2002**, *32*, 2401–2408. [CrossRef]
87. Malmberg, K.J.; Lenkei, R.; Petersson, M.; Ohlum, T.; Ichihara, F.; Glimelius, B.; Frödin, J.E.; Masucci, G. A short-term dietary supplementation of high doses of vitamin E increases T helper 1 cytokine production in patients with advanced colorectal cancer. *Clin. Cancer Res.* **2002**, *8*, 1772–1778. [PubMed]
88. Li-Weber, M.; Weigand, M.A.; Giaisi, M.; Süss, D.; Treiber, M.K.; Baumann, S.; Ritsou, E.; Breitkreutz, R.; Krammer, P.H. Vitamin E inhibits CD95 ligand expression and protects T cells from activation-induced cell death. *J. Clin. Invest.* **2002**, *110*, 681–690. [CrossRef] [PubMed]
89. Neuzil, J.; Svensson, I.; Weber, T.; Weber, C.; Brunk, U.T. α-tocopheryl succinate-induced apoptosis in Jurkat T cells involves caspase-3 activation, and both lysosomal and mitochondrial destabilisation. *FEBS Lett.* **1999**, *445*, 295–300. [CrossRef]