Zinc-based nutritional immunity to lower inflammation, viral load and COVID-19 mortality during SARS-CoV-2 infection.

PRINCIPAL INVESTIGATOR COORDINATOR: Robert Güerri-Fernández M.D. Ph.D. & Silvia Gómez-Zorrilla M.D.Ph.D.

PRINCIPAL INVESTIGATOR SUBPROJECT 1: Rubén Vicente Ph.D.

Version: Version 1.0 (29 march 2021)

SPONSOR: Consorci Mar Parc de Salut de Barcelona, con domicilio en Passeig Marítim 25-29, 08003 de Barcelona. C.I.F S-080047

Principal Investigator: Robert Güerri-Fernández M.D. Ph.D.
Edificio Hospital del Mar. Servicio de Enfermedades Infecciosas**.** Paseo Marítimo 25-29, 08003, Barcelona
Phone number: +34 93 248 3468 / +3493 248 3251
Fax: +34 93 248 3257
E-mail**:** rguerri@psmar.cat

ABSTRACT:

Background: The essential micronutrient zinc balances immune responses to infections and additionally directly inhibits some viruses. We have recently shown a robust correlation between serum zinc levels (SZL)and COVID-19 outcome in patients and a direct effect of zinc levels on SARS-CoV-2 expansion in cell culture.These results suggest that SZL might represent an important risk factor for COVID-19 severity whose adjustment would constitute an early and cost-effective therapeutic intervention.

Objectives: To explore the therapeutic benefit of zinc supplementation for COVID-19 patients and to determine the cellular and molecular basis of the effect of zinc levels on SARS-CoV-2 infections.

Methodology: A randomized clinical trial supplementing COVID-19 patients with zinc will be carried out and viral loads, inflammatory and novel zinc-related clinical markers and SARS-CoV-2-specific T- and B-cell Responses monitored. Humanized-ACE2-mice models will be used to address causal-effect associations between zinc levels and SARS-CoV-2 infection. Infections of human Calu-3 cells will be used to decipher the direct antiviral action of zinc on SARS-CoV-2 life cycle.

Expected results: We will generate a clear insight into zinc functioning in COVID-19 patients that might provide a therapeutic guideline immediately applicable to reduce the severity of SARS-CoV-2 pathogenicity and possibly other virus infections.

LAY SUMMARY:

Infections with SARS-CoV-2 result in a systemic disease with a variety of outcomes, from no symptoms to severe and diverse pathologies. Therefore, it is important to identify risk factors determining COVID-19 severity, especially if those factors might be adjusted, allowing early and effective therapeutic interventions.

Zinc is a trace element essential for human health. Zinc deficiency is common in old adults, vegetarians and patients with chronic inflammatory diseases. This condition causes immune dysfunction leading to increased risk of inflammatory and infectious diseases, including acquired immune deficiency syndrome, measles, malaria, tuberculosis, and pneumonia. Besides, zinc has a direct antiviral activity against specific viruses like rhinovirus, HCV, herpes simplex virus. In this scenario, it has been shown that zinc supplementation has benefits on the recurrence and persistence of acute and chronic viral infections like common cold or HCV, HBV. Moreover, our team has recently done an observational study with 249 COVID-19 patients that showed how COVID-19 patients with lower plasma zinc content had worse prognosis, increased time of hospitalization and mortality.

Therefore, the main aim of the project is to explore the therapeutic benefit of zinc supplementation for COVID-19 patients and to determine the cellular and molecular basis of the effect of Zn levels on SARS CoV-2 infections. For that purpose we will run a clinical trial supplementing with zinc COVID-19 patients. Moreover, we will carry out experiments to understand the association between zinc nutritional status and SARS-Cov-2 infection progression in cellular and animal models.

Given the current knowledge about zinc supplementation toxicity and dosage, we expect that recommendations derived from this study will be rapidly applied by physicians and public health decision makers. The results of these studies will be used as a guideline to administer zinc supplements in COVID-19 patients in order to reduce disease severity and mortality. Moreover, our experiments will clarify whether zinc supplementation as a prophylaxis strategy is useful to protect the population at risk of zinc deficiency, more than 20% worldwide.

Finally, considering the new knowledge that this project will generate about the role of zinc in immune responses and viral expansion, we expect that our results will help researchers and physicians to design novel strategies to boost specific immune cell subpopulations against SARS-CoV2 infection. Thus, this knowledge could be used long-term for designing medicines against SARS-CoV-2 and other viral infections.

- **HYPOTHESIS AND DESCRIPTION OF THE PROJECT OBJECTIVES**

We hypothesize that low zinc nutritional status has an impact on COVID-19 progression and that adjustment of this status might constitute an early therapeutic intervention point by balancing immune responses and acting as an antiviral agent. This hypothesis is supported by the following observations:

-Zinc nutritional status impacts on innate and adaptive immunity and influences inflammatory markers(4).

-Zinc has a direct antiviral activity against specific viruses like rhinovirus, HCV, SARS-CoV(16).

-Zinc supplementation has benefits on the recurrence and persistence of acute and chronic viral infections like common cold, or HCV, HBV (2,6) and reconstitutes the production of interferon-alpha from elderly persons(16).

-Our recent data in a cohort of 249 COVID-19 patients show that COVID-19 patients with lower plasma zinc content have worse prognosis, increased time of hospitalization and mortality and that Zn and IL-6 levels are negatively correlated (13). However, the cause-effect association between nutritional zinc status and COVID-19 inflammatory response remains unproven,

-Our recent data in cell culture studies show that low zinc levels correlate with higher SARS-CoV- 2 expansion(13) .

The main aim of the project is to explore the therapeutic benefit of Zn supplementation for COVID-19 patients and to determine the cellular and molecular basis of the effect of Zn levels on SARS CoV-2 infections. To address these questions, we propose the following aims:

- **To study the impact of zinc supplementation on COVID-19 progression. There are two specific objectives:**
- **To carry out the clinical trial with COVID-19 patients (Subproject 1):**

We will run a randomized clinical trial with 60 COVID-19 patients admitted at Hospital del Mar with a control and a zinc supplementation group.

- **To study the immune responses of the COVID-19 patients enrolled in the clinical trial (Subproject 2):** Viral loads, zinc homeostasis markers and T- and B-cell populations in PBMC from COVID-19 patients in control and zinc supplemented groups will be determined. Disease progression and clinical outcomes will be evaluated.

- **To establish causal-effect associations between zinc levels and SARS-CoV-2 infections (Subproject 3):** We will monitor viral expansion and immune responses in SARS-CoV2 infected

hACE2-mice with different zinc nutritional status (i) to evaluate whether SARS-Cov-2 induces changes in SZL and (ii) to study prophylaxis and therapeutic properties of zinc supplementation.

- **To characterize novel zinc homeostasis reporters upon SARS-CoV2 infection (Subproject 2)** We will characterize the expression of zinc transporters and metallothioneins in PBMC from COVID-19 patients and mice infected with SARS-CoV-2.

- **To decipher the direct antiviral action of Zn on SARS-CoV-2 life cycle steps (Subproject 2)**Infections of human Calu-3 cells will be used to decipher the direct antiviral action of Zn on SARS-CoV-2 life cycle steps. Moreover, zinc homeostatic machinery will be evaluated upon infection.

2. BACKGROUND AND STATE OF THE ART, INCLUDING RELEVANT BIBLIOGRAPHY

Zinc is a trace element essential for human health. Zinc deficiency (ZD) causes impaired body growth, neurological disorders, and immunosuppression leading to morbidity and increased risk of inflammatory and infectious diseases including acquired immune deficiency syndrome, measles, malaria, tuberculosis, and pneumonia (1,2). The human body contains 2–3 g of zinc, 0.1% of which are exchanged daily. Due to the lack of major storage organs of this mineral, the body needs a continuous nutritional supply. Zinc deficiency is considered a major public health problem in developing countries, especially in groups with low zinc intake as well as those with inhibited absorption due to high levels of phytates and fiber in vegetable-based diets. Besides, old adults, vegetarians, and people with gastrointestinal disorders are also considered to be at risk

for hypozincemia(1, 2). Globally, one third of the world population is at risk of mild ZD associated disorders. In the elderly, the incidence ranges from 15 to 31% depending on the age and the country of study (3). Remarkably, older adults are the group at higher risk for severe symptoms and mortality from COVID-19. In this context, ZD might be one of the important underlying factors of COVID-19 heterogeneity.

Cellular zinc homeostasis

Zinc is a structural component of up to 10% of the proteome. Moreover, zinc participates as a second messenger in cell signalling. Zinc binding proteins and 24 zinc transporters work in a coordinated way to tightly regulate cytosolic zinc fluxes. There are two main families of zinc transporters, ZnT and Zip, which extrudes and enters zinc in the cytosol

respectively (1). Within each zinc transporter family, the different members vary in cellular localization, affinity, selectivity, regulation and tissue expression. The expression and function of specific transporters is essential for the function and modulation of immune cells of adaptive and innate immunity (4).

The concentration of free zinc in the cytosol is not only determined by the activity of all these transporters but also by the quenching effects exerted by metallothioneins (MTs), abundant cytosolic proteins that quench metal ions, mainly zinc. These proteins, by releasing zinc, participate actively in the defense against reactive oxygen species (ROS). In humans, there are 4 MTs. Interestingly, the cellular expression of MTs is stimulated by

increases in cytosolic zinc content. In fact, one of the most reliable tests to measure the body zinc content is by measuring MTs expression in leukocytes (5).

Zinc and immune cells

Early studies of zinc deficiency showed that zinc is essential for immune system function (4). Due to its importance for proper immune function, zinc supplementation and fortification programs have been widely carried out for boosting the immune system in populations at risk of ZD. Several clinical trials have proven that zinc supplementation is effective in certain types of viral and bacterial infections (reviewed in (2,6)).

Zinc and inflammation

ZD is known to be associated with proinflammatory responses at infection, showing higher ROS production and inflammatory markers (6,7). An imbalance in cytokine production by cells of both innate and adaptive immunity has also been reported.

Concretely, ZD is associated with higher levels of IL-6, IL-1beta and TNF-alfa (6). In this scenario, zinc supplementation has been shown to decrease the incidence of infection, inflammatory cytokines, including IL-6, and oxidative stress markers in elderly individuals (7,8). Moreover, zinc supplementation has been shown to balance immune responses (9). Remarkably, IL-6 is a key player in COVID-19 patients by triggering severe lung damage during SARS-CoV-2 infection.

Sustained IL-6 promotes immune-mediated lung damage and macrophage activation syndrome (10). Nutritional zinc status and IL-6 are highly interconnected. Thus, IL-6 can reduce zinc bioavailability promoting internalization in hepatocytes and the expression of zinc chelators (11). On the other hand, zinc decreases IL-6 production via inhibition of STAT-3 pathway (12). Our study with COVID-19 patients showed a negative correlation between serum zinc levels and IL-6 production (13). Both parameters were robustly associated with COVID-19 progression. This data is in agreement by another observational study with a smaller cohort (14). However the cause-effect association between zinc status and inflammation markers in COVID-19 patients is not yet clear given the observational nature of the studies (13,14).

Zinc in viral infection

Zinc is an essential trace element for both host and pathogens. In order to grow, pathogens require zinc. Thus, zinc is a structural cofactor of viral proteins and certain viruses have developed strategies to alter cellular zinc homeostasis on its benefit (15). On the other hand, there is extensive evidence that zinc can prevent viral replication and lower cellular invasion (eg. Rhinovirus, HCV, SARS-CoV) (reviewed in 6, 16).

Remarkably, our team has shown that low zinc levels favour SARS-CoV2 expansion (13). These results might indicate either a direct anti-viral action against SARS-CoV2 or that the virus benefits from stress conditions caused by low levels of cellular zinc. Although there are several evidences showing that zinc deficiency leads to increased susceptibility to certain infections, supplementation of zinc in viral infections has not always been seen

as beneficial. For example, zinc supplementation in HIV patients does not improve T-cell counts or reduces viral load. In contrast, zinc supplementation has been shown to be beneficial when administering to HBV and HCV infected patients. Moreover, zinc- based nutritional immunity reduces diarrheal episodes and respiratory track diseases (reviewed in 6,16). Remarkably, zinc supplementation for the common cold caused by rhino- and coronaviruses prevents it and reduces its duration, if the administration starts within 24h after the onset of symptoms (reviewed in 6,16). Thus, zinc supplementation has been proven beneficial against some viral infections. However, whether this was caused by improved local immune response or viral inhibition remains uncertain.

IFNs are key antiviral mediators of the host's immune response. Impaired IFN responses related to aging have been associated with increased susceptibility during certain viral infections. Older adults are more susceptible than younger adults to death resulting from viral infections due to a decline in interferons production. Interferons are antiviral agents produced by the host upon infection. Importantly, zinc supplementation reconstitutes the production of interferon-alpha by leukocytes from elderly persons (17). This is an important aspect when considering zinc based nutritional immunity

because SARS-CoV-2 has been shown to antagonize proinflammatory signals during early infection, particularly type I IFN (IFN-I) signaling. In fact, errors of immunity that diminish IFN-I activity are more commonly detected in patients with severe COVID-19 (18).

Zinc in T lymphocytes activation and exhaustion

Several studies have indicated that T cells have a role both in the early immune response against SARS-CoV-2 and that a functional memory is generated (19). In this context, zinc is known to affect T lymphocyte maturation, differentiation and cytokine production (2,4,7). Activated T cells are known to increase zinc content (20). On the one hand, zinc has been shown to be required for the correct TCR signalling by modulating Lck and Zap-70 activity (20). Our team has demonstrated that zinc positively potentiates the three main signalling pathways, AP-1, NF-kB and NFAT1 (21). In addition we have described that the knock down of Zip6, a zinc transporter up regulated early during cell activation, alters zinc entry and dramatically impairs activation of Jurkat T cells (21). Moreover, recent studies on CD8+ T cell dysfunction during cancer and chronic infections have started to elucidate the transcriptional pathways involved in this phenomenon and zinc homeostasis has emerged again as a key element. Thus, metallothioneins 1 and 2 levels and zinc dependent transcription factors are differentially expressed in exhausted CD8+ cells (22,23,24). Nonetheless, there are no studies investigating whether zinc supplementation/chelation might directly affect the CD8+ dysfunctional profile and how these learnings could be translated into novel therapeutic approaches for these diseases including the current COVID-19 pandemic.

Zinc in B lymphocyte function

B lymphocytes are key players for an effective immune response against SARS-CoV2. Antibodies against the spike and nucleocapsid proteins of the virus are detectable from approximately 6 days after PCR confirmation of infection. A decline of anti-SARS-CoV-2

serum IgG levels occurs beyond 20 days post-diagnosis and it is still unclear how long immunity to SARS-CoV-2 lasts after recovery from infection. Importantly, it has been shown that zinc transporters (Zip7 and Zip10) are essential in B cell function. B cells activation and plasma cell differentiation depends on zinc signalling (25,26). Nutritional zinc status is an important factor determining antibody production given its action on both, B-cells and CD4+ T lymphocytes. Thus, ZD has been shown in mouse models to lower humoral immune response (26).

In summary, there are several cross talks between zinc homeostasis and immune function. Moreover zinc has an antiviral activation against certain viruses. All these connections together with the association found between low plasma zinc levels and COVID-19 severity suggest that zinc supplementation might be an important therapeutic tool in COVID-19 patients. A clinical trial based on zinc nutritional immunity together with a deeper knowledge of the impact of zinc supplementation in immune cells will be useful for the current COVID-19 pandemic and other infection diseases

REFERENCES:

- Kambe T, et al.(2015) The Physiological, Biochemical, and Molecular Roles of Zinc Transporters in Zinc Homeostasis and Metabolism. *Physiol. Rev.* 95: 749–784.
- Gammoh,NZ, et al.(2017) Zinc in Infection and Inflammation. *Nutrients* 9: 624.
- Yasuda H, et al.(2016) Infants and elderlies are susceptible to zinc deficiency. *Sci Rep* 2016;6:21850

• Haase H, et al.(2009) Functional significance of zinc-related signaling pathways in immune cells. *Annu. Rev. Nutr.* 29: 133–52.

• Hennigar SR, et al.(2016) Metallothionein and Zinc Transporter Expression in Circulating Human Blood Cells as Biomarkers of Zinc Status: a Systematic Review. *Adv Nutr*. 7:735-46.

• Overbeck, S. et al.(2008) Modulating the immune response by oral zinc supplementation: a single approach for multiple diseases. *Arch. Immunol. Ther. Exp. (Warsz).* 56: 15–30.

• Prasad AS et al.(2007) 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.

• Bao B, et al.(2010) Zinc decreases C-reactive protein, lipid peroxidation, and inflammatory cytokines in elderly subjects: a potential implication of zinc as an atheroprotective agent. *Am J Clin Nutr* 2010;91:1634–41.

• Rosenkranz, et al.(2016) Induction of regulatory T cells in Th1-/Th17-driven experimental autoimmune encephalomyelitis by zinc administration. *J. Nutr. Biochem.* 29: 116–123.

• Beck FW, et al.(1997) Changes in cytokine production and T cell subpopulations in experimentally induced zinc-deficient humans. *Am. J. Physiol.* 272: E1002-7.

- McGonagle D, et al.(2020) The role of cytokines including Interleukin-6 in COVID-19 induced pneumonia and macrophage activation syndrome-like disease. *Autoimmun Rev.* 19:102537.
- Liuzzi JP, et al.(2005) Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. *PNAS*, 102:6843–8
- Liu MJ, et al.(2014) Zinc regulates the acute phase response and serum amyloid A production in response to sepsis through JAK-STAT3 signaling. *PLoS ONE*. 9:e94934.
- Vogel-Gonzalez M, et al.(2020) Low serum zinc levels are associated with poor COVID-19 outcome. MedRxiv **doi:** https://doi.org/10.1101/2020.10.07.20208645
- Lazarczyk M, et al.(2008) Role of Zn2+ ions in host-virus interactions. *J Virol*. 82:11486-94.
- Read SA, et al.(2019) The Role of Zinc in Antiviral Immunity. *Adv Nutr* 2019;10:696–710.
- Cakman I, et al.(1997) Zinc supplementation reconstitutes the production of interferon-alpha by leukocytes from elderly persons. *J. Interferon Cytokine Res.* 17, 469–472.
- Hadjadj J, et al.(2020) Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients. *Science* 369, 718–724.
- Karlsson AC, et al.(2020) *Sci. Immunol*. 10.1126/sciimmunol.abe8063.
- Yu M. et al.(2011). Regulation of T cell receptor signaling by activation-induced zinc influx. *J. Exp. Med.*208: 775–85.

- Colomar-Carando N, et al.(2019). Zip6 transporter is an essential component of the lymphocyte activation machinery. *J. Immunol* 202(2):441-450
- Singer M, et al.(2016). Distinct Gene Module for Dysfunction Uncoupled from Activation in Tumor-Infiltrating T Cells. *Cell*. 166:1500-1511.
- Doering TA, et al.(2012) Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. *Immunity*. 37:1130-44.
- Dominguez CX, et al.(2015) The transcription factors ZEB2 and T-bet cooperate to program cytotoxic T cell terminal differentiation in response to LCMV viral infection. *J Exp Med*. 212:2041-56.
- Anzilotti C, et al.(2019). An essential role for the Zn2+ transporter ZIP7 in B cell development *Nature Immunology* 20, 350–361
- Hojyo S, et al.(2014) Zinc transporter SLC39A10/ZIP10 controls humoral immunity by modulating B-cell receptor signal strength *PNAS* 111, 11786-11791

3. PRELIMINARY RESULTS:

Our preliminary results are shown in the work entitled "**Low zinc levels at clinical admission associates with poor outcomes in COVID-19"** (13) which is the result of a tight collaboration between the laboratory of Roberto Güerri (Hospital del Mar) and those of Rubén Vicente and Juana Díez (both at DCEXS, UPF).

This work aimed to focus clinical attention on serum zinc content (SZC) in COVID-19 patients. Our analysis showed a robust correlation between low SZC with COVID-19

severity and mortality. The cause is likely to be a combination of immune system imbalance and a direct benefit of viral replication.

A summary of the results obtained:

- In our retrospective observational study with 249 COVID-19 patients, **23% of the patients had at admission SZC lower than 50µg/dl**, the cutoff associated with ZD and development of clinical signs.
- Our study showed that **low SZC correlates with higher IL-6 and C-reactive protein at admission**, higher peak IL-6 during the episode.
- We detected in the low SZC group that subjects needed higher time to recover (25 days) versus the control group (8 days). In the same direction, the **mortality in low SZC patients was higher (21%) than in the control group (5%)** with normal zinc concentration.
- **SARS-CoV2** Infections in cell culture demonstrated that **low zinc levels favors SARS-CoV2 viral expansion** in the infected cells.

These results highlighted the urgent need to start clinical trials supplementing with zinc low SZC patients at admission to reestablish zinc homeostasis in order to study the cause-effect association between zinc nutritional status and COVID-19 progression. We expect with this new project that zinc supplementation in low SZC COVID-19 patients will

balance what has been called the cytokine storm caused by SARS-CoV-2 and as a consequence will reduce COVID-19 severity and mortality.

METHODOLOGY:

The methodology is divided into objectives, subobjectives and tasks. Expected outcomes (EO), deliverables (D) and limitations are described for each task.

- **To study the impact of zinc supplementation on COVID-19 progression.**

- **To carry out the clinical trial with COVID-19 patients (Subproject**

1) Clinical trial objectives

Primary objective

To evaluate the effectiveness of oral zinc supplementation (83.91 mg zinc acetate) Zinc+NM QD (~~80mg Zn2+) QD as compared to placebo~~ as compared to control group without zinc supplementation to reduce IL-6 increase in the course of SARS-CoV-2 infection, to decrease days to clinical recovery and to decrease the percentage of people with COVID-19 who have disease progression by Week 2, defined as inpatients, worsening of ≥1 level on the WHO 9-point ordinal scale

Secondary Objectives

To evaluate the effects of zinc supplementation QD on fever, dyspnea, diarrhea, and clinical parameters of disease, and time to worsening clinical status.

Study Endpoints

Primary Endpoints

- Number of days since admissions to clinical recovery
- Change in IL-6 levels from baseline to Week 2.
- Percentage of patients with disease progression by Week 2 based on WHO 9-point ordinal scale

Secondary Endpoints

- Change in Covid-19 signs/symptoms: dyspnea, diarrhea, temperature, neutrophil count... at Week 2

Safety Endpoints

- Incidence of AEs and serious adverse events

Study Design

This is a randomized, open-label clinical study evaluating the effectiveness of zinc supplementation as compared to standard of care on need disease severity and on IL-6

levels in people with COVID-19. Subject eligibility will be based on inclusion and exclusion criteria

Population of study: Sixty eligible adult subjects at one clinical site in Spain (Hospital del Mar) will be randomized in a 1:1 ratio to:

- ~~Zinc acetate 80mg QD~~ 83.91 mg zinc acetate (Zinc+NM) QD + standard of care for 2 weeks
- Standard of care for 2 weeks

Study phases (4 weeks):

- Screening + Randomization + Baseline Visit (Visit 1, same day as or up to 1 day before Baseline Visit)
- Treatment (Day 1 to Day 14) Starting on Day 1 (Baseline/Randomization), Randomized subjects will receive 80mg zinc for 2 weeks. Day 7 for clinical state. Day 14 follow up visit.
- Follow up phase : Day 28 for clinical state.

Inclusion Criteria

To be eligible to participate in this study, all of the following criteria must apply:

- Subject is ≥18 years of age.

- Male or female.
- Subject with diagnosis of COVID-19 based on + RNA or IgM test or compatible clinical presentation.
- Ability to take oral zinc supplementation.
- Subject or surrogate decision maker is capable of understanding and willing to sign the informed consent form.

Exclusion Criteria ~~and withdrawals are detailed in the document sent to the Ethics Committee.~~

Subjects are not permitted to enroll in the study if any of the following criteria apply:

- Female subjects who are pregnant
- Female subjects who are breast-feeding.
- Difficulty or inability to take oral medication.
- Subject is enrolled in another randomized clinical trial in the last 3 months.
- Life expectancy less than 72 hours at hospital admission.
- Conditions that in the opinion of the investigator may interfere with the subject's ability to comply with this protocol (e.g drug abuse, psychiatric disorders).

Task 1.1.1 Visits and sample collection

1.- Visit 1. Screening. Randomization. Baseline. Phase / Visit 0 or 1 (Day 1 or up to 1 day Prior to Baseline/Randomization; In Person)

Visit performed at admission (+1). Written informed consent obtained. Medical history. Vital signs assignment. Clinical labs (CBC, CMP, CRP, ferritin, D-dimer,IL-6, zinc)

2.-Visit 2 / Week 1 (Day 7 ± 2 days)

A complete record of AEs and concomitant therapies will be taken into account. 3.-

Visit 3 / Week 2 (Day 14 ± 2 days), End of Treatment

A complete vital sign assessment (sitting blood pressure, heart rate, respiratory rate, O2 sat, and oral temperature) review and record AEs and concomitant therapies, clinical labs (stated above) and PBMCs. Collect blood samples for future studies.

4.-Visit 4 / End of Study (Day 28 ± 4 days)

Clinical labs (stated above) and PBMCs. Collect blood samples for future studies.

Task 1.1.2 Assessment of Efficacy

Outcomes measured.

- Clinical recovery
    - Defined: Sat O2 (0.21) >94% or to baseline Sat O2 in case of chronic respiratory

    - Temperature <37C
    - Heart Rate <90 bpm & Systolic blood pressure >100
    - Oral tolerance
    - Baseline consciousness

- Assessment of Covid-19 Disease Severity as per the WHO 9-point ordinal scale Ordinal scale, 9 points
- IL-6 levels

EO: We expect to prove the effectiveness of zinc supplementation to reduce COVID-19 severity and mortality

D1: Guideline for zinc-based nutritional supplementation to treat COVID-19 patients admitted in hospitals

Statistical analysis

Randomization: Subjects will be randomized in a 1:1 ratio to:

- 83.91 mg zinc acetate (Zinc+NM) QD + standard of care for 2 weeks
- Standard of care for 2 weeks

Determination of sample Size: Sample size calculations are aimed at comparisons of days to clinical recovery between zinc supplementation and standard of care alone. Assuming alpha = 0.05 and a 10% dropout rate, randomization of 60 subjects will

provide 80% power to detect a difference in time to recovery between arms of 3 days assuming a SD of 4 days. This sample size will also provide at least 80% power to detect a change in percentage of people with clinical progression of 5% based on an estimated

clinical progression rate of 20% in the standard of care arm.

Analysis Populations: The analysis population for efficacy will be per protocol which includes all randomized subjects who complete 2 weeks of therapy and/or return for Week 2 visit. All safety analyses will include all randomized subjects who are

administrated at least 1 packet of the investigational product.

Baseline Characteristics: Demographics and Baseline characteristics will be summarized descriptively by group and overall using per protocol population. The following Baseline

characteristics will be summarized: sex, race, age, comorbilities, ethnicity

Clinical Laboratory Assessments: Blood chemistry, hematology parameters will be summarized at Baseline as well as visit 3 and 4. Laboratory values at Baseline and subsequent changes in laboratory values will be summarized descriptively overall and

by therapy group.

Vital signs: Vital sign measurements will be summarized at Baseline, Week 2 and Week 4. In addition, changes from Baseline to Week 2 or 4 will be summarized. For each summary the number, mean, median, standard deviation, minimum, and maximum

values will be presented overall and by therapy group.

Safety Analyses: All safety analyses will be based on the Safety Population, comprising all subjects who are randomized to the therapy group and subsequently receive at least one packet of investigational product. Safety assessments will be analyzed by frequency of events/abnormalities for categorical values or summarized using descriptive statistics

(mean, standard deviation, median, range, and number of observations).

Adverse events: Adverse events will be coded using the Medical Dictionary for Regulatory Activities (MedDRA) coding dictionary. Adverse events will be defined as any event with a start date occurring on or after initiation of therapy on Day 1 or, if pre-existing, worsening after initiation of therapy on Day 1. The incidence of AEs will be

summarized by body system and MedDRA preferred term, overall and by therapy group. If a subject reports the same AE more than once, then that subject will only be counted once for the summary of that AE, using the most severe intensity.

Handling of Dropouts or Missing Data : Dropouts or participants missing data for the primary endpoints will be excluded from the analysis.

Limitation: We might find no benefit in COVID-19 patients supplemented with zinc. That would mean that the low zinc levels detected in COVID-19 patients with worse prognosis are a consequence of the inflammatory status. The results will be also very helpful for physicians worldwide.

- **To study the immune responses of the COVID-19 patients enrolled in the clinical trial (Subproject 2)**

Task 1.2.1 Assessment of B-cell responses in COVID-19 patients

We will use sera samples from COVID-19 patients enrolled in the clinical trial described in objective 1.1 in both groups. We will measure antibody production against SARS- CoV2 virus at admission, 2 weeks and 28 days post admission. We will carry out ELISA experiments to measure IgM, IgA, IgG1 and IgG2a immunoglobulins against recombinant SARS-CoV2 spike protein.

Task 1.2.2 Assessment of antiviral T-cell responses in COVID-19 patients

We will use PBMC from COVID-19 patients enrolled in the clinical trial described in objective 1.1 in both groups. We will evaluate T cell responses using intracellular staining and flow cytometry. Subtype population (CD3, CD4, CD8), activation degree (IL- 2, INFg, perforin), exhaustion (PD-1, Tim-3) and memory (CD45RO and CD62L) markers will be evaluated using panels of tagged antibodies. We will stimulate T-cells using a commercial pool of overlapping spike peptides of SARS-CoV2. We will study activation degree and exhaustion at admission and at 2 weeks. We will evaluate memory at 28 days post admission.

EO: We expect to know whether zinc supplements might strengthen humoral and cellular adaptive immunity in COVID-19 patients. This will be of special interest for immunologists and pharma companies

D2: Publication describing the results obtained in the clinical trial with COVID-19 patients and the influence of zinc supplementation in their immune response

2. To establish causal-effect associations between Zn levels and SARS-CoV-2 infections (Subproject 3)

Task 2.1 To monitor SARS-CoV-2 infection progression in susceptible mice with different Zn supplementations.

We will use as an infection model our own colony of transgenic mice expressing human ACE2 (K18-hACE2). Animals will be fed with different supplemented zinc diets (Research diets Inc, or similar). Groups of mice (n=21) will be fed with standard diet, zinc deficiency (ZD) diet (10mg Zn/kg), adequate (AD) diet (30mg Zn/kg) and supplemented (SD) diet (150 mg Zn/kg) for 30d before infection to the end of the experiments. Zn levels in the serum of mice will be determined before challenge with infectious virus. Mice will be anesthetized and intranasally challenged with a lethal dose of a Spanish SARS-CoV-2 D614G(S) isolate and followed for 3 weeks.

Evaluation will be performed by assessing the mice's clinical health score (appearance, mobility and alertness, body weight and temperature) and mortality rates after being challenged with SARS-CoV-2 following standardized guidelines. Mice will be bled at different times post-infection and the viremia will also be compared between different treatments.

Determination of sample size

Group size was calculated according to the equation in (PMID: 24250214) for quantitative endpoint data in order to detect a significant 45% change in survival and considering a 5% of significance level ($P<0.05$) and 80% power of the analyses.

Task 2.2 To study impact of zinc nutritional status on adaptive immune responses against SARS-CoV2

Groups of K18-hACE2 mice (n=30) will be fed and challenged as in task 2.1. Ten mice/group will be anesthetized and sacrificed 7, 15, and 21 d.pi., and spleen and PBMC will be used. T-cell responses will be evaluated using intracellular staining and flow cytometry. Subtype population (CD4, CD8, ...), activation degree (IL-2, INFg, perforin), exhaustion (PD-1, Tim-3) and memory (CD45R0, CD44m cd103a, CD62L) markers will be evaluated using panels of tagged antibodies. We will stimulate T-cells using a commercial pool of overlapping spike peptides of SARS-CoV2. Antibodies against SARS- CoV-2 elicited in sacrificed mice will be also evaluated by ELISA to compare humoral immune response.

Sample size

Group size was calculated using Lehr's formula to have a power of 80%, considering a 5% level of significance, and a difference of 13 MFI in the CD4+ T cells between the two group means and assuming a combined standard deviation of 10 MFI (PMID: 18341999).

EO: We expect to answer the question whether zinc nutritional status before SARS-CoV2 infection determines COVID-19 progression. Moreover, we expect to understand the correlation between serum zinc levels and disease progression.

D3: Scientific communication addressed to physicians and public health decision makers describing the potential benefits of using zinc supplementation as a prophylactic strategy against severe COVID-19.

3 To characterize novel zinc homeostasis reporters upon SARS-CoV2 infection (Subproject 2) We will run RT-PCR for the 14 Zip and 10 ZnT transporters and for MT1 and MT2. The expression analysis will be correlated to serum zinc levels measured.

Task 3.1 Characterization of machinery for cellular zinc handling in PBMCs from COVID- 19 patients

We will study the expression in PBMCs from COVID-19 patients enrolled in the clinical trial (objective 1.1) in both groups at admission and at 2 weeks.

Task 3.2 Characterization of machinery for cellular zinc handling in PBMCs from SARS- CoV-2 infected mice. from mice

We will study the expression in PBMCs from SARS-CoV-2 infected mice at 7 days post infection (objective 2.1) in the three groups of mice (deficient, adequate and supplemented diet).

EO: Zinc plasma levels are very dependent on inflammatory signalling. We expect to find certain genes differentially regulated depending on the nutritional zinc status and COVID-19 progression.

D4: A novel and reliable diagnostic tool in the hospitals to check zinc nutritional status and define therapeutic strategies with COVID-19 patients and for other infectious diseases.

4. To decipher the direct antiviral action of Zn on SARS-CoV-2 life cycle steps (Subproject 2 UPF team)

In both tasks we will use infections of SARS-CoV-2 in the human lung Calu-3 cell lines already established in our group. Cells will be infected at high mois to mimic synchronize infections.

Task 4.1 To determine virus-induced changes in the zinc homeostatic machinery and in the intracellular zinc levels.

At time points after infections representing early, middle and late events in the infection cycle we will first measure zinc levels at the cytosol (pCDNA Zif-CY1)and the endoplasmic reticulum (pcDNA-ER-Zap-CY1), the site of SARS-CoV-2 replication. For this, we will transfect, fix cells and measure by fluorescence microscopy. Second, we will test whether SARS-CoV-2 infection induces changes in the expression of a set of host genes related to Zn homeostasis described in objective 3.

Task 4.2. To decipher the direct Zn antiviral action on the different SARS-CoV-2 life cycle.

We will carry out infection kinetics to study whether Zn levels affect early or late steps during cell cycle following protocol previously described (13). Moreover, to study specific effects in entry, we will use Pseudotyped GFP rSARS-CoV-2 Spike and to study effects in assembly/exit defects we will compare differences in intracellular/extracellular of SARS-CoV-2 RNA. Once the precise step of the cycle

affected by Zn levels have been detected, we will address the molecular mechanism involved.

EO: We expect to comprehensively determine the molecular mechanisms involved in the direct antiviral potential of zinc against SARS-CoV-2

D5. Scientific communication on the effect of Zn on the SARS-CoV-2 life cycle and its antiviral activity.