Hospital del Mar
Barcelona

CLINICAL RESEARCH PROTOCOL

Role of Zinc supplementation in immunological non-responders HIV individuals: exploring pathways for persistent inflammation

Protocol version and date	Version: 2.1 date: 04/24/2024
Protocol code	
Abbreviated title or acronym	*VIHZn*
Promoter	*Hospital del Mar, Barcelona*
CRO (where applicable)	*If applicable*
Research Team	**Principal Investigator** *Robert Guerri-Fernandez* *Infectious Diseases Service.* *Hospital del Mar* *Tel: 932483251* *e-mail: rguerri@imim.es* **Collaborating researcher(s)** *Sonia Footwear* *Infectious Diseases* *Sabadell-Parc Taulí Hospital* *Tel: 932483251* *e-mail:scalzado@tauli.cat* *Marta Navarro Vilasaro* *Infectious Diseases* *Sabadell-Parc Taulí Hospital* *Tel: 932483251* *e-mail:mnavarro@tauli.cat* *Maria del Carmen Navarro Saez* *Infectious Diseases* *Sabadell-Parc Taulí Hospital* *Tel: 932483251* *e-mail:mcnavarro@tauli.cat*

	Maria Esperanza Canas Ruano *Infectious Diseases* *Hospital del Mar* *Tel: 932483251* *Email: mariadelaesperanza.canas.ruano@psmar.cat* *Maria Jose Fernandez Quiroga* *Infectious Diseases* *Hospital del Mar* *Tel: 932483251* *E-mail:* *Cecilia Canepa* *Infectious Diseases* *Hospital del Mar* *Tel: 932483251* *Email: ccanepa@psmar.cat* *Juan Du* *Infectious Diseases* *Hospital del Mar* *Tel: 932483251* *Email: dujuan@researchmar.net* *Mariano Pascual Aranda* *Infectious Diseases* *Hospital del Mar* *Tel: 932483251* *Email: mpascualaranda@psmar.cat* *Augustin Marcos Blanco* *Infectious Diseases* *Hospital del Mar* *Tel: 932483251* *Email: amarcos@psmar.cat* *Natalia Garcia-Giralt* *E.Infectious* *Hospital del Mar* *Tel.: 932483251* *Email: ngarcia@researchmar.net* *Itziar Arrieta* *Infectious Diseases* *Hospital del Mar* *Tel 932483251* *Email: iarrieta@psmar.cat* *Alicia Gonzalez Mena* *Infectious diseases* *Hospital del Mar* *Tel. 932483251*

	Email: agonzalez@psmar.cat

SUMMARY

Qualification	*Role of Zinc supplementation in immunological non-responders HIV individuals: exploring pathways for persistent inflammation*
Goals	**Aim 1:** To design an open label randomized double-blinded multicentric 2-arm study The following parameters will be measured at baseline and at week-16 in zinc-supplemented individuals vs controls: - Quality of life changes through EuroQol-5D-3L -Immune function (including CD4+ T-cell count, CD4/CD8 ratio. T-cell subsets balance: naïve, effector memory, central memory, TEMRA, and exhausted T-cells and Torque Teno virus viral load) -Immune activation (CD38+ CD4+ and CD38+ CD8+ T-cells) -Systemic inflammatory biomarkers -Transcriptomic changes in peripheral blood -Mitochondrial function and ROS **Aim 2.** To measure persistence in changes quality of life, immune function, immune activation, inflammatory biomarkers, and mitochondrial function and ROS at week 24 respect to week-16 and baseline.
Design	Double blinded randomized multicentric 2-arm study
Inclusion-exclusion criteria	Inclusion criteria: **1)** confirmed HIV infection; **2)** >18 years; **3)** Serum zinc levels <150ug/dl (normal range considered 75-150 ug/dl); **4)** HIV-1 infection on stable ART for at least 3 months with cumulative ART duration of at least 6 months; **5)** Undetectable (<50 copies/ml) persistently (isolated transient increases in viremia of less than 1000 copies/ml were accepted); **6)** persistence of CD4 <500 cells/mm3 at enrollment or an increase of less than 80 cells/mm3 after one year of viral undetectability Exclusion criteria: **1)** Pregnancy; **2)** Lactancy; **3)** Active infectious or inflammatory condition; **4)** Uncontrolled diabetes; **5)** Serum Zinc levels >150ug/dl
Treatments or interventions	**120** eligible HIV INRs under ART adult individual at two clinical sites in Spain (Hospital del Mar and Hospital Parc Taulí) will be randomized in a 3:1 ratio to: (TO) 30 participants in Control group. They will continue the SoC + placebo (3 tablets)(B) 90 participants in Zinc supplementation group. This group will keep receiving SoC and separated 12h (just in case the ART is based on Integrase Inhibitors (INSTI)) will be supplemented for 16 weeks with 3 tablets of zinc (83mg Zinc acetate/tablet).
Variables	Plasma Zn levels, Inflammatory biomarkers (IL-6, C-Reactive protein, CD14, soluble TNF-alpha receptor, I-FABP, LBP), T-cell subpopulation, Torque Tenovirus Replication Immunoactivation Exhaustion markers in lymphocytes ROS and mitochondrial function

	QoL quesionnarie EuroQoL-5D-3L Changes in transcriptromic profiling
Population and number of subjects	120 HIV individuals
Participating centers	Hospital del Mar. Barcelona Sabadell-Parc Taulí Hospital
Study Description	Study to evaluate the impact of Zinc supplementation in immunological non-responders with HIV.
Overall duration of the study	February 2024-February 2026
Duration of the study for the patient	24 weeks

Table 1. Study schedule/procedures for a subject

	Screening **(max 28 days before baseline visit)**	**Baseline visit (randomization)**	**W16** **(+/-7d)**	**W24** **(+14-7d)**
Type of visit **Intervention group** **Control group**	In person	In person	In person	In person
Informed consent	**x**			
Inclusion and exclusion criteria	**x**			
Demographic data	**x**			
Medical history	**x**			
HIV-related history	**x**			
Physical examination		**x**	**x**	**x**
Anthropometric data		**x**	**x**	**x**
Questionnaires **(EQ-5D and IPAQ)**	**X**	**x**	**x**	**x**
Concomitant medication	**x**	**x**	**x**	**x**
Adverse effects		**x**	**x**	**x**
Pregnancy test **if applicable**		**x**	**x**	**x**

Analytical studies	**x**	**x**	**x**	**x**
Sample storage (PBMCs, whole blood, plasma, serum)		**x**	**x**	**x**

INDEX

1. LIST OF ABBREVIATIONS

HIV	Human immunodeficiency virus
PLHIV	Person living with HIV
TAR	Antiretroviral treatment
AIDS	Human acquired immunodeficiency syndrome
LDL	Low-density lipoproteins
MRI	Nuclear magnetic resonance
TMAO	Trimethylamine N-oxide

2. VERSION HISTORY

Version	Date	Description of change	Brief justification
1.0	Dec 15, 2023	First version	
2	23-Apr-2024	Version 2	We changed the study design to a double-blind study as recommended by the CEIm to improve the robustness of the study.

3. THEORETICAL FRAMEWORK

3.1 BACKGROUND AND JUSTIFICATION OF THE STUDY

Studies of people living with HIV show that although antiretroviral treatments (ART) decreases inflammation (1) , a number of inflammatory markers remain persistently elevated and this chronic inflammation can play an important role in cardiovascular disease (CVD). Furthermore, some individuals with HIV initiating ART do not recover CD4+ count as expected, and are described as Immunological Non-Responders (INRs). INRs present with severely altered immunological functions, including malfunction and diminished production of cells within lymphopoetic tissue, disturbed frequencies of immune regulators such as regulatory T cells and Th17 cells, and increased immune activation, immunosenescence, and apoptosis. Importantly, INRs have an increased risk of morbidity and mortality compared to HIV-infected patients with an optimal immune reconstitution. Our group has demonstrated how this group responds poorly to mRNA vaccination against SARS-CoV-2 showing an impaired immune function among these individuals (2).

However, the definition of Immunological Non-Responders (INRs) suffers from lack of consensus impeding the comparison of findings. Most often INRs are defined as having CD4+ cell counts <200 cells/ μ L while the treatment duration needed for categorizing patients as INRs is variable. Furthermore, some study groups define INRs by the CD4+ cell increase in percentages, most commonly <20% increase from baseline (3). On the other hand, there seems to be agreement that an adequate immune response to HAART should include a CD4+ cell count >500 cells/ μ L, mainly because HIV-infected patients with this level of immune restoration have a morbidity and mortality rate approaching or comparable to those of HIV negative individuals (4). Patients with CD4+ cell counts <500 cells/ μ L are consequently classified as inadequate responders, included in the INRs

group. And, although they are a group poorly described, their morbidity and mortality seem to be more similar to INRs than to adequate responses (5).

The underlying mechanism that explains INRs phenomena is not well understood. Several hypotheses have been raised as an older age, a long duration of HIV infection prior to HAART, coinfection with hepatitis C, and a low CD4 nadir predispose to immunological nonresponse (6). The CD4 nadir specifically appears to be critical for the recovery of CD4+ cells (7). However, none of these factors provide a full explanation for the lack of immune reconstitution in INRs. Probably this is the consequence of several factors and their interaction. Interestingly, low plasma zinc levels and inadequate zinc intake were found in up to 50% of participants in several cohorts of HIV infected adults (8,9), and were independently associated with faster HIV disease progression (10) and increased mortality (11). Zinc deficiency reduces generation of T-cells, depresses humoral and cell-mediated immunity, leads to lymphopenia, and thymic atrophy, and increases the frequency and number of infections (8). Thus, the zinc status could also have a role in the mechanisms involved in INRs.

Zinc in viral infection

Zinc is an essential trace element for both hosts 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 (12). 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 13).

Remarkably, our team has shown that low zinc levels favor SARS-CoV2 expansion (14). 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. Zinc supplementation has been shown to be beneficial when administering to HBV and HCV infected patients. Furthermore, zinc-based nutritional immunity reduces diarrheal episodes and respiratory track diseases (reviewed in 13). 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 13). 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.

Zinc as an immunomodulator

Zinc deficiency (ZD) is known to be associated with proinflammatory responses at infection, showing higher reactive oxygen species (ROS) production and inflammatory markers (12,13). An imbalance in cytokine production by cells of both innate and adaptive immunity has also been reported.

Specifically, ZD is associated with higher levels of IL-6, IL-1beta and TNF-alpha (13,15). In this scenario, zinc supplementation has been shown to reduce the incidence of infection, inflammatory cytokines, including IL-6, and oxidative stress markers in elderly individuals (12). Furthermore, zinc supplementation has been shown to balance immune responses (16).

Nutritional zinc status and IL-6 are highly interconnected. Thus, IL-6 can reduce zinc bioavailability by promoting internalization in hepatocytes and the expression of zinc chelators (17). On the other hand, zinc decreases IL-6 production via inhibition of STAT-3 pathway (18). Our study with COVID-19 patients showed a negative correlation between serum zinc levels and IL-6 production (14).

Zinc in T lymphocytes activation and exhaustion

Zinc is known to affect T lymphocyte maturation, differentiation and cytokine production (19). 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 signaling by modulating Lck and Zap-70 activity (20). Our collaborators Vicente et al. have demonstrated that zinc positively potentiates the three main signaling pathways, AP-1, NF-kB and NFAT1 (21). 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 (22). Furthermore, 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 (23). Nevertheless, there are no studies investigating whether zinc supplementation/chelation could directly affect the CD8+ dysfunctional profile and how these learnings could be translated into novel therapeutic approaches for these diseases including HIV infection.

Zinc in B lymphocyte function
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 signaling (24). 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 (25).

In summary, Zinc has anti-oxidative and anti-inflammatory properties and is involved in immune regulation including cell maturation, cell differentiation, and apoptosis. Zinc homeostasis is important for several aspects of the immune system since there are several cross talks between zinc and immune function. Zinc deficiency results in an increased sensitivity to the effects of oxidative stress. Furthermore, zinc has an antiviral activation against certain viruses. In this regard, we propose a clinical trial based on the effect of zinc supplementation, together with a deeper study of the impact and mechanisms of this supplementation in immune cells and inflammation, on the HIV infection and especially in those cases with less response to ART and less immune recovery capacity.

Preliminary Results

Our group has been working in the immunomodulatory effect of zinc in viral infections and in the immune response since 2020. Also, we are actively working in the immune response in PWH INRs (2). We have several publications and an active research line putting these two research lines together.
We previously carried out a study analyzing the association between zinc plasma levels at hospital admission and COVID-19 prognosis, during the first wave of the pandemics. We found a negative correlation between the zinc levels and IL-6, as well as with mortality. We also found that zinc acted on viral replication in vero cell cultures (14). We have recently conducted a clinical trial (NCT05778383) whose preliminary results have been reported in CROI2023 #542. In this trial, we observed that high-dose Zinc supplementation in the acute phase of SARS-coV-2 infection is associated with a better prognosis of the disease. We also showed an abnormal immune response to vaccination among INRs (2). Therefore, we have demonstrated in a viral infection context that zinc has an antiviral and anti-inflammatory effect, which may have a benefit in people living with HIV, especially in the INR situation.

3.2.HYPOTHESIS

This study has several hypotheses:

1. Relative low zinc levels are frequent in our cohort of HIV patients and have a role in worse immune response and in higher inflammatory response.
2. We hypothesize that zinc has an impact on the immune system restoring the lymphocyte count in INRs HIV individuals and improving immune function and inflammation associated with chronic viral infection.
Therefore, zinc supplementation in physiological concentrations and recommendations has a beneficial impact in immune function, reducing immune activation and reducing chronic inflammation in INRs HIV individuals.
3. Zinc supplementation protects gut mucosa of cytopathic effect of viruses, partially restoring the gut permeability induced by HIV
4. Zinc supplementation improves oxidative stress and mitochondrial function

4. GOALS

The main objective is to study the clinical and immune benefits of 16 weeks of supplementation with zinc acetate in HIV immunological non-responders' individuals. To address this objective, a Randomized Clinical Trial (RCT) will be designed.

Aim 1: To design a double blinded randomized multicentric 2-arm study The following parameters will be measured at baseline and at week-16 in zinc-supplemented individuals vs controls:

- Quality of life changes through EuroQol-5D-3L
-Immune function (including CD4+ T-cell count, CD4/CD8 ratio. T-cell subsets balance: naïve, effector memory, central memory, TEMRA, and exhausted T-cells and Torque Teno virus viral load)
-Immune activation (CD38+ CD4+ and CD38+ CD8+ T-cells)
-Systemic inflammatory biomarkers
-Transcriptomic changes in peripheral blood
-Mitochondrial function and ROS

Aim 2. To measure persistence in changes quality of life, immune function, immune activation, inflammatory biomarkers, and mitochondrial function and ROS at week 24 respect to week-16 and baseline.

5. METHODOLOGY

5.1. Study Design

The methodology is divided into tasks.

Task 1. Randomized Clinical Trial.
An **open label randomized multicentric 2-arm study** will be designed. Participants will be prospectively enrolled at two sites Hospital del Mar and Hospital Parc Taulí. In the Zinc Supplementation group (Zn), participants will receive the Standard of Care (SoC) and separated 12h (just in case the ART is based on Integrase Inhibitors (INSTI)) will be supplemented for 16 weeks with 75mg of elemental Zn (3 tablets (83mg Zn acetate) 249mg of Zinc Acetate Zinc-NM; Laboratorios Cantabria (National code 156252.4)). We have selected 75mg of elemental Zn/d as oral supplementation because it has been used safely in non-HIV studies with 1 year duration at this concentration with no serious adverse effects (15). End of Treatment (EoT) will be at 16 weeks, but a follow up to 24-weeks will be done to assess the persistence of the effects of Zn.

Primary outcome
Change from baseline to week 16 (primary endpoint) and to week 24 (secondary endpoint) in the following parameters:

- Plasma Zn levels
- Inflammatory biomarkers (IL-6, C-Reactive Protein, CD14, soluble TNF-alpha receptor, I-FABP, LBP)
- Immune Function:

Torque Teno Virus (TTV) Replication : The TTV is from the family of *Anelloviridae* and is detectable in >90% of healthy people (27,28) (3 to 6 log10 copies/ml). The viral load reflects the interplay between TTV replication and antiviral immune response, with an estimated daily clearance rate of more than 90% of virions (29). Higher viral loads respect to HIV negative and an elevated correlation between TTV viral load and the CD4+ count has been described in PWH (30). Significantly TTV plasma concentration in patients with diminished CD4+ cell recovery indicates a more profound immune defect. TTV presence and replication could be used as a surrogate of immune function and response to the intervention with Zinc supplementation (31)

T-cell subpopulations : Studying the T-cell compartment also can provide an idea of the balance and function of the immune system. CD4+/CD8+ T-cells and naïve, central memory, effector memory, TEMRA will be measured.

Immune activation: Immune activation levels can be determined by measuring the expression of CD38 and Ki67 in CD8+ and CD4+ T lymphocytes

Exhaustion: PD-1 conveys inhibitory signals to T cells, and PD-1 is selectively upregulated by exhausted T cells during chronic viral infection (32). Elevated levels of PD-1 in INR compared to normal responses have been reported (33).

ROS and mitochondrial function : Mitochondrial dysfunction has been implicated in a growing number of disorders. ROS generated by mitochondria has been implicated in several processes such as cancer, aging, myocardial infarction, cardiovascular disease (38)

Quality of life (QoL) questionnaire (EuroQol-5D-3L): The EQ-5D-3L is an established and validated questionnaire of QoL used in many therapeutic settings (34,35,36). With its small size of 5 items and a visual analog scale, its brevity and ease of use make it well suited for use in clinical research and practice. There is a version validated in the Spanish population (37).

Secondary outcomes

- Change in the transcriptome profile between baseline and week 16 respect to week 24 in whole blood samples

Security endpoints

- Incidence of adverse events (AEs)

5.2.Study population, inclusion/exclusion criteria

Study groups
Population of study : 120 eligible HIV INRs under ART adult individual at two clinical sites in Spain (Hospital del Mar and Hospital Parc Taulí) will be randomized in a 3:1 ratio to:

(TO) 30 participants in Control group. They will continue the SoC + 3 tablets of placebo.
(B) 90 participants in Zinc supplementation group. This group will keep receiving SoC and separated 12h (just in case the ART is based on Integrase Inhibitors (INSTI)) will be supplemented for 16 weeks with 3 tablets of zinc (83mg Zinc acetate/tablet).

Inclusion Criteria
1) confirmed HIV infection; **2)** >18 years; **3)** Serum zinc levels <150ug/dl (normal range considered 75-150 ug/dl); **4)** HIV-1 infection on stable ART for at least 3 months with cumulative ART duration of at least 6 months; **5)** Undetectable (<50 copies/ml) persistently (isolated transient increases in viremia of less than 1000 copies/ml were accepted); **6)** persistence of CD4 <200 cells/mm3 at enrollment or an increase of less than 80 cells/mm3 after one year of viral undetectability, in which the latter case maintained a CD4 <350 cells/mm3 at enrollment.

Exclusion Criteria
1) Pregnancy; **2)** Lactation; **3)** Active infectious or inflammatory condition; **4)** Uncontrolled diabetes; **5)** Serum Zinc levels >150ug/dl

6. Study Procedures

Tasks that will take place during the study.

Task 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)
Randomization: Randomization will be performed using REDCap, randomization that is done by enabling the randomization module in Project Setup. Previously we will upload a predefined randomization scheme. Participants will be randomized by clicking the "Randomize" button on their record screen once required data fields are completed, following the scheme's criteria.

Visit performed at Outpatient's Clinic. Written informed consent will be obtained. Medical history. Vital signs. Clinical Laboratory (CD4+ T-cell count, HIV Viral load, CBC, C-Reactive Protein, ferritin, D-dimer, Vitamin D, zinc).

Quality of Life Questionnaire: measured by EuroQol-5D-3L
Samples 10cc serum tube, 10cc plasma EDTA tube, one tempus RNA tub, and 2 tubes for Peripheral Blood Mononuclear Cells (PBMCs).

2.-Visit 2. Week 16 **(+/-7d)**). End of Treatment.

Vital signs. Clinical Laboratory (CD4+ T-cell count, HIV Viral load, CBC, C-Reactive Protein, ferritin, D-dimer, Vitamin D, zinc). A complete record of AEs and concomitant therapies will be considered.
Quality of Life Questionnaire: measured by EuroQol-5D-3L
Samples 10cc serum tube, 10cc plasma EDTA tube, one tempus RNA tub, and 2 tubes for Peripheral Blood Mononuclear Cells (PBMCs).

3.-Visit 3. Week 24 **(+14/-7d).** Extension of Study.

Vital signs. Clinical Laboratory (CD4+ T-cell count, HIV Viral load, CBC, C-Reactive Protein, ferritin, D-dimer, Vitamin D, zinc). A complete record of AEs and concomitant therapies will be considered.
Quality of Life Questionnaire: measured by EuroQol-5D-3L
Samples 10cc serum tube, 10cc EDTA plasma tube, and 2 tubes for Peripheral Blood Mononuclear Cells (PBMCs).

Task 2. Assessment of T-cell subsets in HIV INRs

We will use PBMCs from patients enrolled in the clinical trial described in Task 1 in all groups.

PBMCs will be obtained by separation from heparinized peripheral blood by density gradient centrifugation (Ficoll-Paque). PBMCs will be isolated and aliquoted in $1x10^{6}$ cells/ml and preserved at -80ºC. All samples will be sent to IMIM for further processing. A circuit will be established to send the samples in safe conditions and guaranteeing their preservation.

Flow cytometry. Panel with antibodies will be used including CD45, CD4, CD8, CD45RO, CCR7, CD69, CD103, Ki67, FOXP3, CD25, TCRVd2, TCRVd1) and exhaustion (PD-1, Tim-3). **Mononuclear cells** will be identified by CD45+ then separated into CD4+ and CD8+ and then **memory** subsets by CCR7+ and CD45RO+. **Regulatory T - cells** will be identified as FOXP3+CD25+. **Gamma-Delta 2 T-cells** will be defined as CD3, CD4/8-TCRVd2+. Appropriate positive and negative controls (eg fluorescence minus one on blood) will be conducted with assays to ensure rigorous results. Cell populations will be acquired on the LSR-Fortessa (UPF core facilities) and analyzed with Flowjo software (Treestar Inc. CA).

Task 3. A multiplex ELISA for inflammatory biomarkers
At entry, and week 16 and week 24, fasting (for at least 8 hours) will be obtained and stored at -80ºC for inflammatory and gut integrity biomarkers. The panel will consist at least: marker of monocyte activation soluble CD14 (sCD14) and soluble CD163 (sCD163), of systemic inflammation [high sensitivity C reactive protein (hsCRP) and soluble tumor necrosis alpha receptor I and II (sTNFR-I and II)] and Lipopolysaccharide binding protein ((LBP) (a marker of microbial translocation) and intestinal fatty acid binding protein ((IFABP) a marker of gut integrity) will be measured by ELISA

Task 4. Measurement Torque Teno Virus (TTV) Viral load.

The TTV viral load will be determined using the TTV R-GENE kit (bioMierieux). TTV will be quantified prospectively per protocol in the peripheral blood at baseline, at week 16 and at week 24.
TTV DNA will be extracted from 200 µL of plasma using the NucliSENS easyMAG platform (bioMerieux, France) as recommended by the manufacturer and eluted in 50 µL of elution buffer. TTV DNA will be quantitated by TaqMan real time-polymerase chain reaction (PCR) as described. The quantitative PCR reactions will be performed in a volume of 25 µL using 2x TaqMan Universal PCR Master Mix, containing 5 µL of extracted DNA, 400 nM of each primer and 80 nM of the probe. Thermal cycling will be started for 3 minutes at 50°C, followed by 10 minutes at 95°C, and then by 45 cycles at 95°C for 15 seconds, at 55°C for 30 seconds, and at 72°C for 30 seconds using the 7300 Real Time PCR System (Applied Biosystems, CA, USA). Results will be recorded in copies/mL.

Task 5. Measuring ROS and mitochondrial function by flow cytometry
PBMCs are isolated at entry, week 16 and week 24 as described. PBMCs will be incubated overnight at a concentration of 1×10^{6} cells/ml in RPMI media containing 10% FBS, 2mM L-glutamime, and 100 U/ml penicillin, 100 µ g/ml streptomycin, 10 µ g/ml amphotericin B . PBMCs will be treated with the nitric oxide (NO) donor NOC-18 for 20 h. NAC will be titrated to pH 7.4 and used at a final concentration of 3 mM. Rotenone will be used at a final concentration of 3 µ M. Both NAC and rotenone will incubated on PBL for either 2 h or 15 min. Treated PBMCs will be washed twice with PBS before mitochondrial isolation.
Flow Cytometry Analysis . All flow cytometry experiments will be performed on a LSR-Fortessa (UPF core facilities). The fluorescent probes will be: ΔΨ m will be measured using 10 nM tetramethylrhodamine, methyl ester (TMRM) and 40 nM 3,3′-dihexyloxacarbocyanine iodide (DiOC6). Mitochondrial mass will be evaluated with 150 nM MitoTracker Green (MTG) and 2.5 µ M nonylacridine orange (NAO). The concentration of NO, a reactive nitrogen species (RNS), will be measured by 1 µ M 4-amino-5-methylamino-2′,7′-difluorescein (DAF-FM). H2O2 levels will be evaluated using 10 µ M 2′,7′-dichlorofluorescin diacetate (DCF-DA). Dihydrorhodamine 123 (DHR) and dihydroethidium (HE) will also be used. Data will be analyzed with FlowJo version 19 software (Tree Star Inc., OR, US).

Task 6 Transcriptomics analysis

The peripheral blood samples will be collected in tempus RNA tubes (ThermoFisher, MA, USA) at baseline and week 16 and stored at -80°C for further processing. For the transcriptomic analysis, the RNA from 10 controls and 30 zinc-treated participants will be extracted and an expression microarray will be made using Clariom_Standard (WT Plus, Affymetrix). This process and the bioinformatics analysis will be carried out on the MARGenomics platform, IMIM.

7. Study timeframe: Stages of the project

In order to carry out the RCT, the project has been divided into different stages:

1. First stage. Elaboration and validation of the RCT protocol
This phase will be carried out by all the researchers and coordinated by the PI Roberto Güerri. Pre-selection of potential participants will be done according to the inclusion/exclusion criteria among the different centers.
A CRO will be contracted to make the start-up and monitoring of the study.
A CRD using the RedCap platform will be prepared.

In this phase, the sample collection procedure (labeling of samples, aliquots, storing in MarBiobank, etc) will be established.
Sample storage and processing will be carried out at Hospital del Mar Research Institute.
A courier service will be put in place in order to transport samples between centers.

2. Second stage: Recruitment of patients (from March 2024 to March 2025).
The recruitment will be carried out in the Infectious Department of Hospital del Mar (HM) and in the Infectious Diseases Department at Hospital Parc Taulí, Sabadell (HpT). The reference investigator in the centers will be Robert Güerri-Fernández (PI of the project) for HM and Dra Sonia Calzado for HpT. The researchers who will carry out the coordination in the recruitment will be Dra. A. González and Dra. Sonia Calzado. The pre-selected patients will be summoned in the outpatient clinics and the study will be proposed. Once the participants are identified, they will be sent to a first visit in the outpatient clinic for the entry visit with Dr. Fernandez or Dr. Arrieta and Agustin Marcos at HM and Dra. Sonia Calzado and Dra Navarro at HpT. Clinical variables and EuroQol-5D-3L will be obtained. Randomization will be applied at this first visit (baseline). Hospital Pharmacy will provide the Zinc Supplementation and placebo to the study participants for the whole treatment (16 weeks).
Sample storage and processing will be carried out at Hospital del Mar Research Institute.
A courier service will be put in place in order to transport samples between centers.
The blood processing and preservation of samples (PBMCs, plasma, serum) will be carried out by Dra. Juan Du at Hospital del Mar and by Dra Calzado at Hospital Parc Taulí. Aliquots will be preserved at -80C in the MarBiobank.

3.Third stage. Follow-up
Participants will be visited at week-16 (end of treatment) and week-24 (extension time) in the respective Hospital. EuroQol-5D-3L, clinical variables and samples will be obtained equally to second stage

4.Four Stage. Sample analysis at the end of follow-up
A) Preparation of the samples for FACS (T-cell subsets, mitochondrial function, and ROS)
Once studied is finished, samples will be stained and prepared to undergo FACS (Dr Juan Du, Dr Güerri-Fernández, Dra García-Giralt). Data reading and interpretation will be done by Dr. Güerri-Fernández and external collaborators: Prof Sumgin Kim and Prof David Asmuth (University of California, Davis) and Dr Ruben Vicente (Universitat Pompeu Fabra).
B) Preserved plasma samples will be prepared and analyzed by a multiplex ELISA panel for inflammatory markers. (Dr Natalia Garcia Giralt, Dr Juan Du)
C) qRT-PCR for TTV will be performed by Dra Natalia Garcia Giralt at IMIM-Lab.
D) Transcriptomics will be coordinated by Dra. Garcia Giralt and performed on the MARGenomics platform.

5.Fifth stage. Analysis and publication of results (from March 2026 (preliminary) to December 2026) will be done by all the researchers

8. Statistical Analysis

7.1. Sample Size calculation
To calculate the sample size we considered the least favorable scenario and the one we found the greatest evidence that was among those supplemented with Zinc (39, 40).

When calculating the sample size needed to assess the changes in C-Reactive protein (according to 39.) Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, 26 subjects are necessary in the control group and 78 in the zinc supplementation group to recognize as statistically significant a difference greater than or equal to 1.27mg/dl. The standard deviation is assumed to be 1.87mg/dl. It has been anticipated a drop-out rate of 10%.

We also calculated the sample size needed to assess whether the relative risk of a CD4 increase above 30%. Based on the study by Asdamongkol et al (41) and accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, 28 subjects are necessary in the control group and 84 in the zinc supplemented group as statistically significant a proportion difference, expected to be of 0.48 in zinc supplemented group and 0.22 in control group. It has been anticipated a drop-out rate of 10%. The ARCSINUS approximation

7.2. Statistical methodology

Descriptive analysis of the results will be presented accordingly to the nature and distribution of variables. Comparisons with Student's t test, and categorical variables using Fisher's exact test.
A multivariable regression model will be fitted for every outcome adjusting by age, gender and current ART to study the impact of the intervention.
Frequencies of adverse events and complications will be compared with the chi-square test. All analyzes will be done according to the intention-to-treat principle, without adjustment for multiple comparisons. Two-sided P values of less than 0.05 will be considered to indicate statistical significance. The study will be registered with ClinicalTrials.gov.

For flow experiments and T-cell analysis subsets and inflammatory markers cross-sectional pairwise comparisons between groups will be performed using the non-parametric Kruskal-Wallis and Mann Whitney U tests. The Spearman rank correlation coefficient will be used to analyze the correlation between continuous variables. The immunological parameters logs (eg the percentage of T-cells) will be represented in these data points as a dichotomous or ordinal variable (but much less magnitude than the sequencing) with tertiles and compared against the TTV replication, inflammation, gut damage markers.

7.3. Limitations

The main limitation is the loss in follow-up of our patients. According to prior studies we have done a conservative estimate 10%. The participant centers have identified more than 480 patients that fit the inclusion criteria for INRs considered in the study, we expect to recruit 120 participants in a low intensity intervention, we are confident that the recruitment process is going to be fast.
Another limitation is the intervention with zinc that at high doses might interfere with cupper metabolism and at low doses might not be effective. We have widely studied prior studies and we have found that the chosen dose is the best dose possible because it delivers high amount of zinc element, but it has been previously shown to be safe at a longer periods of time (even to one year) as a supplementation, regardless of zinc levels. Also zinc might have an interaction with integrase inhibitors ART, patients will be instructed to separate the Zinc dose from ART 12h.

9. Ethical Aspects

The study will be subject to the ethical standards of Biomedical Research in humans. The study will be conducted in accordance with the ethical principles derived from the Declaration of Helsinki (Fortaleza, Brazil, October 2013). Additionally, the study will be carried out according to the protocol, Good Clinical Practice (GCP) in line with the guidelines of the International Conference on Harmonization (ICH), and regulatory requirements for participating institutions. The study will be conducted according to a protocol reviewed by a Committee. The benefits of the study will be considered in proportion to the risks, and the rights and well-being of the subjects will be respected.

Patients will be informed, and they will be given an approved informed consent document in compliance with GCP. Similarly, all participants will sign the consent of the PsMar Biobank. Confidentiality aspects will follow established regulations. Before including subjects in the study, the researcher will review the information sheet with the potential participant, and if they agree to participate, the informed consent document will be signed and dated. The researcher will provide a copy of the signed informed consent form to each subject and retain a copy in the study file.

This study and all the procedures followed will be in accordance with the ethical standards of the responsible committee on human experimentation (CODE OF ETHICS OF THE COMB) and with the Helsinki Fortaleza Declaration of October 2013. This study will follow the provisions of Law 14/2007, of July 3, on Biomedical Research and Organic Law 3/2018, of December 5, on the Protection of Personal Data and Guarantee of Digital Rights and the Protection of Personal Data and Regulation (EU) No. 2016/679 of the European Parliament and the European Council of April 27, 2016 on Data Protection (GDPR) and its development rules.

No financial compensation or reimbursement of expenses related to participation is anticipated for the participants. Recruitment will be done from the clinic, so it will not require.

10. Sample processing

Sample storage and processing will be carried out at Hospital del Mar Research Institute. A courier service will be put in place in order to transport samples between centers.
The blood processing and preservation of samples (PBMCs, plasma, serum) will be carried out by Dra. Juan Du at Hospital del Mar and by Dra Calzado at Hospital Parc Taulí. Aliquots will be preserved at -80C in the MarBiobank.

11. Confidentiality

This study will be conducted in accordance with the General Data Protection Regulation 2016/679 and Organic Law 3/2018, of December 5, on the Protection of Personal Data and guarantee of digital rights.
Our main goal is to preserve anonymity of participants while making data available to researchers who request them under a reasonable premises.
To preserve anonymity De-identification will be performed. All researchers will remove or mask all identifying information from the data, such as names, addresses, and other personal information, date of birth, to ensure that individuals cannot be identified from the data. An independent list with the ID of the study and the data will be kept by the researchers in a separated and coded file.
Once the study results have been published and analyzed we will make them available upon a reasonable request. We will establish clear procedures for data access, outlining who can access the data and under what conditions. Access will be limited to

individuals who have a legitimate need for the data and who have agreed to use the data in accordance with ethical and legal guidelines.
Data will be stored securely using appropriate encryption and access controls, to prevent unauthorized access and ensure data integrity.
As a CRD we will use RedCap because it allows to work online with several researchers at the same time, and it allows traceability of the access, and also allows to grant access to different parts of the dataset without compromising the security and the anonymity of participants.
We will consider to deposit the data in a publicly accessible data repository, such as the National Institutes of Health (NIH) Data Archive or the Inter-university Consortium for Political and Social Research (ICPSR), which provide secure data storage and access to researchers .
Overall, data management and sharing strategies will be developed with the goal of protecting participant anonymity and ensuring ethical and legal compliance, while also making data available to researchers who can use them to advance scientific knowledge.

The study data must be verifiable with source data, requiring access to all original records, laboratory reports, and subject records. The confidentiality of data and patient identity will be maintained during the study and after its completion. Only the principal investigator and authorized study personnel will have access to these confidential records. The data storage location and the person responsible for the file will be explained below.

No data used in the analysis and subsequent disclosure of study results will contain any identifiable reference to patient names.

Once the study is concluded, the results will be communicated to the relevant authorities in an appropriate manner, following local legislation. Additionally, the generated data will be published in conferences and scientific journals.

12. Insurance

Since it is a marketed supplement, approved for use, and no treatment will be conducted outside of clinical indications, the insurance provided for the study is the liability insurance held by the hospital. The study is classified as Low Intervention Level (LIL).

13. REFERENCES

1. Funderburg NT. Current opinion in HIV and AIDS 2014; 9(1):80 -86
2. Sisteré-Oró M et al. Front Immunol. 2022 Aug 26;13:994173
3. G. Marchetti et al. AIDS, vol. 24, no. 10, pp. 1455 -1460, 2010
4. C.Lewden,G.Chene, et al., Journal of Acquired Immune Deficiency Syndromes, vol. 46, no. 1, pp. 72 -77, 2007
5. Antiretroviral Therapy Cohort Collaboration Lancet, 2008. 372, no. 9635, pp. 293 -299,
6. RD Moore and JC Keruly, Clinical Infectious Diseases, 2007. 44, no. 3, pp. 441 -446,
7. R. D'Amico,et al., Journal of Clinical Immunology, vol. 25, no. 2, pp. 106 -115, 2005
8. Endre L, et al. TJ Trace Element Exp Med. 1990;3:337-75
9. Jones CY, et al.. J Acquir Immune Defic Syndr. 2006 Dec 1;43(4):475 -82
10. Graham NMH, et al. J Acquir Immune Defic Syndr. 1991;4(10):976 -980
11. Baum MK, et al. Clin Infec Dis. 2003;37(Suppl 2):S117 -23
12. Prasad AS et al. Am J Clin Nutr 2007;85:837 -44.
13. Overbeck, S. et al.Arch. Immunol. Ther. Exp. (Warsz). 2008. 56: 15 -30.
14. Vogel-Gonzalez M, et al. Nutrients. 2021 Feb 9;13(2):562.
15. Bao B, et al. Am J Clin Nutr 2010;91:1634-41
16. Rosenkranz, et al. J. Nutr. Biochem. 2016. 29: 116 -123.
17. McGonagle D, et al. Autoimmun Rev.2019 19:102537.
18. Liuzzi JP, et al. PNAS, 2005.102:6843 -8
19. Haase H, et al. Annu. Rev. Nutr.2009. 29: 133 -52.
20. Yu M. et al. J. Exp. Med.2011; 208:775-85
21. Colomar-Carando N, et al. J. Immunol 2009; 202(2):441-450
22. Singer M, et al. Cell. 2016; 166:1500-1511.
23. Doering TA, et al. Immunity.2012; 37:1130-44.
24. Anzilotti C, et al. Nature Immunology 2019; 20, 350 -361
25. Hojyo S, et al.PNAS 2014; 111, 11786-11791
26. Okamoto H. Curr Top Microbiol Immunol. 2009;331:1 -20
27. Maggi F, J Virol. 2010;84(13):6891 -3.
28. Burra P, et al. Transpl Int. 2008;21(10):972 -9.
29. Tyagi AK,et al. Virol J. 2013;10:191
30. Taheri M, et al. Int J High Risk Behavior Addict. 2017;6(3):e59363.
31. L. Schmidt, et al. Infection. 2021 Jun;49(3):501-509.
32. MEKeir,MJ et al.,Annual Review of Immunology, 2008; 26, pp. 677 -704.
33. K. Grabmeier-Pfistershammer, et al. Journal of Acquired Immune Deficiency Syndromes, 2011; 56, no. 2, pp. 118 -124,
34. EuroQol G. . Health Policy 1990;16:199-208
35. Schweikert B, et al. Heart 2006;92:62-7.
36. EQ-53-3L Manual Guide [Internet]. Euroqol.org. 2021 [cited 28 March 2021]. Available from: https://euroqol.org/wp-content/uploads/2019/10/EQ-5D-3L-User-Guide_version-6.0.pdf
37. Badia X. Qual Life Res. 1999 Jun;8(4):303-10
38. Yang Y et al. STAR Protocol 2021 Apr 20;2(2):100466. doi: 10.1016/j.xpro.2021.100466. eCollection 2021 Jun 18.
39. Dirajlal-Fargo et al. J Acquir Immune Defic Syndr. 2019 Nov 1;82(3):275-280.
40. Silva M et al. PLoS One. 2021 Jan 22;16(1):e0244823. doi: 10.1371/journal.pone.0244823. eCollection 2021.
41. Nakhon Asdamongkol, et al. Jpn J Infect Dis. 2013;66(6):469-74.

14. ANNEXES:

- Scales
- Questionnaires