UNIVERSITY OF GUADALAJARA

UNIVERSITY CENTER FOR HEALTH SCIENCES

BIOMEDICAL SCIENCES RESEARCH INSTITUTE

Project title:

"Evaluation of the effect of vitamin E (α-tocopherol) supplementation on clinical activity and inflammation in patients with rheumatoid arthritis"

NCT: 06915701

April 7, 2025

Summary

Background: Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic inflammation and joint pain. The etiology of RA is multifactorial; genetic, environmental, hormonal, and epigenetic factors have been associated with it. Vitamin E is the term used for a group of tocopherols and tocotrienols, of which α-tocopherol has the greatest biological activity. The antioxidant and immunoregulatory effects of this vitamin are currently being investigated in chronic degenerative diseases, including RA.

General objective: To evaluate the effects of vitamin E (α-tocopherol) supplementation in patients with RA, as well as its relationship with the clinical activity and inflammation of the disease.

Materials and methods: A randomized, controlled, double-blind clinical trial was conducted. 46 volunteer patients with RA who met the selection criteria were recruited and divided into two groups of 23 patients: a control group (conventional treatment plus placebo) and an intervention group (conventional treatment plus 800 mg/day of vitamin E) for one month. A peripheral blood sample was obtained from both groups to be used for laboratory studies and to quantify proinflammatory cytokines IL-1β, IL-6, and TNF-α using a multiplex assay. Serum α-tocopherol was quantified using gas chromatography (GC), and antioxidant capacity was also quantified using spectrophotometry and fluorescence at baseline and after one month. At the end of the study, the blinding will be unblinded, and both groups will be compared.

Infrastructure: The study will be conducted at the Institute for Research in Biomedical Sciences (IICB) of the University Center for Health Sciences (CUCS), which has the necessary equipment such as microplate readers, centrifuges , refrigerators, ultra-freezers, clinical chemistry equipment, among others, for the implementation of the project and the storage of samples and reagents. The Clinical Analysis and Translational Research Laboratory (LACIT) of the University Center for Exact Sciences and Engineering (CUCEI) of the University of Guadalajara in the state of Jalisco has gas chromatography equipment and microplate readers for the quantification of vitamin E and its antioxidant capacity. The Rheumatology Service of the Civil Hospital "Fray Antonio Alcalde" in Guadalajara, Jalisco has consulting rooms for clinical and nutritional evaluations of patients.

Time to develop: November 2024 to January 2027.

1. Background

1.1 Rheumatoid Arthritis (RA)

RA is one of the most common autoimmune diseases, characterized by chronic inflammation of the synovium, primarily in the small joints of the hand, wrist, and foot. Chronic inflammation can lead to the destruction of articular cartilage and bone, resulting in joint deformity, functional disability, depression, and significant economic costs for the affected individual. [1] The most recent RA classification criteria are those of 2010 by the ACR/EULAR (American College of Rheumatology and European Alliance of Rheumatology Associations). These criteria include the evaluation of the involved large or small joints, painful and swollen, in addition to clinical laboratory tests such as rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPAs), C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR), and the duration of symptoms less than or greater than six weeks. [2]

1.2 Epidemiology of RA

The global prevalence of RA is 1% to 2%, and this percentage is distributed differently depending on the populations studied. In some regions of the world, the prevalence of RA may be higher or lower than average; this is suggested to be due to the involvement of genetic and environmental factors in the development of the disease. In Asia, the prevalence of RA is 0.30%, followed by Africa with 0.52%, Europe with a prevalence of 0.50% to 0.59%, and America with 0.57% to 0.86%. [2] In the case of the United States of America, the prevalence is 0.6%; however, there is a higher prevalence in indigenous populations such as the Yakima (3.4%), Chippewa (5.3%), and Pima (5.3%). [3] In Mexico, the prevalence of AR is 0.5 to 1.6% in the adult population. Within the states of the Mexican Republic studied, the highest prevalence is found in the state of Yucatan with 2.6%, followed by Chihuahua with 1.9%, Sinaloa with 1.6%, Mexico City with 0.9%, and the lowest prevalence is Nuevo Leon with 0.77%. [4]

RA most frequently occurs between the ages of 45 and 55. [4] It primarily affects women, with a ratio of 4:1. However, in patients over 75 years of age, this ratio is evenly balanced. [5] RA has one of the highest mortality rates (1.5 to 2.98) of any cardiovascular disease. Life expectancy decreases by 3 to 10 years, depending on the severity of the disease and the age at which it occurs. [4]

1.3 Etiology of RA

The etiology of RA is currently unknown, but several factors are recognized as being involved in the development of RA, including genetic, environmental, and hormonal factors. The interaction of these factors promotes the development of autoimmunity and the onset of joint inflammation.[6]

One of the most studied factors is the genetic factor, within this factor we find the *HLA* (Human Leukocyte Antigen) which is found on the surface of most cells, its main function in the immune response is antigen presentation. [7] The *HLA-DRB1 * 01, * 04* and ** 10* alleles have been associated with a high risk of developing RA while *HLA- DRB1 * 13* has been associated with protection. [8] Other risk genes for RA have been identified, such as *PADI4, PTPN22, MIF, TNFR2, CTLA4, CD28* and other molecules involved in the immune response whose degree of association with RA varies between populations. [6]

Furthermore, several environmental factors have been associated with the development of RA, such as smoking, exposure to bacterial and viral infections, and exposure to certain chemicals and metals. Smoking is the risk factor most strongly associated with the development of RA; tobacco has been shown to be associated with an increased risk of positive ACPAs and RFs. [9] It has also been suggested that exposure to wood smoke may increase elevated ACPA levels. [9]

Likewise, it is suggested that hormones exert specific and dose-dependent immunoregulatory effects: such as cortisol and androgens, which suppress humoral and cellular responses; progesterone and estrogens, which stimulate humoral responses and inhibit cellular responses; and prolactin, which exerts immunostimulatory and lymphoproliferative effects on both humoral and cellular responses. Thus, the hormonal profile can regulate the immune system through complex interactions. [9]

1.4 Pathogenesis of RA

Among the factors that may promote the development of RA, the citrullination process has been described. The antigen presentation of citrullinated peptides by antigen-presenting cells activates T cells, which differentiate into Th1 cells that activate macrophages found in the joint and have the capacity to secrete proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α). On the other hand, Th17 cells produce IL-17, IL-1, and TNF-α, which activate osteoblasts, chondrocytes, and fibroblasts. [10] Chondrocytes secrete enzymes called matrix metalloproteinases (MMPs); fibroblasts transform into fibroblast-like synoviocytes (FLS) and produce proinflammatory cytokines that destroy the extracellular matrix. [10] T cells also activate B cells to differentiate into plasma cells, which generate a wide variety of autoantibodies such as RF and ACPAs. The autoantibodies bind to their targets and form immune complexes that are recognized by neutrophils and macrophages. This entire inflammatory process generates the formation of *pannus* , which, in addition to pain, causes destruction of cartilage and bone in the joint. [10]

1.5 Pharmacological treatment of RA

Synthetic DMARDs

There is a multi-therapy regimen with conventional synthetic DMARDs, such as monotherapy, mainly with methotrexate, or triple therapy, which refers to hydroxychloroquine, sulfasalazine, and methotrexate or leflunomide. In addition, it can be approached from a "treat to target" perspective, which refers to a systematic approach that involves frequent monitoring of disease activity using validated instruments and treatment modification to minimize disease activity with the goal of achieving a predefined objective (low disease activity or remission). [23] The use of glucocorticoids in patients with RA is limited to a maximum of 8 weeks because their prolonged use is associated with a significant risk of serious adverse effects. [23] Specific indications for NSAIDs: at the beginning of treatment with DMARDs, while they take effect (usually 6 to 12 weeks). Their prolonged use is associated with a significant risk of serious adverse effects. [23]

Recommendations for the use of DMARDs

Drug	Use
Methotrexate	Patients without previous DMARD treatment with moderate to high disease activity
Hydroxychloroquine	Patients with no prior DMARD treatment and low disease activity. Used alone or in combination with methotrexate or sulfasalazine in triple therapy.
Sulfasalazine	It is recommended in patients with moderate disease activity in combination with methotrexate and hydroxychloroquine in patients who do not achieve adequate disease control with monotherapy.
Leflunomide	An alternative to methotrexate for patients who cannot tolerate it or have contraindications. Indicated for moderate to severe RA, especially erosive disease.

csDMARDs, Conventional synthetic disease-modifying drugs;

(Fraenkel et al., 2021)

Glucocorticoids (bridging therapy)

The efficacy of glucocorticoids in the treatment of rheumatoid arthritis (RA) is widely documented, demonstrating their ability to control inflammatory activity of the disease and delay the progression of joint damage. However, their use is associated with significant adverse effects, such as hyperglycemia, osteoporosis, cataracts, increased susceptibility to infections and cardiovascular events, raising concerns about their long-term safety. In recent years, the availability of targeted biologic and synthetic disease-modifying antirheumatic drugs (DMARDs) has led to a shift in RA management strategies. Current clinical guidelines advise against prolonged use of glucocorticoids, especially at high doses, and recommend limiting them to short-term bridging therapy in specific cases. [24]

1.6 Vitamin E

Vitamin E was discovered by Herbert McLean Evans in 1922 as a fat-soluble micronutrient essential for reproduction in rats. Vitamin E groups a series of compounds divided into two main ones: tocopherols and tocotriols. The natural form contained in food is called Vitamin E. [11] α -Tocopherol is the most abundant structure in the human body and the most active since it has a great antioxidant capacity, its chemical structure is composed of $C_{29}H_{50}O_2$. [12] Vitamin E is only synthesized in vegetables, mainly in vegetable oils, such as sunflower seeds, soybean oil, walnuts, peanuts and avocado. [13]

1.6.1 α-Tocopherol acetate

α -Tocopherol acetate is the most widely used analogue in dietary supplements because esterification gives it stability. Its main function is as an antioxidant, preventing lipid oxidation of cell membranes. For this reason, α -tocopherol is considered a possible protector against diseases related to oxidative processes such as chronic degenerative diseases such as diabetes mellitus, cancer, cardiovascular diseases, rheumatic diseases, neurological diseases, and aging. [14] The antioxidant capacity of vitamin E is due to the fact that its chemical structure contains a hydrocarbon tail and a chromanol ring that provide lipophilicity to be incorporated into lipid membranes or lipoproteins. [14] Its phenolic group chromanol effectively removes reactive free radicals by reducing one electron, preventing the spread of free radical reactions that can lead to lipid peroxidation, it has been reported that vitamin E has been shown to be an effective antioxidant to neutralize free radicals and protect against oxidative damage in assays such as ABTS (2,2'-azinobis (3- ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (Ferric Reducing Antioxidant Power), ORAC (Oxygen Radical Absorbance Capacity) In addition, α -tocopherol acetate is involved in the immune system by regulating prostaglandins and leukotrienes, this due to its ability to eliminate free radicals that can damage DNA and its indirect effect on the generation of T cells. [14]

The recommended daily intake of vitamin E is 15 mg/day through food for people over 19 years of age; however, to correct vitamin E deficiencies or in some pathologies, an intake of up to 450–800 mg/day is recommended, as is the case with liver disease. [11]

Specific effects of vitamin E, including gene regulation, have been studied, and now the non-antioxidant properties of tocopherols are of interest. The anti-proliferative effect of vitamin E has been observed in various in vivo and in vitro studies. [15] Regarding the kinetics of vitamin E, once administered orally, it reaches the small intestine and, together with the chylomicrons, passes through the intestine and reaches the bloodstream. In the blood, lipoprotein lipases separate

vitamin E and chylomicrons. The remaining chylomicrons carry vitamin E to the liver, mainly through the LDL receptor pathway, and then α -tocopherol is specifically recognized by the α -tocopherol carrier protein, is incorporated into VLDL and redistributed to peripheral tissues in LDL and HDL via the LDL receptor or (scavenger receptor) SR-BI receptor, and are respectively metabolized and eliminated. The α -tocopherol binding protein-mediated pathway is essential for maintaining adequate plasma α-tocopherol concentrations, as shown in Table 1.

Table 1. Serum levels of Vitamin E

Levels	Values
Sufficiency	12 and 20 µg/mL (27.9-46.4 µmol/L)
Insufficiency	5 and 12 µg/mL (11.6-27.9 µmol/L)
Deficiency	Less than 5 µg/mL (11.6 µmol/L)
(Modified from Traber, et al. 2014.)	

1.7 Vitamin E in RA

Vitamin E is attributed with pharmacological properties such as antioxidant action, cardiovascular protection, immune system support, anti-inflammatory effects, skin care benefits, and potential neurological protection. [16] Several studies have identified that cardiovascular disease is the main cause of mortality in patients with RA. However, it has also been suggested that vitamin E may have a protective role in RA. Recent research suggests that vitamin E supplementation could prevent cardiovascular disease and its complications. Główka AK et al. found that more than 85% of patients with cardiovascular disease had significant α-tocopherol deficiencies. [17] Tianyi Zhang, in a systematic review from the same year, observed that a higher intake of vitamin E was associated with a significant reduction in cardiovascular risk. [18]

Various investigations have studied the role of vitamin E as a conjugated therapy for the treatment of RA. In the study by Al-Okbi SY, the findings suggest that vitamin E could act as an inflammation modulator , improving levels of inflammatory biomarkers (ESR and pCr), seromucoids, fibrinogen, TNF-α, prostaglandin E2, oxidative stress (malondialdehyde), among others. In addition, vitamin E is attributed the ability to restore intestinal flora and improve the gastrointestinal tract, which could be related to the response to RA treatment. [19] Hama S. in 2022 analyzed the effect of tocopherol on the expression of RA-related genes in fibroblast-like synoviocytes (FLS) in a synoviocyte cell culture, where he observed that α-tocopherol inhibited the mRNA expression of *IL-6, TNF-α, MMP-3 and MMP-13* , in a murine model of laminarin-induced arthritis. [20] In 2021 Kyoung, evaluated the compound tocotrienol in a synoviocyte cell

culture and found that this molecule was associated with the decrease of the receptor activator of nuclear factor Kappa B ligand (RANKL), a protein that at high levels triggers inflammatory processes. [21] The association of vitamin E with RA has been the subject of various investigations in systemic reviews, murine models of arthritis and clinical trials, however, studies remain inconsistent. Table 2 shows the clinical trials conducted in patients with RA and vitamin E supplementation.

Table 2. Clinical trials in patients with RA and supplemented with vitamin E

Reference	Population (patients)	Dose / Time	Observations
Kolarz, G, et al (1990)	Germany (21/21)	800 mg/ day 3 weeks	Decrease in Ritchie Index
Aryaeian N, et al. (2009)	Iran (26/25/25/26)	400 mg/day 3 months	Reduction in pCr, ESR, DAS28
R, Porkodi & J. (2014).	India (43/4 2)	800 mg/day 3 months	Decrease in pCr, ESR, painful joints
Vaidya B, et al. (2020)	Nepal (106/124)	800 mg/ day 3 months	Vitamin E significantly attenuates transaminitis methotrexate-induced RA

2. Problem statement

RA is a chronic, systemic, inflammatory autoimmune disease that significantly affects the adult population worldwide, with a prevalence of approximately 1% to 2%. In Mexico, its prevalence ranges from 0.77% to 2.8%, depending on the region. Although the etiology of RA is not fully understood, its development is recognized as influenced by a combination of genetic, environmental, and hormonal factors that result in a loss of immunological tolerance to self-antigens, triggering autoimmunity.

In RA, the chronic inflammatory state promotes disease progression. Immune system cells and proinflammatory cytokines such as IL-1, IL-6, and TNF-α participate in this process. It has been proposed that vitamins play an important role as immunomodulators in pathologies associated with immune system disorders.

α-tocopherol supplementation has been observed to decrease the expression of proinflammatory cytokines such as *IL-6, TNF-α, and MMPs.* However, the mechanisms involved are unknown. There are few studies in patients with RA in which the effect of α-tocopherol supplementation has been analyzed. In clinical trials, it has been observed that, starting after 3 weeks of α-tocopherol supplementation, clinical activity scales, as well as morning stiffness and joint pain, and even biochemical parameters such as acute phase reactants such as pCr and ESR, decrease. However, the effect on proinflammatory cytokines, autoantibodies, and the antioxidant effect has not been analyzed.

Based on the above, the following research question was posed: Is supplementation with Vitamin E (α -tocopherol) for one month associated with decreased clinical activity and inflammation in patients with RA?

3. Justification

RA is a chronic, inflammatory autoimmune disease that primarily affects the joints, causing damage to cartilage and bone. It is the most common autoimmune inflammatory disease worldwide, affecting approximately 1–2% of the adult population. However, in some ethnic populations in North America, a higher prevalence is recorded, reaching 6.8%. In Mexico, the overall prevalence is 1.6% in the adult population, although in the southern region of the country, such as Yucatán, the prevalence is higher, reaching 2.4%.

RA treatment is based on the use of disease-modifying antirheumatic drugs (DMARDs), nonsteroidal anti-inflammatory drugs (NSAIDs), and glucocorticoids. Furthermore, nutritional therapy and supplementation have been proposed as complementary treatment options for patients with RA. This is because a deficiency in certain vitamins can lead to a chronic inflammatory state, as these act as inhibitors of inflammatory cytokine secretion. Recent research indicates that the majority of the Mexican population suffers from nutritional deficiencies, primarily of vitamin E.

In fibroblast cell cultures from RA patients stimulated with IL-17 plus α-tocopherol at different concentrations, inhibition of RANKL production and osteoclast formation has been observed. Meanwhile, in murine models of arthritis, it is suggested that vitamin E (α -tocopherol) could decrease the expression of proinflammatory cytokines such as *IL-6* and *TNF-α,* consequently contributing to a decrease in the activation of immune response cells such as macrophages and fibroblasts, as well as the synthesis of matrix metalloproteases (MMPs) responsible for the destruction of cartilage and articular bone.

Vitamin E supplementation at a dose of 800 mg/day has been studied in populations from India, Iran, Germany, Egypt and Nepal as an adjuvant to the treatment of RA, observing a significant decrease in biochemical parameters such as ESR, pCr, SGTP, SGOT, stiffness and joint pain.

This project is a randomized, double-blind clinical trial that aims to supplement RA patients with vitamin E insufficiency with vitamin E for one month and evaluate the effect of vitamin E on clinical activity and inflammation in these patients. The risk is considered greater than minimal due to the use of randomized methods for assigning patients to vitamin E supplementation or placebo. This decision is based on the need to ensure the rigor and objectivity of the study. It is important to note that to date, no harmful interactions between vitamin E and standard RA treatment, which is based on DMARDs, glucocorticoids, and NSAIDs, have been reported, according to the PubMed, UpToDate, Drugs.com, and DrugBank platforms.

The suggested dose for supplementation is 800 mg/day is based on previous clinical trials that have demonstrated its potential anti-inflammatory effect and decreased RA activity. In addition, a study conducted by Dysken MW et al. in 2014 evaluated the safety of vitamin E for one year and no adverse effects were observed with supplementation. The supplement intended to be used is Tocofersolan (α-tocopherol), which has health registration with COFEPRIS (006V2002 SSA) and is manufactured by Gelpharma, SA de C. V (The researchers declare no conflict of interest). This supplement will be covered by the researchers. Supplementation of patients with vitamin E capsules will be from the same batch to ensure consistency, reproducibility, and validity of the results.

Based on the above, we are interested in determining whether supplementation with Vitamin E (α -tocopherol) for one month has effects on clinical activity and inflammation in patients with RA from Western Mexico.

4.Objectives

a. General objective

Evaluate the effects of supplementation with Vitamin E (α-tocopherol) in patients with RA, as well as its relationship to clinical activity and inflammation of the disease.

b. Specific objectives

1. To analyze the clinical characteristics and diet quality of patients with RA
2. To determine serum levels of autoantibodies in patients with RA
3. To quantify serum vitamin E levels before and after supplementation in both study groups (vitamin E and placebo)
4. To compare clinical activity before and after supplementation in both study groups
5. To quantify the serum concentration of the proinflammatory cytokines IL1β, IL6 and TNF-α before and after supplementation in both study groups
6. To determine the antioxidant capacity before and after supplementation in both study groups
7. To associate vitamin E levels with clinical and inflammatory parameters in patients with RA

5. Hypothesis

There is an association between supplementation with Vitamin E (α -tocopherol) and the decrease in clinical activity and inflammation in patients with RA from Western Mexico

6. Methodological design

a) Type of study

- Randomized, controlled, double-blind clinical trial.

- Randomization: Simple randomization will be performed. The study will consist of two groups: control and intervention. Participants will be randomly assigned using a random selection process, using the OxMaR system (an acronym for Oxford Minimization and Randomization), a validated software. Randomization will be performed by a researcher independent of the project.
- Definition of patients:
- Female patients with RA will be included due to the prevalence and characteristics of the disease: a higher incidence in women and differences in clinical presentation, as they tend to present more severe signs and symptoms. Furthermore, the influence of hormonal factors is considered, given that female sex hormones have a significant impact on the modulation of the immune system. The study also seeks to improve the validity and applicability of the results through sample homogeneity, which reduces variability in treatment response associated with sex-related hormonal and genetic factors.
- Patients with vitamin E deficiencies (<15 mg/day) who are receiving conventional synthetic DMARDs (methotrexate, hydroxychloroquine, leflunomide, sulfasalazine, and their combinations) for more than one month. The intervention group will receive vitamin E at a dose of 800 mg/day, divided into two doses of 400 mg each. The control group will receive a magnesium oxide placebo at a dose of 200 mg/day. Participants will consume one capsule of vitamin E or placebo after their morning and evening meals.
- Patients receiving glucocorticoids will not be included, as current clinical guidelines advise against prolonged use of glucocorticoids, especially at high doses, and recommend limiting them to short-term bridging therapy in specific cases. Furthermore, it is suggested that treatment with these drugs may mask symptoms such as joint pain and decrease inflammatory parameters (acute phase reactants and proinflammatory cytokines).
- Use of placebo: Participants will be informed that they could be taking either vitamin E or placebo. Both vitamin E and placebo will have similar excipients and will be masked by similar packaging.
- Double-blinding: To minimize bias in the study, it is important that both the researchers involved and the participants be blinded as to who is receiving the active treatment and who is receiving the placebo. Once the one-month supplementation period and data collection are

over, the study will be unblinded. A researcher independent of the project will maintain the double-blinding information.

- During the study, potential adverse events will be identified through scheduled consultations and spontaneous reports from participants, who will have access to a 24-hour communication channel. All events will be recorded in a standardized format (Naranjo Questionnaire and Adverse Event Recording Form), evaluated by the principal investigator or a safety committee, and classified according to their severity and relationship to treatment. Mild or moderate events will receive symptomatic treatment, while severe events will be reported to the appropriate authorities, with treatment suspension if necessary. Each affected participant will be monitored until the event is completely resolved, ensuring their safety and an appropriate response at all times.

b) Research venues

- Institute for Research in Biomedical Sciences (IICB) of the University Center for Health Sciences (CUCS)

- Clinical Analysis and Translational Research Laboratory (LACIT), at the University Center for Exact Sciences and Engineering (CUCEI)

- Rheumatology Service of the “Fray Antonio Alcalde” Civil Hospital in Guadalajara, Jalisco.

c) Study period

November 2024 to January 2027.

d) Inclusion criteria

Female sex, RA classification (ACR/EULAR 2010), early RA ≤ 2 years, treatment with conventional synthetic DMARDs (methotrexate, hydroxychloroquine, leflunomide, sulfasalazine and their combinations) for more than one month , DAS28 ≥3.2, vitamin E deficiency (<15 mg/day), no comorbidities: arterial hypertension, type 2 diabetes, dyslipidemias, severe interstitial lung disease, active or recent cancer (less than 5 years in remission), chronic infections (active tuberculosis, hepatitis B or C, HIV) , age > 18 years, voluntary participation and signing of informed consent.

e) Exclusion Criteria

Liver and kidney diseases, overlap syndrome with other autoimmune diseases, coagulation disorders, pregnancy, consumption of supplements (iron, vitamin E and/or K), and medications such as aspirin, amlodipine, estrogens , glucocorticoids and medications used in the treatment of dyslipidemia in the last three months.

f) Elimination Criteria

Errors in supplement administration ≥20% : The treatment adherence form (Annex 14) will be used as the tool to be used, recording daily vitamin/placebo intake. Patients who fail to consume at least 80% of the total daily intake (48 capsules) will be eliminated from the study. Patients with adverse effects from the supplement (Annexes 15 and 16), pregnancy during the study, insufficient blood sample collection, and voluntary withdrawal of informed consent may be excluded.

g) Sample Size

The two-means statistical formula was used to calculate the sample size. To do this, the following data are required: Identify whether the hypothesis is unilateral or bilateral. Establish alpha and beta levels, and use them to determine the K value (Zα and Zβ). The difference in measurements we would expect to find or want to be able to detect in our study (μ1 - μ2), the expected standard deviation for each group .

Calculations were performed using data from the 2001 clinical trial by Mona Helmy et al., resulting in a sample size of 19 patients plus a 20% increase to cover potential losses.

Data: Serum vitamin E levels: start: 0.64 mg/dL and end: 0.86 mg/dL

K value: 15.8 with a statistical power of 95%.

Standard deviation of vitamin E levels: start: 0.16 mg/dL and end: 0.18 mg/dL

Twenty-three patients were included in the control and intervention groups. Both groups had RA from the Rheumatology Service of the “Fray Antonio Alcalde” Civil Hospital in Guadalajara, Jalisco.

$$n = \frac{K(\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)^2}$$

Formula substitution:

$$n = \frac{15.8(0.16^2 + 0.18^2)}{(0.86 - 0.64)^2} = 18.93$$

18.93 + 20% in case of loss = 22.7 = **23 patients per group**

h) Variables

Independent variables

- Vitamin E

Dependent variables

- Inflammatory cytokines (IL-1β, IL-6, and TNF-α). Antioxidant capacity (DPPH, ABTS, FRAP, and ORAC). Clinical course: DAS-28. Acute phase reactants: pCr and ESR. Autoantibodies: Anti-CCP and Rheumatoid factor.

Operationalization of variables			
Variable	**Interrelationship**	**Nature**	**Indicator**
Vitamin E	Independent	Quantitative	µg/dl
Proinflammatory cytokines (IL-1β, IL-6 and TNF-α)	Dependent	Quantitative	pg/ml
VSG	Dependent	Quantitative	mm/hr
C-reactive protein	Dependent	Quantitative	mg/l
Anti-CCP	Dependent	Quantitative	UR/ml
Rheumatoid factor	Dependent	Quantitative	IU/ml
Antioxidant capacity (DPPH, ABTS, FRAP and ORAC)	Dependent	Quantitative	µmol/L
DAS28	Dependent	Quantitative	Remission: < 2.6 Low activity: ≥ 2.6 <3.2 Moderate: ≥ 3.2 < 5.1 High: ≥ 5.1

i) Biosecurity considerations

In this study, the guidelines established in the Mexican Official Standards, NOM-052-SEMARNAT-2005, will be applied. NOM-054-SEMARNAT-1993, NOM-003-SSA2-1993 and NOM-087-ECOL-SSA1-2002 which refer to the classification, handling, storage and disposal of hazardous waste, chemical reagents and biological-infectious, in order to guarantee the protection of people who are in contact with them and the environment (annex 1 and 2).

The biological material to be used will be whole blood, plasma, and serum. These biological samples will be obtained from RA patients in an in vivo experimental model (clinical trial). Blood sampling will be performed by trained personnel. Sharps will be disposed of in rigid red

polypropylene containers, based on the Classification of Establishments Generating Hazardous Biological-Infectious Waste in NOM-087-ECOL-SSA1-2002. This procedure will be performed by a PhD student and a Rheumatology resident in the Rheumatology Service of the Fray Antonio Alcalde Civil Hospital.

The blood collection tubes will be discarded in red polyethylene bags for final disposal (Annex 1) and the residues from the ESR determination will be inactivated with sodium hypochlorite with a concentration of 4 to 7% free chlorine, and added in such a proportion to the blood or its components, a final concentration of free chlorine of 0.4 to 0.7% is achieved, maintaining it in this way for one hour, prior to disposal based on NOM-003-SSA2-1993. The quantification of ACPA and cytokines will be carried out using the ELISA and MAGPLIX techniques with commercial kits. The hazardous chemical waste is 3,3 ', 5,5'-Tetramethylbenzidine and sulfuric acid and will be treated according to the specifications (annexes 3 and 6). Once the experiment is finished, the ELISA plates will be discarded in a red polyethylene bag for final disposal (Annex 1) . Furthermore , the technique requires the use of sulfuric acid, which will be neutralized with sodium bicarbonate before being discarded. Commercial kits will be used for the quantification of PCR and RF by turbidimetry; none of these kits contain chemical reagents that require special attention for handling and disposal (Annexes 4 and 5).

In the case of antioxidant capacity using the ABTS, DPPH, ORAC, and FRAP methods, residues such as peroxodisulfuric acid, 2,2-diphenyl-1-picrylhydrazyl acid, 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH), TPTZ (2,4,6-Tris (2-pyridyl) -s-triazine) and ferrous sulfate are generated (annexes 7, 8, 9, 10), these residues are inactivated with ascorbic acid and sodium bicarbonate. Finally, the plate is discarded in a red polyethylene bag for final disposal (Annex 1).

For the inactivation and disposal of reagents for vitamin E (α-tocopherol) quantification by gas chromatography in accordance with the provisions of NOM-052-SEMARNAT-2005 and NOM-054-SEMARNAT-1993, the solvents used, such as hexane, methanol, and isopropanol, will be collected in specific containers designed to contain liquid hazardous waste, clearly labeled with the contents, date of generation, and hazardous characteristics (flammability and toxicity) (annexes 11, 12, and 13). Likewise, the glass vials must be stripped of as much liquid waste (solvents) as possible. These vials must be deposited in rigid, chemical-resistant plastic containers with lids, clearly labeled as glass hazardous waste. In the case of storage and disposal of nitrogen and hydrogen gases in gas chromatography, appropriate cylinders for each gas will be used, ensuring that they comply with safety specifications. The cylinders will be

stored in well-ventilated areas, away from heat sources or sparks. Finally, the waste generated will be sent to companies authorized for the treatment of hazardous waste for final disposal.

The IICB and LACIT have the essential materials, equipment, and facilities for the proper development of the project, including sterile materials, gloves, face masks, centrifuges, refrigerators, autoclaves, stainless steel sinks, and tables. 70% ethanol will be used to disinfect the work area and non-disposable materials (e.g., micropipettes). In addition, the laboratories have a dedicated area for the storage and temporary confinement of chemical reagents, as established in NOM-054-SEMARNAT-1993.

All researchers involved in the project will work under the IICB and LACIT regulations, being aware of the standards described above for their personal protection and handling of chemical and biological-infectious products. Collaborators on this project are familiar with the safety codes included on the labels of the reagent bottles that will be used. In the event of an accident, the area will be secured and the Internal Civil Protection Unit will be notified. Building E, CUCS. Tel. 10585200, Ext. 3394, or the CUCEI Emergency Response Security Unit. Calz. Revolución 1500, Olímpica, 44840 Guadalajara, Jal., Mexico. Tel. 3332348411.

The supplement intended to be used is Tocofersolan (α-tocopherol), which has health registration with COFEPRIS (006V2002 SSA) and is manufactured by Gelpharma, SA de CV, while the placebo is manufactured by Botica Toledo and will contain 200mg of magnesium oxide.

The researchers responsible for this project declare that there are no conflicts of interest related to the objectives, methodologies, or results of this study. No member of the research team has any financial, commercial, or personal interests that could influence the conduct, interpretation, or presentation of the project results.

j) Ethical considerations

The project will be conducted in accordance with the ethical standards and principles for medical research involving human subjects, as set out in the Declaration of Helsinki, last revised at the 64th General Assembly in Fortaleza, Brazil, in October 2013. The Declaration also refers to the ethical principles for medical research involving human subjects.

Participation of subjects included in the study will be voluntary, with prior signing of the informed consent letter (Annex 14) prepared in accordance with the provisions of guidelines 9, 10 and 12.

- Guideline 9: Equitable inclusion of research participants, ensuring that the benefits and risks of research are distributed fairly among all groups involved.

- Guideline 10: Respect for informed consent, ensuring that this process is understandable, voluntary, and appropriate to the cultural and social context of the participants.

  Guideline 12: Ensure that research involving vulnerable participants is conducted fairly and ethically, avoiding any type of abuse, and ensuring that these groups receive equal rights.

As well as Appendix 2 (which guarantees fair, safe, and respectful treatment of all participants) of the guidelines developed by the Council for International Organizations of Medical Sciences (CIOMS), Title Two of the General Health Law on Health Research, Articles 21 (research on human beings may only be carried out when the human rights of the subjects are respected), and 22 (informed consent is essential for entering the protocol and must be given freely and voluntarily). Individual treatment of each patient will be the same for all, always with respect and protection of their integrity. Participation in this study will not generate any cost for the patient, and only the essential resources to carry out this project will be used.

All personal data provided by the patient is absolutely confidential and will be treated in accordance with the General Law on the Protection of Personal Data Held by Private Parties. Participants will not be identified in any reports or publications resulting from this study. In all documents, including laboratory test results, the participant will be identified with a code that includes a number and their initials to ensure the anonymity of their data. The personal data provided by the participant may only be reviewed by the principal investigator, a regulatory entity, or an auditor if verification is necessary. Furthermore, we guarantee that the processing of this data will be legitimate, transparent, and appropriate, and always within the purposes established for this project. When the blood sample is collected, the participant will be assigned a unique code, which will be used to label the blood sample tubes.

The researcher in charge of the study is responsible for the processing and protection of the personal data obtained and will ensure that they are protected in accordance with the provisions of the General Law on the Protection of Personal Data Held by Private Parties and the General Law on the Protection of Personal Data Held by Obligated Subjects. The personal data requested will be used exclusively for the purposes outlined in this project, thereby ensuring compliance with data protection regulations and safeguarding the privacy of individuals, both in the private and public spheres, in accordance with applicable laws.

All participants will receive a brochure entitled "Comprehensive Wellness for Patients with Rheumatoid Arthritis," prepared by nutrition and fitness experts, which includes healthy eating

recommendations and physical activity routines tailored to their condition. They will also receive a workshop on physical activity at the Rheumatology Service facilities and nutritional guidance.

In case the patient requires medical attention due to an adverse effect of vitamin E supplementation, he/she will be asked to contact the principal investigator to arrange care in a hospital (agreement in process with the FELMAN Hospital, Annex 18).

This study has been approved by the Research and Ethics Committees of the Fray Antonio Alcalde Civil Hospital (Annex 15). Once the protocol is approved by the committees responsible for evaluating research projects at CUCS, this project will be registered in Clinical Trials, for which information has already been requested and the Workshop for Strengthening Good Practices in Clinical Trials was attended, organized in collaboration with the Good Clinical Trials Collaborative (GCTC) and The Global Health Network (TGHN).

7. Methodology

7.1 Clinical Evaluation

7.2 Medical record

The clinical evaluation will be carried out by a rheumatology specialist, who will perform a general physical examination and apply clinical evaluation indices such as:

DAS28 Clinical Activity Index : This index measures disease activity in patients with rheumatoid arthritis (RA). It is calculated using four variables: the red blood cell sedimentation rate (ESR), the number of swollen joints, the number of painful joints (evaluating a total of 28 joints), and a visual analog pain scale. A mathematical formula is then applied, providing a score from 0 to 10. A value greater than 5.1 indicates high disease activity, a value less than 3.2 indicates low activity, and a value less than 2.6 indicates disease remission.

SDAI (Simplified Disease Activity Index): The SDAI is a more simplified disease activity index compared to the DAS28. It is based on five parameters: the number of swollen joints, the number of tender joints, the visual analogue pain scale score, the ESR (or C-reactive protein), and the patient's global status. The total score can range from 0 to 86, and like the DAS28, values above 26.0 indicate high disease activity, while values below 3.3 reflect disease remission.

CDAI (Clinical Disease Activity Index): The CDAI is similar to the SDAI, but differs in that it does not include laboratory measurements, i.e., it does not use ESR or C-reactive protein. It is

calculated by adding the following parameters: number of swollen joints, number of tender joints, the score on the visual analog pain scale, and the patient's overall condition. The total score ranges from 0 to 76, and like the SDAI, values less than 10 indicate disease remission, while values greater than 22.0 indicate high disease activity.

HAQ-DI (Health Assessment Questionnaire Disability Index): The HAQ-DI is a tool used to measure the level of physical disability in patients with chronic diseases, such as rheumatoid arthritis. It assesses a patient's functional ability in eight categories: dressing, bathing, eating, getting out of bed, walking, reaching, bending, and performing activities of daily living. Each category is scored from 0 to 3 (with 0 being no difficulty and 3 being maximum difficulty), and the total score is obtained by adding the scores for each category and dividing by the total number of categories assessed. A higher score indicates greater functional disability.

7.3 24-hour reminder

It will be performed by the nutritionist, who will ask about the foods consumed the previous day and thus assess the quality of the diet and vitamin E intake.

The 24-Hour Recall (Appendix 17) is a subjective, retrospective method conducted through an in-person or telephone interview. This method relies on accurate recall, describing and quantifying the foods consumed the day before the visit. All food intake, including time and place, must be described in detail. To ensure greater reliability of the survey results, questions about food consumption will be asked on three days (Tuesday, Thursday, and Saturday). The information provided is then analyzed using specialized software (Nutritionist Pro™) to count calories, macronutrients, and micronutrients.

7.4 Supplementation

Study participants with vitamin E deficiency, as determined by a 24-hour recall, will receive either 800 mg of α-tocopherol in capsules daily, divided into two 400 mg doses (one after breakfast and one after lunch), or a placebo consisting of 200 mg of magnesium oxide. Both the supplement and placebo will be packaged identically to ensure double-blinding, preventing both participants and researchers from knowing the treatment assignment. In addition, each participant will be given a diary to assess treatment adherence, as detailed below.

7.5 Patient Diary: Treatment Adherence

The World Health Organization (WHO) defines adherence as the degree to which a patient's medication-taking behavior corresponds to the recommendations agreed upon with the

healthcare professional. This document allows for monitoring adherence to treatment. Indirect methods are used: **Self-reporting diaries** : Participants document their supplement intake; and **Capsule counting** : The remaining capsules are compared with the expected number according to the prescribed regimen.

7.4 Clinical analysis

7.4.1 Blood sampling

The invitation to participate will be sent **before** the vitamin E intake assessment, as this is part of the clinical trial screening. Subsequently , a BD Vacutainer SST™ tube with a gold cap and a capacity to collect 5 ml of blood will be collected. It contains a polymer gel, silicone coating, and will be protected from sunlight or UV exposure to obtain serum. In addition, another BD Vacutainer tube with anticoagulant (EDTA) with a capacity to collect 4 ml of blood will be collected for ESR determination.

7.4.2 Quantification of serum vitamin E

Serum vitamin E quantification in patients with RA will be performed using the gas chromatography (GC) method. It will start with serum obtained from a peripheral blood sample. Once the serum is separated, aliquots will be made in 0.6 mL amber microtubes to prevent vitamin E degradation due to exposure to UV light, they will be labeled and stored at -80 ° C until the day of the assay. This assay uses a mobile phase which is a carrier gas (nitrogen), a pump that has the function of exerting a constant flow of gases and a sample of the mobile phase, an injector where the patient's serum (10 µL) will be placed. This technique also consists of a stationary phase in which there is a fused silica capillary column, with a non-polar stationary phase, where the separation of the analytes of interest will take place according to the non-covalent interactions of the compounds with the column. It also has a flame ionization detector (FID) that detects the amount of analyte present in the sample. It also has specialized software for programming the flow rate, run times, and retention times.

7.4.3 Acute phase reactants

Clinical analyses will be performed on samples from RA patients with vitamin E deficiency using automated methods at the IICB. These analyses will include:

- PCR, by turbidimetry. (Mindray, BS-120, Chemistry Analyzer, Shenzhen, China) Serum C-reactive protein (CRP) causes agglutination of latex particles coated with anti-human C-reactive protein antibodies. Latex particle agglutination is proportional to the PCR concentration and is quantified by turbidimetry.

- ESR by Wintrobe. One milliliter of the anticoagulated sample is transferred to each Wintrobe tube and held upright at 90° ^{C} for one hour. ESR is measured visually, and the result is corrected for the patient's hematocrit using the nomogram.

7.4.4 Immunological analysis

To quantify serum levels of cytokines and autoantibodies in the study groups, serum will be obtained from a peripheral blood sample. The blood will be collected in a vacuum tube with a separating gel and centrifuged for 10 minutes at 3,000 rpm. Once the serum is separated, aliquots will be made into 0.6 mL microtubes, labeled, and stored at -80°C until the day of the assay.

7.4.5 Quantification of RF antibodies

RF quantification will be performed by turbidimetry using semi-automated equipment (Mindray, BS-120, Chemistry Analyzer, Shenzhen, China). This assay relies on the agglutination of latex particles coated with immunoglobulin G, to which the RF binds.

7.4.6 Quantification of anti-CCP antibodies

The anti-CCP assay (EA 1505-9601G, EUROIMMUN Medizinische Labordiagnostika AG) will be used, which allows the detection of IgG autoantibodies in human serum directed against the synthetic cyclic peptide containing citrulline residues. The plate wells are coated with the highly purified citrullinated peptide.

7.4.7 Quantification of cytokines IL-1β, IL-6 and TNF-α

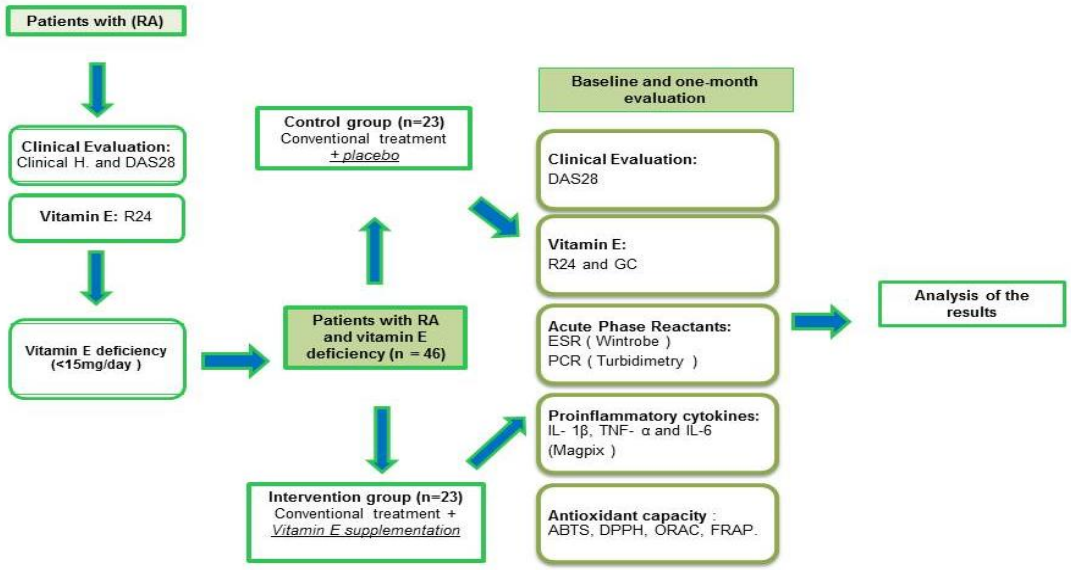
Cytokine quantification will be performed using a multiplex assay (15-Plex #171-AA001M Bio-Plex BIO-RAD), using the MAGPIX platform. This assay uses paramagnetic microspheres whose interior is differentially labeled with combinations of fluorophores within the red and infrared range. This allows each group of spheres (corresponding to each cytokine) to be identified at a certain wavelength by the equipment. On its external surface, each sphere is covered with a capture antibody specific for the cytokine of interest. This allows the simultaneous quantification of all cytokines in a single sample, since the reaction well contains thousands of microspheres with different specificities for the cytokines.

7.4.8 Quantification of antioxidants (DPPH, ABTS, FRAP and ORAC)

The antioxidant capacity measured by these assays is crucial for understanding the potential of various compounds to neutralize free radicals and other oxidants, which is important in nutritional and pharmacological research, and in the development of food and supplement

products. For the quantification of DPPH (2,2-diphenyl-1-picrylhydrazyl): It is based on the reduction of the free radical DPPH, which changes color from violet to yellow when reduced. For ABTS (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]): It involves the generation of the ABTS+ radical, which is reduced in the presence of antioxidants. In the case of FRAP (ferric reducing power): It measures the antioxidant capacity based on the reduction of the ferric ion (Fe^3+) to ferrous (Fe^2+), forming a blue ferrous-TPTZ complex. And finally, the change in absorbance is analyzed. For ORAC (Oxygen Radical Absorbance Capacity): This assesses antioxidant capacity based on the inhibition of free radical-induced oxidation on a fluorescent substrate (usually fluorescein). Free radicals are generated (usually by AAPH) and attack the fluorescent substrate. The presence of antioxidants in the sample protects the substrate, and the decrease in fluorescence is measured.

7.5 General diagram of the methodology



8.0 Project viability

a) Funding: The project will be supported in part by funding from the Support Program for the Improvement of Production Conditions for SNII Members and SNCA-PROSNII, which the researchers comprising the working group will apply for. The project also intends to participate in calls for proposals such as PIN, FRONTERA, and/or FODESIJAL.

b) Infrastructure and equipment: The IICB and LACIT have the necessary equipment and

supplies to perform various tests. The IICB uses equipment such as the Thermo Fisher Scientific Multiskan Go for autoantibody determination, the Mindray BS120 for acute phase reactants, and the MAGPIX system for proinflammatory cytokines. The LACIT is equipped with Agilent Technologies gas chromatography to measure serum vitamin E levels and Thermo Fisher Scientific equipment to evaluate antioxidant capacity markers. In addition, the Rheumatology Service at the Fray Antonio Alcalde Civil Hospital has dedicated consulting rooms for clinical and nutritional evaluations.

c) Human Resources Training: Students of the PhD in Pharmacology and the Rheumatology Specialty at the University of Guadalajara

d) Deliverables resulting from the research: This will be planned to encompass different platforms and formats, ensuring the dissemination of these findings and their accessibility to the scientific community and the general public. This strategy will include:

1. Conferences, congresses, and symposia: both national and international, offering an opportunity to interact with expert researchers, receive valuable feedback, and establish future collaborations.
2. Presentations at academic events: such as doctoral seminars, workshops, and conferences both within and outside the University of Guadalajara.
3. Publications in scientific journals: Publications in high-impact journals will be prioritized. Partial results will focus on specific aspects of the research, while the final results will integrate the main findings and their implications.
4. Technical reports: These reports will be shared with collaborators and regulatory bodies, enabling the progress and results of the research to be seen, ensuring transparency and compliance with objectives.

References:

1. Cush JJ. Rheumatoid arthritis: early diagnosis and treatment. Med Clin North Am. 2021;105(2):355-65.
2. Aletaha D, Neogi T, Silman AJ, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62(9):2569-81.
3. Castillo-Cañón JC, Trujillo-Cáceres SJ, Bautista-Molano W, et al. Rheumatoid arthritis in Colombia: a clinical profile and prevalence from a national registry. Clin Rheumatol. 2021.
4. Moreno-Montoya J, Alvarez-Nemegyei J, Sanin LH, et al. Association of regional and cultural factors

with the prevalence of rheumatoid arthritis in the Mexican population: a multilevel analysis. J Clin Rheumatol. 2015;21(2):57-62.

5. Almutairi K, Nossent J, Preen D, Keen H, Inderjeeth C. The global prevalence of rheumatoid arthritis: a meta-analysis based on a systematic review. Rheumatol Int. 2021;41(5):863-77.

6. Muñoz-Valle JF, Padilla-Gutiérrez JR, Hernández-Bello J, et al. PTPN22 -1123G>C polymorphism and anti-cyclic citrullinated protein antibodies in rheumatoid arthritis. Med Clin (Barc). 2017;149(3):95-100.

7. Busch R, Kollnberger S, Mellins ED. HLA associations in inflammatory arthritis: emerging mechanisms and clinical implications. Nat Rev Rheumatol. 2019;15(6):364-81.

8. Padyukov L. Genetics of rheumatoid arthritis. *Semin Immunopathol*. 2022;44(1):47-62.

9. Orellana C, Saevarsdottir S, Klareskog L, et al. Oral contraceptives, breastfeeding and the risk of developing rheumatoid arthritis: results from the Swedish EIRA study. *Ann Rheum Dis*. 2017;76(11):1845- 52.

10. Mueller AL, Payandeh Z, Mohammadkhani N, et al. Recent advances in understanding the pathogenesis of rheumatoid arthritis: new treatment strategies. *Cells*. 2021;10(11):3017.

11. Traber MG, Head B. Vitamin E: how much is enough, too much and why? *Free Radic Biol Med*. 2021;177:212-25.

12. Mohd Zaffarin AS, Ng SF, Ng MH, et al. Pharmacology and pharmacokinetics of vitamin E: nanoformulations to enhance bioavailability. *Int J Nanomedicine*. 2020;15:9961-74.

13. Brigelius-Flohé R. Vitamin E research: past, now and future. *Free Radic Biol Med*. 2021;177:381-90.

14. Kemnic TR, Coleman M. Vitamin E deficiency. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023.

15. Lewis ED, Meydani SN, Wu D. Regulatory role of vitamin E in the immune system and inflammation.*IUBMB Life*. 2019;71(4):487-94.

16. Galli F, Azzi A, Birringer M, et al. Vitamin E: emerging aspects and new directions. *Free Radic Biol Med*. 2017;102:16-36.

17. Główka AK, Kowalówka M, Burchardt P, et al. Selected psychosocial factors, nutritional behavior, and the analysis of concentrations of selected vitamins in patients with cardiovascular diseases. Nutrients. 2024;16(12):1866.

18. Zhang T, Yi X, Li J, et al. Vitamin E intake and multiple health outcomes: an umbrella review. Front Public Health. 2023;11:1035674.

19. Al-Okbi SY. Nutraceuticals of anti-inflammatory activity as complementary therapy for rheumatoid arthritis.*Toxicol Ind Health*. 2014;30(8):738-49.

20. Hama S, Kirimura N, Obara A, et al. Tocopheryl phosphate inhibits rheumatoid arthritis-related gene expression in vitro and ameliorates arthritic symptoms in mice. *Molecules*. 2022;27(4):1425.

21. Kim KW, Kim BM, Won JY, et al. Tocotrienol regulates osteoclastogenesis in rheumatoid arthritis. *Korean J Intern Med*. 2021;36(Suppl 1):S273-S82.

22. Velasco R. Víctor Manuel. Sampling and sample size: A practical guide for health professionals

conducting research. 2003.

23. Fraenkel L, Bathon JM, England BR, et al. 2021 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis. Arthritis Care Res (Hoboken). 2021;73(7):924-939.

24. Doumen, M., Pazmino, S., Bertrand, D., Westhovens, R., & Verschueren, P. (2023). Glucocorticoids in rheumatoid arthritis: Balancing benefits and harm by leveraging the therapeutic window of opportunity. *Joint bone spine* , *90* (3), 105491.