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Impacto de una intervención mediante suplementación mineral en mujeres posmenopáusicas sobre la defensa antioxidante y el estatus nutricional de la vitamina D

Impact of a mineral supplementation intervention in postmenopausal women upon antioxidant defense and vitamin D nutritional status

UNIVERSIDAD DE GRANADA

Programa de Doctorado en Nutrición y Ciencias de los Alimentos

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Universidad de Granada

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TESIS DOCTORAL CON MENCIÓN INTERNACIONAL

HÉCTOR VÁZQUEZ LORENTE

Granada, 2023

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Memoria que presenta para aspirar al Grado de Doctor con Mención Internacional por la Universidad de Granada D. Héctor Vázquez Lorente.

La presente Tesis Doctoral ha sido realizada bajo la dirección de:

Dra. Dña. Elena María Planells del Pozo

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Aspirante al Grado de Doctor con Mención Internacional

Granada, 2023

Dra. Dña. Elena María Planells del Pozo, Catedrática de la Universidad de Granada.

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Directores de la Memoria de Tesis Doctoral de Título: "Impacto de una intervención mediante suplementación mineral en mujeres posmenopáusicas sobre la defensa antioxidante y el estatus nutricional de la vitamina D", realizada por D. Héctor Vázquez-Lorente, autorizan su presentación ante el Tribunal que en su día se designe.

Y para que así conste, y en cumplimiento de las disposiciones vigentes, firman la presente Tesis Doctoral el 14 de junio de 2023.

Fdo. Elena María Planells del Pozo Fdo. Jorge Molina López

El doctorando D. Héctor Vázquez Lorente y los directores de la tesis la Dra. Dña. Elena María Planells del Pozo y Dr. D. Jorge Molina López.

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

En Granada, a 14 de junio de 2023

Directores de la tesis

Fdo. Elena María Planells del Pozo Fdo. Jorge Molina López

Doctorando

Fdo. Héctor Vázquez Lorente

El doctorando D. Héctor Vázquez Lorente ha realizado la presente Tesis Doctoral como beneficiario de una beca-contrato con cargo al programa de Formación de Profesorado Universitario (FPU18/03655) del Ministerio de Educación, Cultura y Deporte, por resolución del 12 de junio de 2019, de la Secretaría de Estado de Educación, Formación Profesional y Universidades.

El doctorando D. Héctor Vázquez Lorente ha reflejado en la presente Tesis Doctoral el uso de suplementos de Zinc suministrados por SM Natural Solutions, Sabadell, España (Ref: 0B62713821) y de Magnesio proporcionados por Botánica Nutrients SL, Sevilla, España (Ref: B91070797). A pesar de haber utilizado suplementos de ambas casas comerciales y haber obtenido resultados a consecuencia de su administración, cabe destacar que tanto el doctorando como ningún miembro del grupo de investigación que ha participado en la Tesis Doctoral, no presentaron ningún conflicto de interés ni recibieron algún tipo de retribución económica.

El trabajo de investigación que constituye esta Tesis Doctoral titulada "Impacto de una intervención mediante suplementación mineral en mujeres posmenopáusicas sobre la defensa antioxidante y el estatus nutricional de la vitamina D", se engloba en el marco de los Proyectos de Investigación financiados por el Plan Propio de la Universidad de Granada y por el Fondo de Investigación Sanitaria (FIS) del Instituto de Salud Carlos III, referencias PI07/1228 y PI10/01993, además de la red Zinc-Net-The Network for the Biology of Zinc (FA COST Action TD1304).

La presente Tesis Doctoral se ha realizado en el Instituto de Nutrición y Tecnología de los Alimentos "José Mataix" del Centro de Investigación Biomédica y en el Departamento de Fisiología de la Facultad de Farmacia en colaboración con el Centro de Instrumentación Científica de la Universidad de Granada (Granada, España). Adicionalmente a la Universidad de Granada, este trabajo ha sido realizado en colaboración tanto a nivel nacional con (I) el Hospital Universitario Virgen de la Nieves (Granada, España), (II) la Farmacia Lamenca (Granada, España), y (III) el Departamento de Didácticas Integradas de la Facultad de Ciencias de la Educación, Psicología y Deporte de la Universidad de Huelva (Huelva, España) como a nivel internacional en (I) la Facultad de Medicina de la Universidad de Belgrado (Belgrado, Serbia), y (II) la Facultad de Agrociencias de la Universidad de Mendel en Brno (Brno, República Checa).

"Acepta los desafíos para que puedas

sentir la euforia de la victoria"

(George S. Patton)

A mis padres, Ana y Joaquín, fuisteis,

sois, y seréis mi gran escudo en

momentos de guerra

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ÍNDICE DE CONTENIDOS

1. ÍNDICE DE ABREVIATURAS

- ANOVA / ANOVA = Análisis de la Varianza / Analysis of Variance
- ADN / DNA = Ácido Desoxirribonucleico / Deoxyribonucleic acid
- ARN / RNA = Ácido Ribonucleico / Ribonucleic Acid
- B_{12} / B_{12} = Vitamina B_{12} / Vitamin B_{12}
- Ca / Ca = Calcio / Calcium
- CAT / TAC = Capacidad Antioxidante Total / Total Antioxidant Capacity
- CC / BC = Composición Corporal / Body Composition
- CCI / ICC = Coeficiente de Correlación Interclase / Interclass Correlation Coefficient
- CIC / SIC = Centro de Instrumentación Científica / Scientific Instrumentation Center
- CFCA / FFQ = Cuestionario de Frecuencia de Consumo de Alimentos / Food Frequency Questionnaire
- CL–EMT / LC–MS/MS = Cromatografía Líquida–Espectrometría de Masas en Tándem / Liquid Chromatography–Tandem Mass Spectrometry
- CT / TC = Colesterol Total / Total Cholesterol
- Cu / Cu = Cobre / Copper
- CV / CV = Coeficiente de Variación / Coefficient of Variation
- PUD / DBP = Proteína de Unión a la Vitamina D / Vitamin D Binding Protein

- DE / SD = Desviación Estándar / Standard Deviation
- DMO / BMD = Densidad Mineral Ósea / Bone Mineral Density
- EAAL / FAAS = Espectrofotometría de Absorción Atómica de Llama / Flame Atomic Absorption Spectrophotometry
- ECV / CVD = Enfermedad Cardiovascular / Cardiovascular Disease
- EI / IS = Estándar Interno / Internal Standard
- EO / OS = Estrés Oxidativo / Oxidative Stress
- ERO / ROS = Especies Reactivas del Oxígeno / Reactive Oxigen Species
- Fe / Fe = Hierro / Iron
- Fol / Fol = Folato / Folate
- GGT / GGT = Gamma-Glutamil Transferasa / Gamma-Glutamyltransferase
- Hcy / Hcy = Homocisteína / Homocysteine
- HFS / FSH = Hormona Foliculoestimulante / Follicle Stimulating Hormone
- HL / LH = Hormona Luteinizante / Luteinizing Hormone
- ICC / WHR = Índice Cintura-Cadera / Waist to Hip Ratio
- IDR / RDA = Ingesta Dietética Recomendada / Recommended Dietary Allowances
- IEE / EIA = Inmunoensayo Enzimático / Enzyme Immuno-Assay
- IM / IOM = Instituto de Medicina / Institute of Medicine
- IMC / BMI = Índice de Masa Corporal / Body Mass Index

- K / K = Potasio / Potassium
- LAD / LDL = Lipoproteínas de Alta Densidad / Low Density Lipoproteins
- LBD / HDL = Lipoproteínas de Baja Densidad / High Density Lipoproteins
- LDH / LDH = Lactato Deshidrogenasa / Lactate Dehydrogenase
- MG / FM = Masa Grasa / Fat Mass
- Mg / Mg = Magnesio / Magnesium
- MLG / FFM = Masa Libre de Grasa / Fat Free Mass
- MM / MM = Masa Muscular / Muscular Mass
- Mn / Mn = Manganeso / Manganese
- Na / Na = Sodio / Sodium
- OMS / WHO = Organización Mundial de la Salud / World Health Organization
- THS / HRT = Terapia Hormonal Sustitutiva / Hormone Replacement Therapy
- P / P = Fósforo / Phosphorous
- P-Ca / P-Ca = Fósforo-Calcio / Phosphorous-Calcium
- PA / BP = Presión Arterial / Blood Pressure
- PB / AP = Perímetro del Brazo / Arm Perimeter
- Pb / Pb = Placebo / Placebo
- PC / WP = Perímetro de la Cintura = Waist Perimeter
- PCR / CRP = Proteína C-Reactiva / C-Reactive Protein
- PCC / = Perímetro de la Cadera / Hip Perimeter

- PTH / PTH = Parathormona / Parathyroid Hormone
- RVD / VDR = Receptor de la Vitamina D / Vitamin D Receptor
- R24H / 24HR = Recordatorio de 24 Horas / 24 Hours Recall
- Se / Se = Selenio / Selenium
- TG / TG = Triglicéridos / Triglycerides
- TGO / GOT = Transaminasa Glutámico Oxalacética / Glutamic Oxaloacetic transaminase
- TGP / GPT = Transaminasa Glutámico Pirúvica / Glutamic Pyruvic Transaminase
- X / X = Media / Mean
- Zn / Zn = Zinc / Zinc
- $1,24,25(OH)_2$-D / $1,24,25(OH)_2$-D = 1,24,25-Dihidroxivitamina D / 1,24,25-Dihydroxyvitamin D
- $1,25(OH)_2$-D / $1,25(OH)_2$-D = 1,25-Dihidroxivitamina D / 1,25-Dihydroxyvitamin D
- 25(OH)D / 25(OH)D = 25-Hidroxivitamina-D / 25-Hydroxyvitamin-D

2. ÍNDICE DE TABLAS

3. ÍNDICE DE FIGURAS

4. RESUMEN

Introducción: la mujer posmenopáusica, a causa de la caída de los niveles de estrógenos y los múltiples efectos que este hecho conlleva, constituye una población con un considerable riesgo de presentar alteraciones del estatus nutricional, afectando a su composición corporal, características antropométricas, parámetros bioquímicos y patrones de ingesta dietética, pudiendo derivar, en lo que respecta al estado nutricional, en posibles estados carenciales, fundamentalmente, de micronutrientes. **Objetivos:** la presente Tesis Doctoral plantea como objetivo evaluar el estatus clínico-nutricional general de una población de mujeres posmenopáusicas antes y después de una intervención de 8 semanas mediante suplementación oral mineral con Zinc (Zn) y Magnesio (Mg), así como su relación con la defensa antioxidante y el estatus nutricional de la vitamina D. **Materiales y Metodología:** se realizó una valoración del estado nutricional donde la historia clínica fue autorreportada a través de cuestionarios, la ingesta dietética cuantitativa se midió mediante el Recordatorio 24 Horas, la antropometría y la composición corporal a través de un equipo de bioimpendancia y material especializado, y los parámetros bioquímicos mediante métodos analíticos específicos. **Resultados:** se observó una elevada prevalencia de inadecuación de ingesta en Zn, Mg, y vitamina D junto con una ingesta hipocalórica, además de alteraciones en la antropometría y composición corporal. El porcentaje elevado inicial de mujeres deficientes en Mg plasmático y de Zn y Mg eritrocitarios, fue corregido prácticamente en la totalidad de los casos tras la intervención. Por otro lado, en caso del Zn

plasmático, la prevalencia de deficiencia se mantuvo en un tercio de la población suplementada con Zn. La intervención con Zn mejoró el estatus de la vitamina D_3, siendo mínima su contribución al estatus general de la vitamina D. Por su parte, la suplementación con Mg mejoró los niveles iniciales de vitamina D y redujo el porcentaje de deficiencia en un 20% de la población. En cuanto al efecto sobre los parámetros del estatus antioxidante, a pesar de que se observó una relación directa con el Zn eritrocitario, ninguna de las intervenciones mostró efecto sobre los mismos. **Conclusiones:** la intervención mineral tuvo un efecto positivo sobre los niveles de vitamina D. Aunque hubo una asociación positiva entre los niveles de Zn eritrocitario y los parámetros de estatus antioxidante, la intervención no pareció tener un efecto determinante sobre la misma. No obstante, son necesarios más estudios en los que se realicen intervenciones con una mayor duración y en una población más heterogenea a fin de poder confirmar nuestros hallazgos.

5. ABSTRACT

Introduction: postmenopausal women, due to the decline in estrogen levels and the multiple effects that this fact entails, constitute a population at considerable risk of presenting alterations in nutritional status, affecting their body composition, anthropometric characteristics, biochemical parameters, and dietary intake patterns, which may lead, in terms of nutritional status, to possible deficiency states, mainly of micronutrients. **Objectives:** the aim of this Doctoral Thesis is to evaluate the general clinical-nutritional status of a population of postmenopausal women before and after an 8-week intervention by means of oral mineral supplementation with Zinc (Zn) and Magnesium (Mg), as well as its relationship with antioxidant defense and vitamin D nutritional status. **Materials and methodology:** an assessment of nutritional status was carried out in which the clinical history was self-reported by means of questionnaires, quantitative dietary intake was measured by means of the 24-hour recall, anthropometry, and body composition via bioimpedance equipment and specialized material, and biochemical parameters by means of specific analytical methods. **Results:** a high prevalence of inadequate intakes of Zn, Mg, and vitamin D together with a hypocaloric intake was observed, as well as alterations in anthropometry and body composition. The initial high percentage of women deficient in plasma Mg and erythrocyte Zn and Mg was corrected in practically all cases after the intervention. On the other hand, in the case of plasma Zn, the prevalence of deficiency was maintained in one third of the Zn-

supplemented population. The Zn intervention improved vitamin D_3 levels, with minimal contribution to overall vitamin D status. Mg supplementation improved initial vitamin D levels and reduced the percentage of deficiency in 20% of the population. Regarding antioxidant status, although a positive relationship with erythrocyte Zn was observed, none of the interventions showed an effect on these parameters. **Conclusions:** the mineral intervention had a positive effect on vitamin D levels. Although there was a positive association between erythrocyte Zn levels and antioxidant status parameters, the intervention did not appear to have a determinant effect on antioxidant status. However, further studies with longer duration of interventions and in a more heterogeneous population are needed to confirm our findings.

6. INTRODUCCIÓN

6.1. Climaterio y Menopausia

6.1.1. Climaterio

6.1.1.1. Concepto

El climaterio es una fase del envejecimiento de la mujer que converge un periodo amplio que marca la transición de la fase reproductiva al estado no reproductivo debido al agotamiento folicular ovárico a consecuencia de la caída de la secreción de hormonas sexuales. Se suele confundir con la menopausia, sin embargo, el climaterio abarca una serie de etapas claramente diferenciadas [1].

6.1.1.2. Etapas del Climaterio

El climaterio comprende 4 etapas caracterizadas por una serie de cambios a nivel sistémico de duración variable:

- **Premenopausia:** también conocida como transición menopáusica, es la etapa que precede a la menopausia (10 años antes) y que finaliza con la misma. Es un proceso que suele cursar asintomático, de forma muy lenta y gradual. Aquí se ven los primeros desajustes hormonales y cambios en el ciclo ovárico femenino [2].
- **Perimenopausia:** es el periodo que abarca desde unos años previos hasta 1 o 2 años después de la última menstruación (menopausia). Aquí tienen lugar la mayoría de los síntomas fruto

de la caída hormonal menopáusica, destacando las irregularidades menstruales y apreciándose claramente los efectos a nivel endocrino y patológico. Se produce entre los 45 y los 55 años de edad [3].

- **Menopausia:** es el momento en el que la mujer pierde la fertilidad y comienza una etapa de no fertilidad. Coincide con la última menstruación y su edad de aparición difiere entre países, razas, culturas, hábitos socioeconómicos y nivel sociocultural, entre otros [4].
- **Posmenopausia:** es la fase que viene a continuación de la menopausia y dura hasta el final de la vida de la mujer. En este periodo serán frecuentes las manifestaciones clínicas y síntomas asociados a los desequilibrios hormonales menopáusicos, los cuales se prolongarán durante toda la vejez tanto a corto como largo plazo [5].

6.1.2. Menopausia

6.1.2.1. Concepto

La menopausia es un proceso que se caracteriza por un cambio general progresivo a nivel hormonal caracterizado por el cese en la secreción ovárica, produciéndose una caída hormonal de los niveles de estrógenos y progestágenos, pudiéndose generar un estado de deficiencia en estas hormonas. La menopausia tiene lugar exactamente en el momento en el

que ocurre la última menstruación [6]. Sin embargo, hay que asegurarse a nivel retrospectivo que ha pasado al menos un periodo de 12 meses sin menstruación y que esto no es debido a una causa patológica. En España, la menopausia es un proceso que ocurre de forma natural en la mujer de edades comprendidas entre 45 años y 55 años, aproximadamente, siendo la media de edad a los 51 años [7].

6.1.2.2. Cambios Hormonales en la Menopausia

Durante el proceso menopáusico tienen lugar una serie de cambios en el eje hormonal de la mujer que van a intervenir sobre la sintomatología y cambios fisiológicos observados en este periodo. La mujer menopáusica va a ir perdiendo fertilidad y esto se va a ver manifestado en un número muy reducido de ovocitos. Este suceso irá acompañado de un incremento de las hormonas gonadotropinas, las cuales son las encargadas de estimular los pocos ovocitos activos que tenga la mujer [6]. La edad de la menopausia puede ser más sensible a las tasas variables de atresia de los folículos ováricos que al número absoluto de ovocitos agotados. Se alcanza la menopausia cuando el número de folículos alcanza aproximadamente los 1000 [8].

Cabe destacar que la hormona que más se va a ver alterada y la que más involucrada está en este proceso es el estradiol, el cual es el estrógeno más importante cuya disminución es considerable en esta etapa. El estradiol está estrechamente vinculado con la fertilidad dado

que participa en el aumento del grosor en el endometrio para prepararlo para la fecundación y aumenta la libido o deseo sexual [9].

La inhibina, que es la hormona encargada de inhibir la Hormona Foliculoestimulante (HFS) también va a verse disminuida. Por tanto, la HFS incrementará en este periodo, haciendo a su vez que aumente la Hormona Luteinizante (HL). Aunque este proceso es más evidente al comienzo de la transición menopáusica, con el paso del tiempo perderá efectividad y tanto la HFS como la HL irán disminuyendo. Finalmente, otras hormonas que se verán reducidas en menor medida son los andrógenos, androstenediona y testosterona. La progesterona y la prolactina estarán disminuidas, mientras que el resto de las hormonas no hipofisarias no se verán alteradas [8].

6.1.2.3. Epidemiología de la Menopausia

Para el año 2025 se estima que 1 de cada 7 mujeres en todo el mundo serán posmenopáusicas. El envejecimiento de las sociedades, sobre todo las occidentalizadas, y el aumento de la esperanza y calidad de vida, han originado que la mujer viva más de un 33% de su vida en periodo posmenopáusico [10].

A continuación, se muestra la relación existente entre el número de mujeres que habitan en España, Andalucía, y Granada (provincia y ciudad), así como la proporción de mujeres menopáusicas que hay en cada una de ellas, fijando como menopausia una edad de 51 años, sin

tener en cuenta posibles casos de menopausia precoz o a edades ligeramente inferiores a los 51 años [11]:

- En España, a día 1 de julio de 2022 (últimos datos disponibles), existe una población de aproximadamente 24,304,407 mujeres. Si fijamos el periodo menopáusico en una edad a partir de los 51 años, en España hay una población de 10,602,147 (43.6%) mujeres dentro de ese rango de edad [11].
- En la Comunidad Autónoma de Andalucía, habitan 4,299,068 mujeres de las cuales 1,797,700 (41.8%) superan la edad de 51 años [11].
- En la provincia de Granada, habitan 467,655 mujeres y hay 200,135 (42.7%) que están dentro del rango de menopausia [11].
- En la ciudad de Granada habitan 128,660 mujeres, de las cuales 50,874 (39.5%) son mayores de 51 años [11].

6.1.2.4. Tipos y Causas de la Menopausia

A pesar de que la menopausia es un proceso fisiológico, existen diversos tipos de menopausia y causas de por qué esta se origina en base a la forma y a la edad en la que se presenta.

Según la forma en la que se ha producido:

- **Menopausia artificial:** es aquella que no ocurre de forma gradual y fisiológicamente normal. Es causada por desórdenes

genéticos y autoinmunes, infecciones, quimioterapia, radioterapia, cirugía y causas iatrogénicas [12].

- **Menopausia natural:** es un proceso fisiológico gradual con ausencia de factores externos causado por el envejecimiento normal ovárico. Ocurre en mujeres entre 49 y 52 años, con una mediana de edad de 51 años en mujeres de países desarrollados [13].

Según la edad de aparición:

- **Menopausia precoz:** hace referencia a aquella que ocurre cuando la amenorrea durante un año tiene lugar antes de la edad de 40 años. La padece en torno un 1% de las mujeres [14].
- **Menopausia temprana:** tiene lugar entre los 40-45 años. Ocurre a un 5% de mujeres [13].
- **Menopausia tardía:** es la menopausia que tiene lugar en una edad posterior a los 55 años [13].

6.1.2.5. Síntomas y Consecuencias de la Menopausia

La menopausia está a veces, pero no invariablemente, asociada a una sintomatología. Cuando esto ocurre, puede denominarse síndrome climatérico. El término "síndrome climatérico" hace referencia al conjunto de síntomas y signos tanto a corto como a largo plazo resultantes de la interacción entre factores socioculturales, psicológicos

y endocrinos que se producen en las mujeres menopáusicas conforme envejecen. Su diagnóstico se basa en una anamnesis detallada complementada con una exploración física exhaustiva mediante métodos observatorios, analíticos y exploratorios [15].

6.1.2.5.1. Síntomas a Corto Plazo:

6.1.2.5.1.1. Síntomas Físicos

- **Vasomotores:** los síntomas vasomotores (sofocos, bochornos, y sudoración nocturna), los cuales afectan a 2 de cada 3 mujeres en transición menopáusica y/o menopausia artificial, hacen referencia a sensaciones sofocantes calurosas en el pecho, el cuello y la cara, seguidas de escalofríos, con frecuencia, gravedad y duraciones variables y recurrentes [16]. La diminución de estrógenos reduce el punto de ajuste en el núcleo termorregulador y desencadenan una pérdida inadecuada de calor en el hipotálamo. Esta sensación puede incrementarse con ambientes cálidos, comidas o bebidas calientes y estrés, además de modularse según la zona geográfica [17].
- **Cambios en piel y cabello:** la disminución de estrógenos en el periodo menopáusico disminuye la producción de colágeno. Esto resulta en alteraciones en la piel, grasa subcutánea y en la resorción ósea, aumentando las líneas de expresión, las arrugas, acortamiento de mandíbula, la sequedad, deformidades en la

papada, flacidez y caída de la piel. Además, disminuye la producción de melanocitos apareciendo coloraciones desiguales y manchas. Por otro lado, el aumento de andrógenos generará la caída del cabello y el crecimiento de vello no deseado, normalmente facial, dando lugar a grandes pelos en la barbilla y a un aumento del crecimiento de vello fino en la cara [18].

- **Mamarios:** Las mamas durante la menopausia pierden turgencia e involucionan. La mujeres menopáusicas van a perder colágeno, elastina, tejido adiposo y mamario, viéndose reflejado en una reducción del tamaño de las mamas. Además, el tejido conectivo que las sujeta se atrofia, haciéndolas menos firmes y tendentes a caerse [19].
- **Palpitaciones:** las palpitaciones son frecuentes en mujeres en transición menopáusica y están relacionadas con el aumento de la actividad simpática causada por la menopausia. Se ha demostrado que las palpitaciones pueden conducir a una peor calidad de vida. Las razones de este síntoma no están claras, aunque parece que el déficit de estradiol podría ser el agente causante [20].
- **Musculoesqueléticos:** la sarcopenia, osteoartritis, el dolor y la rigidez aumentan durante la transición menopáusica. La disminución de estrógenos es un factor clave en estas alteraciones ya que el sistema musculoesquelético presenta numerosos receptores de estrógenos. Por otro lado, la hormona del

crecimiento, que mantiene la masa muscular, disminuye a la vez que se reducen los estrógenos [21].

- **Genitourinarios:** incluyen cambios en el tracto genital inferior en respuesta a la privación de estrógenos. Existe una amplia gama de cambios como atrofia de la vulva y la vagina, sequedad vaginal, estrechamiento y acortamiento vaginal, prolapso uterino e incontinencia urinaria. Estos cambios pueden causar dispareunia, irritación y mayor riesgo de infecciones urinarias y son debidos a la reducción de estrógenos que disminuyen el flujo sanguíneo y a la deshidratación del tejido conjuntivo [22].
- **Composición corporal:** la mayoría de las mujeres ganan peso durante la transición menopáusica. Los estudios en animales confirman que la pérdida de estrógenos puede contribuir al aumento de peso y a la redistribución del compartimento graso, con tendencia a aumentar de peso en la zona abdominal. La etiología es compleja, ya que muchas mujeres son menos activas y pierden masa muscular, lo que complica aún más la explicación del aumento de peso con tendencia al sobrepeso y obesidad. Las mujeres pueden ganar aproximadamente entre 4 kg durante ese periodo [23].
- **Fatiga:** la fatiga o astenia, es un proceso muy común que afecta a un 80% de mujeres en transición menopáusica. A pesar de que el propio proceso de envejecimiento incrementa este fenómeno, durante la menopausia la mujer suele afrontar una mayor

responsabilidad familiar tanto en la atención hacia sus padres como a sus hijos [24].

- **Nerviosismo:** las mujeres menopáusicas suelen mostrar actitudes de nerviosismo en forma de irritabilidad y síndrome de piernas inquietas. Estas actitudes son manifestaciones de la queja sobre el estado de ánimo que derivan en problemas en la vida personal y profesional de la mujer, pudiendo afectar a su salud física, mental, y a las relaciones personales [25].

6.1.2.5.1.2. Síntomas Mentales:

- **Depresión:** las mujeres posmenopáusicas son vulnerables a la depresión y presentan más riesgo de padecerla en comparación con etapas anteriores de su vida. Factores como los niveles variables de estradiol, el aumento de los niveles de HFS, la menopausia artificial y la presencia de sofocos podrían ser los motivos, además de otros socioculturales como cambios en el matrimonio y la estructura familiar tales como hijos que no viven en el domicilio familiar, pérdida de trabajo o jubilación, y cuidado de familiares [26].
- **Ansiedad:** durante la menopausia aumentan las preocupaciones y el estrés, haciendo que la mujer sea más vulnerable a padecer ansiedad. En esta línea, el cómo se sienta la mujer consigo misma, así como su manera de afrontar la menopausia, puede influir en su salud mental [27]. A pesar de que la ansiedad haya sido de

menor interés que otros problemas mentales en menopausia, está directamente vinculada a los síntomas vasomotores, al deterioro de la calidad de vida y a la sensación de angustia [28].

- **Cefalea:** la cefalea o migraña es una afección más común en mujeres que en hombres debido a que estas presentan una mayor predisposición genética a padecerla. Durante la menopausia, este proceso se ve adicionalmente incrementado debido a que la disminución de estrógenos está estrechamente vinculado a episodios recurrentes de cefalea [29].
- **Calidad del sueño:** los problemas de conciliación y trastorno del sueño, y los despertares frecuentes han sido reportados por más de un 40% de mujeres que superan la edad de menopausia. En esta línea, al comparar mujeres posmenopáusicas con premenopáusicas, los problemas con el sueño son evidentes en las primeras. Parece ser que los sofocos, unidos a enfermedades asociadas, medicación, dolores crónicos, ansiedad y depresión, son los factores que podrían contribuir a los problemas con el sueño menopáusicos [30].
- **Pérdida de interés sexual:** la disminución de las hormonas sexuales esteroideas durante la transición menopáusica también puede generar disfunción sexual como dispareunia, disminución de la libido, problemas de excitación sexual y dificultades para alcanzar el orgasmo. Los efectos de otros acontecimientos vitales, las fluctuaciones emocionales, la atrofia vaginal (cambios

genitourinarios) y otras enfermedades crónicas pueden desempeñar un papel en la causa de la disfunción sexual [31].

- **Cognición:** durante la transición menopáusica, las mujeres suelen reportar episodios de pérdidas frecuentes de memoria acompañadas de un rendimiento mental más lento y una disminución en la concentración. A pesar de que la caída de estrógenos sea un factor fundamental, los trastornos mentales, el uso de medicamentos y el estrés podrían jugar un papel fundamental [32].

6.1.2.5.2. Síntomas a Largo Plazo:

- **Osteoporosis:** la osteoporosis es la causa más frecuente de morbilidad en las mujeres posmenopáusicas. Las mujeres alcanzan su pico de masa ósea a los 30 años, edad a partir de la cual comienza la pérdida de masa ósea, que se acelera en un 20% finalmente en la menopausia con la pérdida de estrógenos ováricos y otro 20% al alcanzar edades de 80 años [33]. Las fracturas osteoporóticas de cadera, columna y antebrazos limitan la capacidad de movimiento y generan deformidad física, dolor crónico, discapacidad, pérdida de independencia y disminución de la calidad de vida, siendo uno de los factores que más contribuyen a la mortalidad en la mujer [34]. Este proceso ocurre debido a la disminución en un 80% de los niveles de estrona y

estradiol, que incrementan la resorción ósea y se produce un desequilibrio en la remodelación ósea a favor de la destrucción de hueso [35].

- **Enfermedades cardiovasculares:** las Enfermedades Cardiovasculares (ECV) son la primera causa de muerte en los países desarrollados. Su naturaleza es multifactorial, siendo determinantes la hipertensión arterial, el tabaquismo, la inactividad física, el envejecimiento, la dislipemia, la diabetes, la obesidad y los antecedentes familiares, entre otros [36]. Los estrógenos son hormonas vasoactivas que favorecen el remodelado y la elasticidad vascular, y regulan la inflamación [37]. Debido a esto, las mujeres posmenopáusicas presentan un peor perfil de riesgo cardiovascular (mayor riesgo de ECV, cardiopatía coronaria o ictus isquémico) debido a mayores valores de presión arterial sistólica y diastólica, niveles de Colesterol Total (CT) y un Índice de Masa Corporal (IMC) mayor que en etapas anteriores o en comparación con mujeres posmenopáusicas con síntomas vasomotores [38].

6.2. Valoración del Estado Nutricional. Importancia en la Mujer Menopáusica.

La evaluación o valoración del estado nutricional es el proceso en el que se determina si existe un problema con el estado nutricional de una

persona, identificarlo y realizar un examen detallado para determinar la gravedad del problema con la finalidad de establecer intervenciones multidisciplinares para lograr un adecuado estado nutricional [39].

La valoración integral del estado nutricional comprende las siguientes dimensiones [40]:

- **Historia clínica:** parámetros subjetivos y objetivos como la situación fisiopatológica, el historial médico, la función física, la función mental, la calidad de vida y la medicación, entre otros.
- **Hábitos dietéticos:** la evaluación dietética cuantitativa y cualitativa.
- **Valoración corporal:** la valoración antropométrica y de la Composición Corporal (CC).
- **Estado metabólico:** el estudio de parámetros analíticos y de laboratorio.

Ninguna de las herramientas actuales existentes para la valoración del estado nutricional es lo suficientemente fiable como para determinar el estado nutricional de los pacientes debido a las múltiples situaciones diferentes y las alteraciones que puedan presentarse, por lo que debería utilizarse más de un método y tener en cuenta las múltiples dimensiones anteriormente mencionadas para evaluar lo más completamente posible de la forma más adecuada el estado nutricional [41].

La valoración del estado nutricional en las mujeres menopáusicas debe de ir encaminada a contrarrestar los efectos negativos de la carencia de estrógenos sobre el bienestar general y minimizar el riesgo de patologías que deben de ser identificadas a través de una adecuada historia clínica como el síndrome metabólico, la osteoporosis, las fracturas óseas y eventos vasculares, entre otros. Entre los diversos aspectos de la valoración del estado nutricional durante el periodo posmenopáusico, los hábitos nutricionales son esenciales porque conciernen a todas las mujeres, pueden modificarse e influyen tanto en la longevidad como en la calidad de vida [42]. Sin embargo, la medición de esta dimensión puede verse afectada, sobre todo en esta etapa, por variables como la edad y el sexo, la conducta alimentaria (restricción de la ingesta), deseabilidad social, historial de dietas/peso (número de intentos previos de hacer dieta), imagen corporal, psicología (depresión), situación vital (estatus socioeconómico) y la actividad física [43].

La menopausia y el envejecimiento son fenómenos que ocurren de forma simultánea y que modifican la CC y los parámetros antropométricos, los cuales deben de ser evaluados correctamente en menopausia. El aumento en el porcentaje y cantidad de Masa Grasa (MG), y la pérdida de Masa Muscular (MM) aumentan con la edad y el sexo femenino, modificando el Perímetro de la Cintura (PC). Además, la pérdida de estatura con la edad también podría modificar el IMC. En conjunto, estos aspectos complican la valoración antropométrica y de

CC, así como el identificar factores de riesgo claros en la menopausia y el envejecimiento [44]. Adicionalmente a estas alteraciones posibles en la antropometría y la CC, es necesaria una adecuada determinación analítica de parámetros bioquímicos de interés clave, ya que la mujer menopáusica puede sufrir alteraciones en los mismos. Por ejemplo, se han reportado incrementos en los lípidos circulantes y alteraciones en parámetros rutinarios lipídicos asociados a patología cardiovascular [45]. Por otro lado, otros parámetros bioquímicos como los relacionados con el metabolismo de la glucemia y del metabolismo fosfocálcico van a verse alterados, incrementándose así el riesgo de patología a nivel óseo [46]. A continuación, se detallan las dimensiones más relevantes de la valoración del estado nutricional.

6.2.1. Valoración Clínica

El examen de la historia clínica es una dimensión importante en la valoración del estado nutricional. Permite detectar los factores que pueden comprometer un estado nutricional adecuado. Es importante que la historia clínica incluya datos sociodemográficos y socioeconómicos que puedan influir en el estado nutricional de un sujeto, por ejemplo, estructura familiar, nivel educativo, marginación, creencias y estilo de vida, la información sobre la actividad física del paciente (tipo, frecuencia y duración) y el tipo de trabajo realizado [47].

La historia clínica también debe de abarcar la enfermedades que puedan afectar a la ingestión, la motilidad gastrointestinal, la digestión y la absorción, las enfermedades que provocan un aumento de las pérdidas o las situaciones en las que se incrementan las necesidades debido a un aumento del gasto energético y/o del catabolismo proteico, además de otras enfermedades que puedan comprometer la adecuada valoración del estado nutricional o las posibles intervenciones realizadas [48].

6.2.2. Valoración de Ingesta

La información sobre la ingesta dietética de individuos y poblaciones es importante para determinar las asociaciones entre dieta y enfermedad, identificar deficiencias y excesos de nutrientes y evaluar el impacto de las posibles intervenciones. La mayoría de los métodos para evaluar la dieta de las personas implican una entrevista con personal capacitado, la codificación manual de los alimentos, el cálculo del tamaño de las porciones y la comparación con las tablas de composición de alimentos [49]. A continuación, se muestran los dos métodos más utilizados para la valoración de la ingesta:

6.2.2.1. Recordatorio 24 Horas

El Recordatorio 24 Horas (R24H) es un método de valoración de ingesta muy utilizado en estudios poblacionales que valoran la ingesta dietética en numerosas cohortes de riesgo de deficiencia nutricional debido a su alta validez, precisión y tasa de respuesta. Este método cuantitativo y

subjetivo de valoración de ingesta requiere una entrevista directa cara a cara o telefónica por personal cualificado y consiste en recordar, describir y cuantificar con precisión la ingesta de todos los alimentos y bebidas consumidos en las últimas 24 horas antes de la entrevista durante 3 días (2 no festivos y 1 festivo) [50]. A pesar de ser un método que recoge la ingesta cuantitativamente a corto plazo, en la investigación suele generar problemas con la viabilidad y costos asociados con la programación, la capacitación de entrevistadores o encuestados y la codificación de datos [51]. Además, estos cuestionarios son propensos a errores y sesgos ya que su precisión depende en gran parte de la memoria – influyendo el nivel de estudios y la motivación del encuestado – así como de la habilidad y persistencia del entrevistador tanto intencional como no intencional (sesgos de recuerdo, de entrevistador, de deseabilidad social o de aprobación) [52].

6.2.2.2. Cuestionario de Frecuencia de Consumo de Alimentos

El Cuestionario de Frecuencia de Consumo de Alimentos (CFCA) es una herramienta muy utilizada en epidemiología nutricional y se basa en la cuantificación cualitativa de la ingesta Para su validación, se suele utilizar el R24H [53]. Los CFCA se componen de una lista de alimentos, una categoría de frecuencia para determinar el consumo habitual y una medida del tamaño de la ración. A los encuestados se les pide que reporten la frecuencia de consumo y la cantidad de alimentos que consumen regularmente durante semanas, meses y un año natural. Los

CFCA tienen grandes variaciones en las características de diseño, incluidas las diferencias en los alimentos incluidos y las preguntas sobre el tamaño de las porciones; dicha variabilidad, unida a sesgos de memoria, puede afectar en gran medida a las respuestas y los valores de ingesta calculados [54].

6.3. Valoración Antropométrica y de Composición Corporal

Las mediciones antropométricas y de CC son dimensiones de la valoración del estado nutricional que permiten determinar el tamaño y las proporciones corporales de forma sencilla y no invasiva. Los resultados son fácilmente reproducibles por personal cualificado y permiten la comparación con cifras estándar para la población, detectando cambios a lo largo del tiempo en el mismo individuo [47].

6.3.1. Valoración Antropométrica

Las mediciones antropométricas tanto de los perímetros como de los pliegues de la piel representan formas sencillas, no invasivas y económicas de evaluar el estado nutricional. Aunque estas mediciones parecen ser relativamente fáciles, se requiere una habilidad considerable para obtener resultados confiables y existen diferencias individuales entre los operadores. La variación individual y la baja reproducibilidad dificultan el seguimiento de los cambios en el estado nutricional [55].

Las técnicas antropométricas permiten medir la masa corporal, la talla, los perímetros, los pliegues cutáneos y los cocientes derivados de los mismos. La información se procesa aplicando diferentes ecuaciones, obteniendo información sobre el somatotipo, la CC y la proporcionalidad de las diferentes partes del organismo humano [56]. Los parámetros antropométricos más utilizados son:

- **Peso, talla, e IMC:** el peso corporal, la talla y el IMC resultante de dividir el peso (kg) entre la talla (m^2) son parámetros importantes que son relativamente fáciles de obtener. La pérdida de peso involuntaria es primordial para la evaluación del estado nutricional, ya que apunta a una situación metabólica catabólica y se asocia a mayores tasas de morbilidad y mortalidad [57]. El IMC se utiliza ampliamente para estimar la MG, ya que es un método sencillo y barato. El porcentaje de MG para un IMC dado cambia con la edad, y la tasa de este cambio varía en función del sexo, la etnia y las diferencias individuales. Además, el IMC no es sensible a la distribución real de la MG y el riesgo cardio metabólico [58]. Sin embargo, el IMC tiene algunas limitaciones. Por ejemplo, puede estar sesgado por la sobrecarga de líquidos y los edemas, y no describe la CC (por ejemplo, un IMC elevado puede observarse en individuos con elevado peso y también en atletas muy musculosos). Así pues, el IMC no refleja la pérdida

de peso potencialmente patológica ni la ingesta real de alimentos del paciente [57].

- **Perímetros:** las medidas de los perímetros corporales son fáciles de realizar, baratas y no invasivas. Además, requieren solamente una instrumentación mínima (sólo una cinta de antropometría), al tiempo que proporcionan datos precisos cuando las realiza un profesional formado y especializado. Cabe destacar que los perímetros corporales presentan menos problemas de error de medición que los pliegues cutáneos, especialmente en personas obesas [59]. Existen numerosos perímetros a nivel corporal que reflejan el estado de la CC del organismo humano. Algunos ejemplos son el Perímetro de la Cadera (PCC) y el PC, los cuales son indicadores útiles de la acumulación de grasa visceral. Estas sencillas medidas antropométricas pueden detectar a los sujetos de riesgo de alteración de la CC [60]. También cabría destacar el Perímetro del Brazo (PB), el cual es un parámetro antropométrico que a veces se utiliza para detectar el riesgo de sobrepeso y obesidad [61].
- **Índice cintura/cadera:** el Índice Cintura/Cadera (ICC) es el cociente entre el PC y el PCC. Tiene en cuenta las diferencias en la estructura corporal y ha demostrado ser más sensible en la predicción de la mortalidad por varias enfermedades [62]. Como se mencionó anteriormente, el IMC no representa la CC. El ICC puede revelar un aumento desfavorable de la grasa abdominal

mucho mejor, y además es más fácil de calcular que el IMC, siendo dos parámetros antropométricos que deberían medirse simultáneamente [63].

- **Pliegues cutáneos:** son métodos económicos para estimar la CC mediante la medición del grosor de los pliegues cutáneos en diferentes partes del cuerpo utilizando calibradores. Este método se basa en el principio de que la cantidad de grasa subcutánea es proporcional a la cantidad de MG. Los pliegues cutáneos específicos y el número de pliegues varían en función de la ecuación utilizada para estimar la CC la cual es específica para cada sexo [64].

6.3.2. Composición Corporal

Las mediciones de la CC son fundamentales para una evaluación profunda del estado nutricional. El análisis de la CC permite documentar la eficacia del soporte nutricional, elegir terapias nutricionales y evaluar su eficacia y un posible toxicidad. Cabe destacar que pueden ocurrir cambios en la CC independientemente de los cambios en el peso total corporal o el IMC. Además, mientras que los cambios a corto plazo en el peso corporal suelen reflejar cambios en los compartimentos de líquidos, los cambios a largo plazo también reflejan cambios en la masa tisular [55].

El modelo más frecuentemente aplicado para evaluar la CC en la práctica clínica y la epidemiología es un modelo bicompartimental que divide el cuerpo en MG y Masa Libre de Grasa (MLG). La MG indica el componente corporal libre de agua, mientras que la MLG representa los demás componentes corporales (músculo esquelético, órganos internos y masa ósea) [65]. Las mediciones de la CC pueden servir como herramienta de diagnóstico precoz, como cuantificación o como método de seguimiento que ayuda a evaluar el estado nutricional. Además, la CC puede cambiar debido a la enfermedad, la edad, la actividad física y la inanición [57].

6.4. Valoración Bioquímica

Los biomarcadores aportan información acerca de los procesos que ocurren internamente, detectando a menudo la deficiencia de nutrientes mucho antes de que aparezcan los signos y síntomas clínicos. Los marcadores de laboratorio son datos tienen la ventaja de señalar una posible alteración nutricional de forma más precoz y objetiva [66].

Las pruebas bioquímicas miden la concentración de un nutriente o sus metabolitos en fluidos biológicos (la sangre total, el suero y el plasma) o en otros compartimentos específicos como los glóbulos blancos, orina, saliva y pelo. El suero y el plasma reflejan la ingesta dietética reciente o el estado agudo, a menos que el nutriente esté regulado homeostáticamente o amortiguado por fuentes extravasculares.

El eritrocito refleja el estado nutricional a largo plazo, ya que su vida útil es de aproximadamente 120 días. El resto de los compartimentos biológicos serán útiles cuando exista una relación consistente entre la ingesta/estado nutricional y la excreción [67].

Entre los diferentes parámetros rutinarios de laboratorio se incluyen tanto los parámetros relacionados con el metabolismo de la glucosa (por ejemplo, la glucemia), como aquellos que son indicadores del metabolismo proteico (albúmina, prealbúmina, transferrina, ferritina…) y lipídico (CT, Lipoproteínas de Alta Densidad (LAD), Lipoproteínas de Baja Densidad (LBD), Triglicéridos (TG), fosfolípidos totales y algunas apolipoproteínas…) [48]. Además, se suele complementar con el estatus de vitaminas y minerales y biomarcadores asociados, los cuales también pueden proporcionar pistas sobre el estado nutricional. Las concentraciones deben determinarse siempre que se sospechen déficits sobre todo en poblaciones en riesgo de deficiencia de dichos micronutrientes [47]. A continuación, se reflejan una serie de parámetros bioquímicos de interés en la población posmenopáusica que convienen ser comprendidos en profundizad para evaluar su rol en dicha población.

6.4.1. Zinc

6.4.1.1. Concepto, Absorción y Metabolismo del Mineral Zinc

El Zinc (Zn) es el segundo elemento traza esencial en todos los sistemas vivos por detrás del Hierro (Fe) [68]. El organismo humano presenta de 2 a 3 g totales de Zn corporal, repartidos en el músculo esquelético (57%), hueso (29%), hígado (6%), piel (5%), corazón (0,5%), eritrocito (0,9%), plasma (0,1%), y resto de tejidos (1,5%) [69].

El ser humano absorbe en torno a un 33% del Zn dietético a través del intestino delgado. La eficacia en la absorción va a depender del estado de los alimentos consumidos. La absorción de Zn obtenido de alimentos líquidos es más efectiva que a través de alimentos sólidos. Además, el contenido de Zn de una comida es inversamente proporcional a su absorción, debido a la saturación de los transportadores de Zn [70]. Cabe mencionar también, que la absorción en dietas veganas es la mitad que en omnívoras, dado que las proteínas y aminoácidos de origen animal, así como el Fe, aumentan su biodisponibilidad [71].

El Zn absorbido viaja por el sistema portal al hígado, el cual lo libera a la circulación sistémica para que llegue a otros tejidos. En torno al 70% del Zn en circulación está unido a la albúmina. Aunque el Zn plasmático representa sólo el 0,1% de todo el Zn corporal, se renueva rápidamente para satisfacer las necesidades de los tejidos [72], siendo apoyado por el Zn eritrocitario, el cual se encuentra en mayor proporción

que el sérico [73]. Una vez llega a las células, estará presente en 3 formas: (I) Fuertemente unido a metaloenzimas, metaloproteínas, y nucleoproteínas, (II) Débilmente asociado a proteínas o ligandos, y (III) No unido, como ion libre en concentraciones bajas [74].

El Zn se excreta del organismo por tres vías: el intestino, la piel y la orina. La mayor fuente de excreción se produce fundamentalmente por las heces, interviniendo en este proceso las secreciones pancreáticas, biliares e intestinales [75].

6.4.1.2. Funciones del Zinc

El Zn desempeña un papel estructural, regulador o catalítico clave en más 300 enzimas en el organismo. Este mineral interviene en la comunicación, la proliferación, la diferenciación y la supervivencia celular [76]. Además, está involucrado en el crecimiento y el desarrollo, el metabolismo óseo, el correcto funcionamiento sistema nervioso central, la función inmunitaria, el tiroides, la cognición y la cicatrización de heridas [77].

El Zn además es un cofactor vital para la función de más del 10% de las proteínas codificadas por el genoma humano (~3,000 proteínas/enzimas) que a su vez intracelularmente intervienen en la regulación transcripcional, la reparación del ADN, la apoptosis, el procesamiento metabólico, la regulación de la matriz extracelular y la defensa antioxidante [78].

6.4.1.3. Ingestas Recomendadas de Zinc

El Zn necesita ser ingerido diariamente debido a que el organismo humano no precisa de una forma de almacenarlo a largo plazo. Dada su naturaleza de mineral hidrosoluble, este es excretado diariamente, por lo que debe de ser repuesto con relativa frecuencia [79]

La IDR de Zn es de entre 9 y 11 mg al día y sería la ingesta que deberían de seguir las mujeres en proceso posmenopáusico [80]. Cabe destacar que en más del 10% de la población mundial, la ingesta de Zn dietético es inferior a la mitad de las IDR [81].

El estatus de Zn proviene de la ingesta dietética fundamentalmente, sin embargo, puede verse alterado y debe de verse incrementada su ingesta en personas cuya composición de la dieta sea rica en fitatos [70] y alcohol [82]. Por otro lado, enfermedades como la desnutrición, alcoholismo, enfermedad inflamatoria intestinal y los síndromes de malabsorción pueden reducir significativamente la absorción y el almacenamiento de Zn, o aumentar su excreción [83].

6.4.1.4. Fuentes Alimentarias de Zinc

El Zn proviene de fuentes animales (56%) y vegetales (44). En cuanto a las fuentes animales, este mineral va a estar presente fundamentalmente en carnes (rojas) y lácteos, y en menor escala en pescados, mariscos, y huevos. Respecto a su concentración en alimentos vegetales, cabe

destacar que esta varía en base a la cantidad de Zn presente en el suelo, y está fundamentalmente presente en cereales integrales, y en menor concentración en verduras, tubérculos, frutas y legumbres [80], a pesar de que lleven fitatos, oxalatos, y fibra, los cuales disminuyen su biodisponibilidad [71].

Existen otras posibles fuentes de exposición al Zn, ya sea por ingestión (agua potable) o por inhalación (Zn en el aire debido a emisiones industriales, polvo o exposición ocupacional a humos con Zn), aunque su contribución a la carga corporal de Zn, al menos en la población general, sigue siendo incierta [84].

6.4.1.5. Deficiencia en Zinc

La deficiencia de Zn es un problema de Salud Pública a nivel mundial debido a que afecta a más de 2.000 millones de personas en todo el mundo. Según datos de la Organización Mundial de la Salud (OMS), la carencia de Zn es el quinto factor de riesgo para la salud en los países en desarrollo y el undécimo en todo el mundo [76].

A pesar de que los valores plasmáticos recomendados de referencia de Zn sean de entre 0,66 y 1,10 μg/mL, no existe un consenso claro para establecer los mismos, ya que la mayor parte de este mineral está a nivel intracelular [85]. Un punto importante a tener en cuenta en este sentido es la dificultad para detectar el estatus marginal de Zn, ya

que la concentración de Zn en el plasma está estrechamente regulada debido a su importante papel en la señalización celular [86].

Determinar la deficiencia real de Zn es un reto debido a que los síntomas de deficiencia son múltiples e inespecíficos. Además, el Zn en plasma se renueva rápidamente para abastecer a los distintos tejidos y se repone diariamente a partir de la dieta. Por otro lado, los indicadores de estatus de Zn actuales no son fiables, dado que a pesar de que el método más aceptado para evaluarlo e identificar la deficiencia sea analizar el Zn plasmático, seguido de la valoración de su ingesta, no se tiene en cuenta la variabilidad individual, así como la escasa sensibilidad y la inespecificidad de los métodos utilizados [87].

En el organismo humano, la deficiencia grave de Zn presenta graves repercusiones como el retraso del crecimiento, la diarrea infantil, la anemia, alteraciones del sistema inmune, disfunciones neuronales, además de otras enfermedades crónicas como la diabetes, el cáncer, y las ECV [74].

6.4.1.6. Toxicidad en Zinc

Al ser un mineral hidrosoluble, la toxicidad por Zn es muy poco frecuente. Sin embargo, una ingesta excesiva sostenida en el tiempo que supere de 10 a 20 veces las recomendaciones diarias de ingesta podría producir síntomas como las náuseas, vómitos, dolor epigástrico, letargo y fatiga, además de competir con otros minerales como el Cobre (Cu) y

el Fe, induciendo así a enfermedades relacionadas con la deficiencia de dichos minerales [88].

6.4.1.7. Suplementación con Zinc

El Zn se suplementa comúnmente de dos formas: forma orgánica y forma inorgánica. Los minerales ligados a moléculas orgánicas llamados quelatos tienen ventajas sobre la forma inorgánica, con mayor absorción y menor competencia por los sitios de unión con otros minerales [68]. Las preparaciones más usadas en el mercado son el acetato (30% Zn), el gluconato (14.5% Zn), el sulfato (23% Zn) y el óxido (80% Zn) [83]. Hay estudios que reportan que la biodisponibilidad del Zn difiere entre los suplementos orales de Zn, siendo mayor en el Zn unido a aminoácidos como el aspartato, la cisteína y la histidina, seguido del sulfato y el acetato de Zn, y menor medida en el gluconato y en el óxido de Zn [89]. Por otro lado, otros autores reportan que en los ensayos clínicos se suelen utilizar 3 sales solubles de Zn: sulfato, gluconato y acetato de Zn. Todas han demostrado ser eficaces, sin embargo, se ha reportado que el gluconato de Zn podría ser el más eficaz, a pesar de que muestre algunos efectos secundarios [90].

6.4.1.8. Zinc y Menopausia

Las personas que comienzan a entrar en edades avanzadas como las mujeres posmenopáusicas, están en riesgo de presentar deficiencia de Zn, debido a que suelen recurrir a dietas restrictivas, además de limitar

algunos alimentos ricos en este mineral como la carne roja. Por otro lado, existe evidencia de que la eficacia de la absorción de Zn puede disminuir con la edad, siendo el porcentaje de absorción mucho menor que en otras etapas anteriores de la vida [91].

Tanto la ingesta como el estatus adecuado de Zn han demostrado retrasar la edad de menopausia natural. De hecho, aquellas mujeres que son vegetarianas, al ingerir menos Zn, presentan más riesgo de presentar menopausia temprana respecto a aquellas omnívoras [92]. Esto parece ser debido a que las mujeres cuya ingesta y estatus de Zn es inadecuado, presentan mayor riesgo de desarrollar insuficiencia ovárica primaria, disminuyendo así la reserva ovárica [93].

La menopausia provoca un aumento de la resorción ósea, lo que da lugar a la movilización de Zn óseo junto con un aumento de la excreción urinaria de Zn con niveles séricos normales de Zn [94]. En esta línea, en mujeres posmenopáusicas con osteoporosis se ha observado una reducción de las concentraciones de Zn en suero o plasma y un aumento de la excreción urinaria de Zn [95]. Cabe destacar que las mujeres posmenopáusicas que ingieren adecuadamente el Zn presentan un menor riesgo de osteoporosis, dado que el Zn aumenta la densidad mineral ósea y el T-score [96].

La suplementación con Zn ha demostrado reducir la sintomatología de la menopausia, como por ejemplo el síndrome

genitourinario, donde este mineral favorece la remodelación de la estructura vaginal mejorando su funcionalidad [97]. Además, el Zn en mujeres de esta edad puede provocar o intensificar la aparición de palpitaciones cardíacas, temblor de manos y pies, parestesia, trastornos en el funcionamiento del sistema inmunitario, así como síntomas de sequedad y dureza de la piel, caída del cabello, apatía, depresión, trastornos de la concentración, alteraciones de la visión, el gusto, el olfato y el oído [73].

6.4.2. Magnesio

6.4.2.1. Concepto, Absorción y Metabolismo del Mineral Magnesio

El Magnesio (Mg) es un nutriente esencial y el cuarto mineral del cuerpo humano después del Sodio (Na), el Potasio (K) y el Calcio (Ca). El Mg presente a nivel corporal es el resultado de (I) la absorción que se produce a nivel intestinal, (II) su almacén en hueso y tejidos blandos fundamentalmente, y (III) la reabsorción a nivel renal [98]. En esta línea, 24 g de Mg es la cantidad aproximada que presenta el organismo de una persona estándar. Aproximadamente el 99% del Mg es intracelular, con un 50% del Mg presente en huesos (siendo un 30% fijo en hueso y un 70% en constante intercambio con el medio extracelular), y el otro 49% en otros tejidos y órganos blandos, mientras que el 1% restante, el cual se encuentra en la sangre, sería el Mg extracelular [99]. Del 1% del Mg extracelular, un 0,7% estaría presente en el eritrocito y el 0,3% restante

circula en el plasma en tres formas diferentes: libre (no unido; 60%), que representa la forma biológicamente activa; unido a la albúmina (30%); o en complejo con otros iones (10%) [100].

La absorción de Mg es eficaz entre un 20% y un 80%, ya que la absorción no es solo proporcional a la ingesta, sino que el estatus previo de Mg va a influir en este proceso. Por otro lado, cabe destacar que la reabsorción a nivel renal recupera de un 95% a un 99% de Mg. Por tanto, de una ingesta en un día de por ejemplo 370 mg, se aprovecharían 100 mg que posteriormente formarían parte de la excreción urinaria, y se excretarían 270 mg a nivel fecal, manteniendo el ciclo y conservando el Mg total del organismo [101]. Numerosos factores pueden afectar al balance de Mg: Una dieta rica en Na, Ca, y proteínas, el consumo de cafeína y alcohol, los fitatos, el uso de ciertos medicamentos como diuréticos, los inhibidores de la bomba de protones o antibióticos, así como condiciones patológicas (la diabetes, el deterioro de la función renal, trastornos de la conducta alimentaria y el estrés fisiológico) afectarían a la absorción y metabolismo del Mg [102].

6.4.2.2. Funciones del Magnesio

El Mg es cofactor de más de 300 reacciones enzimáticas en el organismo humano. Juega un papel fundamental en numerosos procesos fisiológicos como la síntesis de proteica, el metabolismo energético, de los ácidos nucleicos (manteniendo la estructura del Ácido Desoxirribonucleico

(ADN)) y de la glucosa, la replicación celular, la contracción y relajación muscular, la reducción de la transmisión del impulso nervioso, la liberación de neurotransmisores y hormonas, la regulación de la presión arterial [103], la reducción de la permeabilidad en las membranas celulares, el transporte a través de la membrana plasmática, la mineralización ósea, y procesos anticoagulantes y antiinflamatorios, entre otros [104].

6.4.2.3. Ingestas Recomendadas de Magnesio

Numerosos estudios han reportado unas elevadas tasas de baja ingesta en Mg en países occidentalizados, incluso en mujeres europeas con un elevado estatus socioeconómico y físicamente activas [105]. En esta línea, casi un 70% de la población de Estados Unidos de América no consume las recomendaciones diarias de Mg. La presencia de una gran cantidad de alimentos refinados y procesados en las dietas occidentales puede ayudar a explicar el gran porcentaje de individuos con bajas ingestas de Mg [106].

La IDR para mujeres tanto en transición menopáusica como posmenopáusicas son de 320 mg/día [98], aunque estas recomendaciones pueden aumentar en otras situaciones como la práctica de ejercicio intenso, el envejecimiento y otras condiciones patológicas que cursen con sudor y poliuria [107].

6.4.2.4. Fuentes Alimentarias de Magnesio

A pesar de que el Mg es un mineral bastante presente en los alimentos, sobre todo en los de origen vegetal, no hay ningún alimento que aporte una cantidad muy elevada de Mg. Entre los grupos de alimentos ricos en Mg, va a estar presente fundamentalmente en frutos secos, vegetales de hoja verde, frutas, legumbres, granos integrales, lácteos y pescado [108]. Entre los alimentos a destacar se encontrarían las almendras, los plátanos, las judías, el brécol, el arroz integral, los anacardos, la yema de huevo, el aceite de pescado, las semillas de lino, la leche, las setas, la avena, las semillas de calabaza, las semillas de sésamo, la soja, las semillas de girasol, el maíz dulce, y el tofu [109]. El consumo de Mg procedente de agua rica en Mg también debe de tenerse en cuenta como fuente alternativa de Mg, por lo tanto, el agua puede proporcionar una importante contribución suplementaria a la ingesta total de Mg [110].

A pesar de la amplia gama de alimentos que contienen Mg, el contenido de Mg presente sobre todo en verduras y frutas ha disminuido considerablemente en los últimos 60 años debido a que los suelos cada vez presentan un mayor déficit en minerales esenciales [111]. Por otro lado, es posible que los errores o las diferencias en los sistemas de medición debidos a los avances tecnológicos muestren estos cambios en el contenido Mg, sumado al uso de fertilizantes y pesticidas que también disminuyen su disponibilidad [109]. Además, la dieta occidental hoy en día es más rica en alimentos procesados y refinados, perdiéndose en este

proceso un 90% de su contenido. Por otro lado, la cocción de alimentos es una de las mayores causas de pérdida de Mg y tiene que ser tenida en cuenta junto al refinado de los alimentos como los principales factores que más disminuyen el contenido de Mg en los alimentos [107].

6.4.2.5. Deficiencia en Magnesio

El análisis de Mg extracelular es el método más fácil, disponible, práctico, accesible y rápido para identificar cambios en la homeostasis del Mg en medicina clínica a pesar de no ser el método ideal, ya que la concentración sanguínea de Mg a veces no es un buen reflejo del estatus corporal de Mg total dado que la mayoría del Mg corporal se encuentra a nivel intracelular, aunque puede ser útil para determinar cambios agudos en la ingesta o la excreción de Mg [112].

El déficit de Mg es frecuente entre la población general debido a la disminución de la ingesta sobre todo en países occidentalizados y a las técnicas de cocción y refinado de alimentos. Más del 50% de la población normal presenta deficiencia en Mg, a pesar de la variabilidad entre países y poblaciones [113], definiendo la hipomagnesemia como una concentración plasmática de Mg de < 1,6 mg/dL. Los signos de deficiencia de Mg son inespecíficos e incluyen pérdida de apetito, letargo, náuseas, vómitos, fatiga y debilidad, hipocalcemia, hipopotasemia, además de un incremento en la excitabilidad

neuromuscular (temblores, convulsiones, y tetania) y de arritmias cardiacas [114].

6.4.2.6. Toxicidad en Magnesio

La hipermagnesemia a niveles perjudiciales para el organismo es difícil de lograr a través de la alimentación, ya que habría que ingerir para ello una gran cantidad de alimentos con una elevada densidad nutricional de Mg. Además, el Mg es un mineral hidrosoluble, por lo que está en constante excreción diaria por el organismo [100].

Por tanto, la hipermagnesemia suele ocurrir en casos patológicos como en pacientes en hemólisis ya que los glóbulos rojos contienen 3 veces más Mg que el plasma. La ruptura de estas células vierte Mg en el plasma incrementando su estatus [115]. Existen además otros casos patológicos en los que el riñón no filtra de forma adecuada o en terapias con suplementos o medicamentos con cantidades elevadas de Mg o antiácidos. La hipermagnesemia derivaría en paro a nivel muscular, disminuyendo la capacidad del pulmón para trabajar correctamente y derivando en coma y muerte [116].

6.4.2.7. Suplementación con Magnesio

Las sales de Mg utilizadas en la práctica clínica actual para tratar la deficiencia de Mg pueden ser orgánicas (acetato, aspartato, citrato, gluconato, lactato y pidolato) o inorgánicas (carbonato, cloruro, óxido y

sulfato). Se ha observado que las distintas sales tienen una eficacia de absorción y unas propiedades solubles diferentes, lo que da lugar a una variación de la biodisponibilidad. Las sales de Mg se han estudiado en un número limitado de estudios en humanos con resultados dispares. En algunos estudios no se observaron diferencias entre las sales de Mg orgánicas e inorgánicas; otros demostraron una biodisponibilidad ligeramente superior de las sales de Mg orgánicas en condiciones estandarizadas [117].

6.4.2.8. Magnesio y Menopausia

El Mg es un micronutriente muy importante de cara a aliviar la sintomatología de la mujer posmenopáusica tanto a corto como a largo plazo a pesar de que exista una elevada prevalencia de deficiencia de este mineral en dicha población, habiendo sido reportados casos de deficiencia en Mg sérico tanto en mujeres posmenopáusicas, como en ratas ovariectomizadas [118]. En esta línea, estudios previos han demostrado una prevalencia de deficiencia de Mg en aproximadamente un 40% de mujeres posmenopáusicas [119].

La caída de estrógenos que se produce en la menopausia hace que se fomente la excreción (fundamentalmente urinaria) de Mg, disminuyendo su contenido corporal, y por tanto, limitando los efectos de este mineral a nivel sistémico [120]. Cabe destacar que el Mg puede reducir el riesgo de ansiedad y depresión, así como prevenir la

osteoporosis en mujeres posmenopáusicas [121]. Además, la deficiencia de Mg en mujeres de esta edad puede provocar o intensificar la aparición de palpitaciones cardíacas, temblor de manos y pies, parestesia, trastornos en el funcionamiento del sistema inmunitario, así como síntomas de sequedad y dureza de la piel, caída del cabello, apatía, trastornos de la concentración, alteraciones de la visión, el gusto, el olfato y el oído [122].

6.4.3. Vitamina D

6.4.3.1. Concepto, Absorción, y Metabolismo de la Vitamina D

La vitamina D, también conocida como calciferol, hace referencia a la combinación de un grupo de compuestos liposolubles de naturaleza esteroidea. La vitamina D proviene de la piel (fuente endógena) donde la pro-vitamina D es foto-isomerizada a vitamina D_3 por la luz ultravioleta, y de la dieta (fuente exógena), a través de la cual la vitamina D_3 y la vitamina D_2 son absorbidas a nivel intestinal [123]. Ambas formas son transportadas al hígado donde tiene lugar una primera la hidroxilación y se unen para formar 25-Hidroxivitamina-D (25(OH)D) o calcidiol, la cual refleja el estatus bioquímico de vitamina D al ser más estable en plasma. La vitamina D en forma de 25(OH)D viaja transportada en sangre unida a su proteína de unión y llega al riñón, donde se hidroxila de nuevo para formar 1,25-Dihidroxivitamina D ($1,25(OH)_2$-D) o calcitriol, la forma fisiológicamente activa de la vitamina D [124]. Finalmente, en caso de ser necesario inhibir su actividad, esta es

catabolizada por la 24-hidroxilasa para formar 1,24,25$(OH)_2$D, un compuesto inactivo de la vitamina D. La 1,25$(OH)_2$D aumenta su propio catabolismo al incrementar la expresión de la enzima 24-hidroxilasa [125].

6.4.3.2. Funciones de la Vitamina D

La vitamina D activa ejerce su acción uniéndose al Receptor de la vitamina D (RVD), el cual se encuentra en numerosas líneas celulares como la intestinal, la ósea, la muscular, la renal, la pulmonar, la mamaria, y la cerebral, entre muchas otras [126].

La vitamina D presenta una clara función tradicional sobre el sistema esquelético, ya que favorece la absorción intestinal de Ca, su fijación al hueso y evita su pérdida en orina. Su deficiencia en etapas tempranas de la vida conduce a raquitismo y en edad adulta a osteoporosis, ya que cuando está en déficit, disminuye la densidad ósea y se asocia con un mayor riesgo de fractura [127].

Además del conocido efecto sobre la salud ósea, en las últimas 2 décadas se ha acumulado evidencia sobre el efecto pleiotrópico de la vitamina D en otros compartimentos. En esta línea, un adecuado estatus de vitamina D podría reducir diversas enfermedades como el cáncer, las ECV, la psoriasis, la esclerosis múltiple, la diabetes tipo 1, la artritis reumatoide, la enfermedad inflamatoria intestinal, el lupus eritematoso sistémico, la artrosis y la enfermedad periodontal, entre otras [128].

6.4.3.3. Ingestas Recomendadas de Vitamina D

El hecho de que la vitamina D presente tantas funciones biológicas – algunas de las cuales aún no están esclarecidas – genera controversia acerca del aporte necesario para ejercer todas esas funciones [129]. Diversos organismos como el Institute of Medicine (IOM), el Grupo de Trabajo de Osteoporosis y Metabolismo Mineral de la Sociedad Española de Endocrinología y Nutrición, y el US Task Force hacen recomendaciones para población sana basadas en la ingesta de 15 µg/día en mujeres menores de 70 años y de 20 µg/día para las mayores de 70 años para lograr un estatus suficiente de vitamina D, siempre y cuando el aporte endógeno de vitamina D sea el adecuado [130,131].

6.4.3.4. Fuentes Alimentarias de Vitamina D

En cuanto a la ingesta de vitamina D_3, pescados como el salmón, el atún, la caballa, la sardina, la trucha, la anguila, el bacalao, así como sus aceites, son las mejores fuentes animales de vitamina D_3 [132]. Además, otros alimentos como los hígados de las carnes, leche, huevos, así como sus derivados, son una fuente aceptable de vitamina D. Por otro lado, los alimentos de origen vegetal, que de por sí no presentan vitamina D_3, están fortificados en esta, como los cereales de desayuno, zumos de naranja y las margarinas, que serán resto de alimentos que más vitamina D_3 contengan [133]. Cabe destacar que aquellos animales que tomen pienso enriquecido en vitamina D_3, que estén más expuestos a la luz, y que

presenten una mayor proporción de grasa, presentarán más vitamina D_3 [132].

Respecto a la ingesta de vitamina D_2, ésta está presente en muy pocos alimentos tanto de origen animal como vegetal. En cuanto a los de origen animal, algunas carnes como la de cerdo, lácteos, y sus derivados, van a presentar vitamina D_2. Respecto a los de origen vegetal, algunos zumos y margarinas están fortificados en vitamina D_2 [134]. Por otro lado, el consumo de setas ha aumentado recientemente y es la principal fuente de vitamina D_2, siendo la fuente vegetal que más contribuye al estatus total de vitamina D [135]. Por otro lado, existen otras fuentes como los granos de cacao, los cuales son susceptibles a la contaminación por hongos. Dado que los hongos tienen una alta concentración de ergosterol, tras la fermentación del cacao, son secados al sol, lo que podría resultar en la conversión de ergosterol en vitamina D_2 [136]. En esta línea, los hongos y las levaduras se tratan con luz ultravioleta para inducir la conversión de ergosterol en vitamina D_2 [135].

6.4.3.5. Deficiencia en Vitamina D

A pesar de que la aportación a través del sol ha sido reportada como un factor diferencial en el estatus de vitamina D contribuyendo entre un 60% y 80%, ésta suele repercutir en un 25% al estatus total de vitamina D [137]. Por otro lado, la ingesta, libre de suplementos, constituiría el 20%-40% restante, a pesar de que no se suela llegar a este porcentaje [132].

La vitamina D presenta numerosas funciones biológicas y muchas de ellas aún no han sido del todo elucidadas, por tanto, existe controversia acerca de los rangos de concentración de vitamina D sérica que definan suficiencia y deficiencia, además de la variabilidad entre laboratorios y los distintos métodos disponibles de medición [129] Los valores suficientes de vitamina D se establecen en base a los niveles necesarios de vitamina D para lograr la máxima absorción intestinal de Ca y para prevenir el riesgo de hiperparatiroidismo secundario y de fracturas [138].

Las recomendaciones de los niveles suficientes de vitamina D varían según el organismo. El IOM propone valores de 20 ng/mL para población sana [130], mientras que otros organismos como la Fundación Internacional de la Osteoporosis [139] y la Sociedad de Endocrinología de los Estados Unidos de América [140] proponen concentraciones por encima de los 30 ng/mL.

Cabe destacar que existen determinados factores ambientales (la estación del año, la latitud, el clima, la polución y la duración del día), las características personales (contenido de melanina en la piel, envejecimiento) y el comportamiento humano (uso de protector solar, ropa, obesidad, alteraciones gastrointestinales, hospitalización, religión, institucionalización, actividad física en interiores...) que limitan la obtención de vitamina D en humanos a través de la exposición a los rayos ultravioleta del sol [141].

6.4.3.6. Toxicidad en Vitamina D

La vitamina D al ser liposoluble presenta riesgo de toxicidad y muerte cuando se alcanzan ingestas superiores a 2 mg. No obstante, alcanzar esas dosis de forma natural es meramente imposible. Nunca ha sido reportada una intoxicación por exposición al sol. Además, se han reportado valores plasmáticos de vitamina D superiores a 200 ng/mL, y suplementaciones prolongadas en el tiempo de 1,25 mg, siendo totalmente compatibles con la salud y sin riesgo de hipercalcemia e hipercalciuria [142]. No existe una razón médica conocida para establecer los límites de las dosis que se acerquen a un nivel que pueda derivar en una patología, por lo tanto, existe un cómodo margen de seguridad entre las ingestas terapéuticas y tóxicas, siendo la máxima ingesta tolerable establecida en 250 μg/día, y unos valores de referencia plasmáticos límite de 100 ng/mL [143].

6.4.3.7. Vitamina D y Menopausia

Los niveles bioquímicos y las ingestas adecuadas de vitamina D deben de ser promovidas en grupos de riesgo de deficiencia como la menopausia [144]. Las mujeres posmenopáusicas presentan una serie de cambios asociados al metabolismo de la vitamina D, como la reducción de la síntesis cutánea de vitamina D, o cambios en la CC como el aumento del peso y de la MG que son relevantes para el estatus y la fisiología de la vitamina D [142].

Los estrógenos activan más vitamina D, por tanto, la disminución de estrógenos deriva en deficiencia de vitamina D, la cual guarda relación con la sintomatología a corto plazo de la menopausia, ya que evita el agotamiento de la melatonina, la cual previe sofocos, además de promover el estado de ánimo [146].

La vitamina D juega también un papel fundamental en la sintomatología a largo plazo de la menopausia, ya que previene y está inversamente relacionada con aquellos parámetros que favorecen el desarrollo de osteoporosis y las ECV, sugiriéndose en algunas ocasiones la posibilidad de suplementarla en menopausia para prevenir o corregir estas patologías [45].

6.4.4. Estatus Antioxidante

6.4.4.1. Concepto de Estatus Antioxidante

El metabolismo implica procesos oxidativos vitales para la supervivencia celular. Durante este proceso se generan diversas especies reactivas del oxígeno que derivan en numerosos daños sobre todo a nivel macromolecular como el Ácido Desoxirribonucleico (ADN), el Ácido Ribonucleico (ARN), los lípidos, las proteínas y los carbohidratos, que, en última instancia, alteran la función de las células y los tejidos y provocan patologías que aceleran el envejecimiento [147].

Para contrarrestarlo, existen diversos mecanismos de defensa antioxidante que intentan balancear el equilibrio oxidante/antioxidante

previniendo el daño oxidativo causado por el exceso de radicales libres. Se conoce como "antioxidante" a toda sustancia capaz de retrasar o incluso prevenir el daño irreversible de otras sustancias/macromoléculas y, por lo tanto, promover beneficios para la salud evitando parte de los procesos fisiopatológicos derivados del Estrés Oxidativo (EO) [148]. Un adecuado estatus antioxidante, podría reducir el grado de envejecimiento y el daño oxidativo asociado, a pesar de que nunca va a predominar completamente [149].

6.4.4.2. Capacidad Antioxidante Total

La Capacidad Antioxidante Total (CAT) es el contenido total de antioxidantes en un fluido biológico [150]. La CAT medida en plasma humano podría definirse también como CAT extracelular. En esta línea, también podría denominarse capacidad antioxidante no enzimática, al no reflejar la acción enzimática antioxidante [151] de enzimas como la Superóxido Dismutasa (SOD), la catalasa o la Glutatión Peroxidasa (GPx). Dada la información reduccionista que aporta, y no representar la concentración de una sustancia específica, es necesario tener precaución en la interpretación de los resultados [152].

Evaluar la CAT en plasma es más útil que medir los antioxidantes individualmente, ya que podrían determinarse sus interacciones sinérgicas [153]. Una de las principales ventajas de la CAT es la medición de la actividad de todos los antioxidantes de la muestra

biológica en un único valor, proporcionando así un parámetro integrado en lugar de la simple suma de sustancias medibles. De este modo, los valores obtenidos se consideran el efecto acumulativo de todos los antioxidantes de las muestras biológicas [154].

6.4.4.3. Tipos de Antioxidantes

Los antioxidantes se pueden clasificar en función de la forma que tienen de actuar en el organismo (primarios, secundarios y terciarios), y también en base a su naturaleza en el organismo humano (endógenos o exógenos) [155].

6.4.4.3.1. Clasificación en Función de su Forma de Actuación

- **Antioxidantes primarios:** impiden la formación de radicales libres, especialmente las Especies Reactivas del Oxígeno (ERO), convirtiéndolos en moléculas menos perjudiciales antes de que puedan reaccionar o evitando la formación de radicales libres a partir de otras moléculas. Algunos ejemplos de estos son: la vitamina E, los polifenoles o las enzimas antioxidantes [156,157].
- **Antioxidantes secundarios:** eliminan el radical libre cuando éste se forma, es decir, capturan los radicales libres evitando la reacción en cadena (vitamina E, vitamina C, β-caroteno, ácido úrico, bilirrubina, albúmina) [158].

- **Antioxidantes terciarios:** reparan el daño causado por los radicales libres o eliminan moléculas dañadas, balanceando el potencial redox y reparando el ADN. Algunos ejemplos son: endonucleasas, exonucleasas, enzimas lipolíticas, proteolíticas y transferasas [159].

6.4.4.3.2. Clasificación en Base a su Naturaleza

- **Antioxidantes exógenos:** los antioxidantes exógenos son los que se obtienen de fuentes externas ya sea por alimentación o suplementación. Son importantes para contrarrestar las ERO cuando los compuestos endógenos no son capaces de garantizar una protección completa. Ejemplos son las vitaminas como la A, C, D y E, y minerales antioxidantes como el Cu, Zn, Mn, Se, además de otros compuestos como los carotenoides, los tocoferoles, los ácidos fenólicos, los flavonoides y los taninos [160].
- **Antioxidantes endógenos:** Los antioxidantes endógenos son aquellos producidos en el organismo y su vez pueden ser de naturaleza no enzimática y enzimática [155].
  - **No enzimáticos:** Los antioxidantes no enzimáticos son agentes rompedores de cadenas fundamentalmente [161]. Ejemplos son el ubiquinol, la melanina, el piruvato, el glutatión, el ácido alfa-lipoico, la coenzima Q, la ferritina, el

ácido úrico, la bilirrubina, la metalotioneína, la l-carnitina, la melatonina, y la albúmina [162].

- **Enzimáticos:** Los antioxidantes enzimáticos actúan descomponiendo los radicales libres [161]. Además, tienen efectos protectores más eficaces contra el EO debido a su capacidad para descomponer las ERO [163]. Existen varios sistemas enzimáticos siendo los más importantes la SOD, la catalasa y la GPx [164].

6.4.4.3.2.1. Superóxido Dismutasa

La SOD es una enzima que cataliza la dismutación del superóxido en oxígeno molecular y peróxido de hidrógeno. Existen tres tipos de SOD en los seres humanos: la SOD citosólica dependiente de Cu y Zn, la SOD mitocondrial dependiente de Manganeso (Mn) y la SOD extracelular. [165]. Esta presencia de isoformas específicas de SOD en distintos compartimentos subcelulares subraya la necesidad de un control estricto de la homeostasis de las ERO, las cuales presentan un papel fundamental en la señalización entre compartimentos [166].

La SOD parece ser la primera línea de defensa contra los radicales libres derivados del oxígeno y puede inducirse rápidamente en algunas condiciones cuando se expone al EO. Además, se ayuda posteriormente de la catalasa o la GPx, las cuales utilizan el peróxido de hidrógeno

generado por la SOD, descomponiéndolo en oxígeno molecular y agua [167].

6.4.4.3.2.2. Catalasa

La enzima catalasa está presente en el peroxisoma de las células aerobias y se encarga de descomponer el peróxido de hidrógeno en agua y oxígeno molecular. Esta enzima presenta una de las tasas de recambio más altas: una molécula de catalasa puede convertir 6 millones de moléculas de peróxido de hidrógeno en agua y oxígeno molecular por minuto [168].

6.4.4.3.2.3. Glutatión Peroxidasa

La GPx es el nombre general de una familia de múltiples isoenzimas que catalizan la reducción de peróxido de hidrógeno en agua o alcoholes, utilizando glutatión reducido como donante de dos electrones. [169]. Además, modifica la actividad de proteínas y vías influidas por las ERO. La GPx puede ser dependiente o no de Selenio (Se), actuando esta última sobre los peróxidos de ácidos grasos formados por peroxidación lipídica en membranas celulares, generando hidroperóxidos fácilmente metabolizables. [170]. La GPx, por tanto, reduce las ERO y los productos de la peroxidación lipídica, y se regula al alza en respuesta al EO [171].

6.4.4.4. Estatus Antioxidante en Menopausia

La pérdida gradual de estrógenos durante la menopausia se asocia a un aumento del EO, como demuestra la disminución de la expresión de

enzimas antioxidantes como la SOD, la catalasa y la GPx durante este proceso [172]. Se sabe que los estrógenos actúan como antioxidantes y eliminadores de radicales libres. Este efecto es incluso evidente en la mujer premenopáusica, debido a las variaciones del estatus antioxidante durante las fases del ciclo menstrual, donde se observa un aumento de la actividad de la GPx en las fases de mayor concentración de estrógenos [173].

El agotamiento de estrógenos debido a una ovariectomía provoca una disminución de la expresión de enzimas antioxidantes. Sin embargo, este efecto se ha visto contrarrestado por el incremento de estrógenos derivados de la repleción ovárica o la Terapia Hormonal Sustitutiva (THS), que restauran la CAT y disminuyen la peroxidación lipídica [174].

Existen también síntomas característicos del periodo posmenopáusico prooxidantes como los episodios de depresión, ansiedad y baja autoestima que contribuyen al EO [175]. Este deterioro del estatus antioxidante debido a la disminución de los niveles de estrógenos aumenta el riesgo de enfermedades relacionadas con el EO en la menopausia [175]. Una disminución en los niveles de antioxidantes se relaciona con la reducción de la Densidad Mineral Ósea (DMO), incrementando el riesgo de desarrollar osteoporosis. En este sentido, los antioxidantes no enzimáticos han demostrado mejorar y prevenir el desarrollo de osteoporosis en mujeres posmenopáusicas [176]. Por otro

lado, el incremento del EO durante el periodo menopáusico va a incrementar el riesgo de ECV, debido a que los estrógenos presentan un efecto antioxidante sobre las LBD, disminuyendo la oxidación de dichas proteínas [177].

6.5. Referencias de la Introducción

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7. JUSTIFICACIÓN

El aumento de la esperanza de vida y el envejecimiento de la población en España hace que el número de mujeres posmenopáusicas cada vez sea mayor. Durante esta etapa, los diversos cambios que se suceden a nivel fisiológico, así como otras circunstancias (situación socioeconómica, relaciones sociales, o factores que afectan a la salud mental), pueden tener un efecto negativo en la salud general de estas mujeres, y, en definitiva, en su estado de bienestar.

Cabe destacar que, dada la tendencia a la ganancia de peso y a la redistribución de la MG durante esta etapa, es frecuente que se lleven a cabo patrones de alimentación más restrictivos, con el consecuente riesgo de aporte insuficiente de determinados nutrientes, en especial de micronutrientes como el Zn y el Mg de los cuales se ha reportado una elevada prevalencia de deficiencia a nivel generalizado, especialmente, en este grupo de riesgo como la mujer menopáusica.

Para identificar, prevenir o revertir en la medida de lo posible las alteraciones a nivel sistémico anteriormente mencionadas, es necesaria una valoración del estado nutricional a nivel individualizado, cuyo abordaje se realice de la forma más completa posible por un grupo multidisciplinar especializado, de forma que se abarquen todas las dimensiones que comprenden la misma. Es decir, una adecuada historia clínica que identifique la situación socioeconómica y sociodemográfica, así como los diversos hábitos de estilo de vida, a la que hay que sumar información de una correcta valoración de la ingesta cualitativa y

cuantitativa, un perfil detallado a nivel antropométrico y de CC, y la adecuada determinación de parámetros bioquímicos más relevantes. Todo ello, podría ser determinante a la hora de identificar cuáles son las alteraciones y las posibles complicaciones y consecuencias que cada mujer en particular puede presentar. Esto permitiría un abordaje más individualizado, con una intervención más adaptada a las necesidades y situaciones particulares, con la finalidad de mejorar la calidad de vida durante la vejez.

Entre las múltiples intervenciones que se podrían llevar a cabo en la mujer posmenopáusica se encuentran las intervenciones mediante suplementación oral, concretamente con aquellos nutrientes que suelen presentar estados carenciales. Entre ellos, destacan los minerales Zn y Mg, los cuales presentan una amplia gama de funciones en el organismo y son esenciales para un adecuado estado de salud. A pesar de la amplia evidencia existente sobre la esencialidad y la necesidad de mantener un estatus adecuado de estos minerales, su suplementación en la mujer menopáusica ha sido escasamente investigada. Además, dada la elevada prevalencia de deficiencia en esta población, la intervención mediante suplementación con Zn y Mg podrían ser una herramienta útil y eficaz en la mejora del estado nutricional y, por ende, en la calidad de vida durante esta etapa.

8. HIPÓTESIS

El presente estudio plantea como hipótesis que, debido a la prevalencia de deficiencia de Zn y Mg reportadas previamente en la literatura científica, las mujeres posmenopáusicas de nuestro estudio podrían presentar una deficiencia de estos, la cual podría ser corregida mediante suplementación oral con dichos minerales. Esta intervención permitiría no sólo mejorar su estatus, sino que también tendría un potencial efecto beneficioso sobre determinados parámetros que con frecuencia se ven alterados en el período posmenopáusico, concretamente sobre parámetros antropométricos, de CC, del estatus antioxidante y de niveles de vitamina D, los cuales podrían guardar relación con los niveles de Zn y el Mg dada la implicación de estos minerales con múltiples procesos biológicos y su esencialidad para un adecuado estado de salud, logrando así una mejora en la calidad de vida en esta población.

9. OBJETIVOS

9.1. Objetivos Generales

- Evaluar el estatus clinico-nutricional en mujeres posmenopáusicas, así como el efecto sobre el mismo de una intervención de 8 semanas mediante suplementación con Zn y Mg, tanto al inicio como al final, haciendo hincapié en la valoración del estatus de estos minerales y su relación con los niveles de antioxidantes y de vitamina D.

9.2. Objetivos Específicos

Al comienzo de la intervención

- Valorar el estatus clínico-nutricional general previo en la mujeres posmenopáusicas incluidas en el estudio, evaluando la ingesta de nutrientes, la antropometría, la CC y los parámetros bioquímicos rutinarios.
- Determinar los niveles circulantes de Zn y Mg a fin de conocer la prevalencia de estados carenciales en la población de estudio.
- Conocer el estatus de vitamina D y la defensa antioxidante previos a la intervención nutricional con dichos minerales, así como su relación con los parámetros del estudio.

Intervención

- Realizar una intervención controlada mediante suplementación por vía oral con Zn o Mg durante 8 semanas a las mujeres incluidas en el estudio, controlando la adherencia al tratamiento durante este periodo.

Al final de la intervención

- Valorar nuevamente el estatus clínico-nutricional y el resto de parámetros considerados al inicio; analizar los posibles cambios producidos en los mismos una vez finalizado el estudio.
- Evaluar la evolución de los niveles circulantes de Zn, Mg, y vitamina D, así como de los parámetros del estatus antioxidante, en respuesta a la intervención mediante suplementación.
- Identificar posibles alteraciones, deficiencias y relaciones existentes considerando distintos biomarcadores, los niveles de vitamina D y los parámetros antioxidantes tras la intervención.
- Analizar y difundir los resultados obtenidos junto con sus limitaciones, y elaborar un protocolo de perspectivas futuras para prevenir y corregir posibles carencias nutricionales en la mujer menopáusica.

10. OBJECTIVES

10.1. General Objectives

- To evaluate the initial clinical-nutritional status in postmenopausal women and the effect of an 8-week intervention through supplementation with Zn and Mg, both at baseline and follow-up, with emphasis on the assessment of the status of these minerals and their relationship with antioxidant and vitamin D levels.

10.2. Specific Objectives

At the beginning of the intervention

- To assess the previous general clinical and nutritional status of the postmenopausal women included in the study, evaluating the nutrient intake, anthropometry, BC, and routine biochemical parameters.
- To determine the circulating levels of Zn and Mg to know the prevalence of deficiency states in the study population.
- To determine the vitamin D status and the antioxidant defense prior to a nutritional intervention with these minerals and their relationship with the study parameters.

During the intervention

- To carry out a controlled intervention by oral supplementation with Zn or Mg for 8 weeks in the women included in the study, while monitoring adherence to the treatment during this period.

At the end of the intervention

- To reassess the clinical-nutritional status and the rest of the parameters considered at the beginning; to analyze the possible changes produced in them once the study is finished.
- To evaluate the evolution of circulating levels of Zn, Mg, and vitamin D, as well as the antioxidant status parameters, in response to the intervention through mineral supplementation.
- To identify possible alterations, deficiencies, and the existing relationships considering different biomarkers, as well as other possible relationships between vitamin D levels and antioxidant parameters after the intervention.
- To analyze and disseminate the obtained results along with their limitations, and to develop a protocol of future perspectives to prevent and correct possible nutritional deficiencies in menopausal women.

11. MATERIALES Y METODOLOGÍA

11.1. Participantes y Diseño del Estudio

El presente estudio es un ensayo de intervención aleatorizado, doble ciego, controlado con Placebo (Pb), de 8 semanas de duración (***Figura 1***) realizado en 78 mujeres posmenopáusicas sanas de entre 44 y 76 años de la provincia de Granada (España). Las participantes fueron asignadas aleatoriamente entre 3 grupos de intervención: (I) Grupo Pb (GP: 25 mujeres), Grupo Zn (GZ: 26 mujeres) – 50 mg/día de Zn (600% de las IDR) –, y el Grupo Mg (GM: 27 mujeres) – 500 mg/día de Mg (160% de las IDR) –. Los suplementos de Zn fueron suministrados por SM Natural Solutions, Sabadell, España (Ref: 0B62713821) y los suplementos de Mg fueron suministrados por Botánica Nutrients SL, Sevilla, España (Ref: B91070797). Ambos suplementos siguieron el periodo de 8 semanas, la dosis y el modo de aplicación recomendados por el fabricante con la intención de asegurar los efectos de la suplementación. Las cápsulas de Pb contenían lactosa y se fabricaron del mismo tamaño y color que los suplementos de Zn y Mg para que su aspecto, color y sabor fueran idénticos. La intervención se llevó a cabo en invierno, del 15 de enero al 15 de marzo.

Los criterios de inclusión fueron (I) aceptar participar en el estudio tras ser informadas del mismo, (II) presentar un estado posmenopáusico natural (con al menos 12 meses de amenorrea), (III) estar bajo un estado saludable normal basado en un análisis de laboratorio hospitalario rutinario previo (es decir, parámetros bioquímicos de rutina

normales valorando perfiles de glucosa, proteínas, y lípidos), (IV) fumar y beber alcohol en cantidades moderadas y ocasionalmente, o no hacerlo (V) no ser sedentarias, y (VI) presentar un nivel basal bajo de Zn y Mg en plasma (de 1 a 5 días previos a la intervención) determinados por Espectrofotometría de Absorción Atómica de Llama (EAAL). Los criterios de exclusión fueron (I) rechazar participar en el estudio, (II) tomar suplementos minerales y vitamínicos, (III) presentar cualquier condición patológica relacionada con trastornos en la absorción y metabolismo de nutrientes que pudieran afectar al estado nutricional (celiaquía, los principales componentes del síndrome metabólico, bulimia o anorexia), (IV) estar bajo tratamiento con THS, (V) presentar estado inflamatorio sistémico (se incluyó la Proteína C-Reactiva (PCR) como biomarcador de referencia para evaluar el estado inflamatorio de las pacientes), (VI) presentar menopausia por causas no naturales (por ejemplo, cirugía o cáncer), (VII) no aceptar el protocolo de aleatorización, y (VIII) ser intolerante a la lactosa.

Se obtuvo el consentimiento informado por escrito de todos los pacientes teniendo en cuenta la aprobación del Comité Ético y del Comité de Investigación del Centro. Se garantizó en todo momento la confidencialidad de todos los datos utilizados y recogidos, cumpliendo los principios de la Declaración de Helsinki (últimas directrices revisadas, 2013) [1] y la aprobación del Comité de Ética de la Universidad de Granada (149/CEIH/2016), de acuerdo con las Normas

de la Conferencia Internacional de Armonización/Buenas Prácticas Clínicas. El estudio se registró en los Institutos Nacionales de Salud de Estados Unidos (ClinicalTrials.gov) NCT03672513.

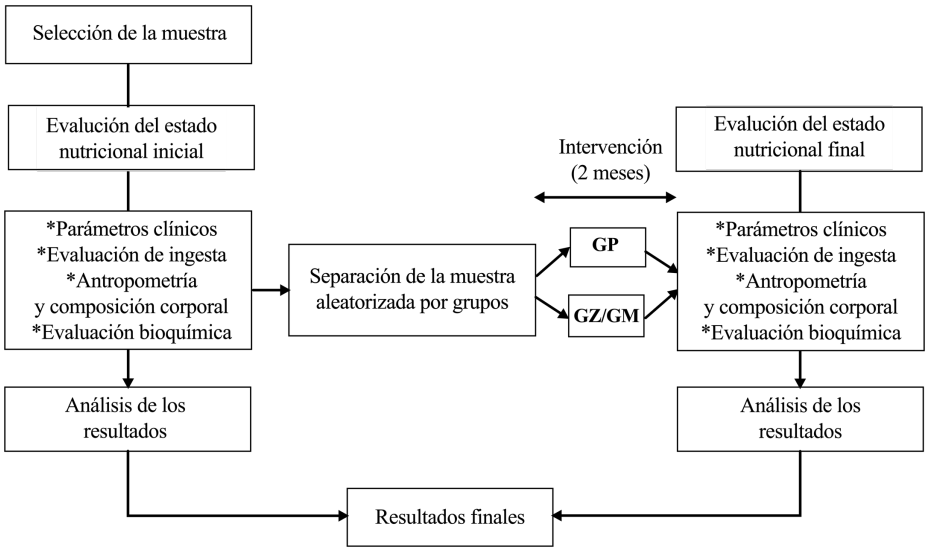

Figura 1. Diseño del estudio. Abreviaturas: GM = Grupo Magnesio; GP = Grupo Placebo; GZ = Grupo Zinc.

11.2. Aleatorización y Cegamiento

Las mujeres fueron asignadas aleatoriamente (aleatorización simple) a los grupos de estudio (diseño paralelo). Para garantizar una distribución comparable entre los grupos de intervención, se estratificó a las mujeres para equilibrar las covariables basales incluyendo la edad (≥ 58 o < 58 años). Tanto las participantes en el estudio como los investigadores estaban cegados a la asignación de grupos. La aleatorización se realizó en una proporción 1:1:1 utilizando una tabla de números aleatorios, preparada por un investigador que no participó en la recogida de datos.

La asignación se ocultó no revelando el código de aleatorización hasta que los participantes fueron reclutados en el ensayo, una vez completadas todas las mediciones basales.

Las participantes elegibles de este estudio están representadas en la ***Figura 2***. De un total de 142 mujeres elegibles, 39 mujeres menopáusicas fueron excluidas porque 18 mujeres no cumplían los criterios de inclusión y 21 mujeres se negaron a participar en el estudio tras la entrevista inicial, por lo que 123 mujeres menopáusicas fueron incluidas en el estudio y asignadas aleatoriamente entre los 3 grupos de intervención. De las 41 mujeres posmenopáusicas que fueron asignadas al GP, un total de 16 mujeres se retiraron del estudio debido a falta de tiempo (n = 9) y no seguir la suplementación (n = 7). En referencia a las 41 mujeres posmenopáusicas asignadas al GZ, un total de 12 mujeres fueron excluidas durante la intervención con Zn debido a falta de tiempo (n = 3), no seguir la suplementación (n = 8) o no dar ninguna razón (n = 1). Hubo 3 mujeres excluidas del análisis de datos en el GZ debido a una recogida insuficiente de sangre. En referencia a las 41 mujeres posmenopáusicas asignadas al GM, un total de 13 mujeres posmenopáusicas se retiraron debido a falta de tiempo (n = 6), no seguir la suplementación (n = 3), a una enfermedad severa (n = 1), o a que no dieron ninguna razón (n = 3). De las 28 mujeres incluidas en el análisis de datos, 1 mujer fue excluida del análisis debido a que no se recogió

sangre suficiente. Por lo tanto, 25 mujeres en el GP, 26 en el GZ y 27 en el GM constituyeron el tamaño muestral del presente estudio.

11.3. Evaluación de la Adherencia al Tratamiento

La adherencia/cumplimiento de la intervención nutricional se determinó como el porcentaje de todas las cápsulas de suplementos ingeridas a lo largo de las 8 semanas que duró el estudio. Se pidió a los sujetos que llevaran un registro diario de los efectos secundarios u otros problemas derivados de la suplementación. Además, se tomaron parámetros bioquímicos y clínico-nutricionales al inicio y durante la intervención para evaluar la seguridad del producto y verificar que no hubiera efectos adversos.

11.4. Valoración del Estado Nutricional

11.4.1. Historia Clínica

La Presión Arterial (PA) (mmHg) clasificada como presión normal/presión alta se midió 3 veces en intervalos de 30 s en participantes sentados utilizando un esfigmomanómetro electrónico (HBP-9020, OMRON Co. Ltd., Kioto, Japón) después de que las participantes hubieran descansado durante 10 min, y se utilizó el valor promedio para el análisis. Los valores de PA superiores a 120 mmHg/80 mmHg, para la PA sistólica y diastólica, respectivamente, se consideraron valores de presión alta. Los siguientes datos se obtuvieron mediante cuestionarios manuales administrados por el investigador: (I) edad, (II) el ejercicio

físico se definió como sedentaria/no sedentaria, clasificando como no sedentaria a la mujer que reportara menos de 30 min/día de ejercicio regular, menos de 3 días/semana, (III) el hábito de fumar se clasificó como no fumador/fumador; siendo un fumador aquel que fumaba más de 1 cigarrillo/día, (IV) el nivel educativo se dividió en básico/ educativo secundario o alto; los individuos con el nivel educativo básico eran los que sólo habían terminado los estudios primarios.

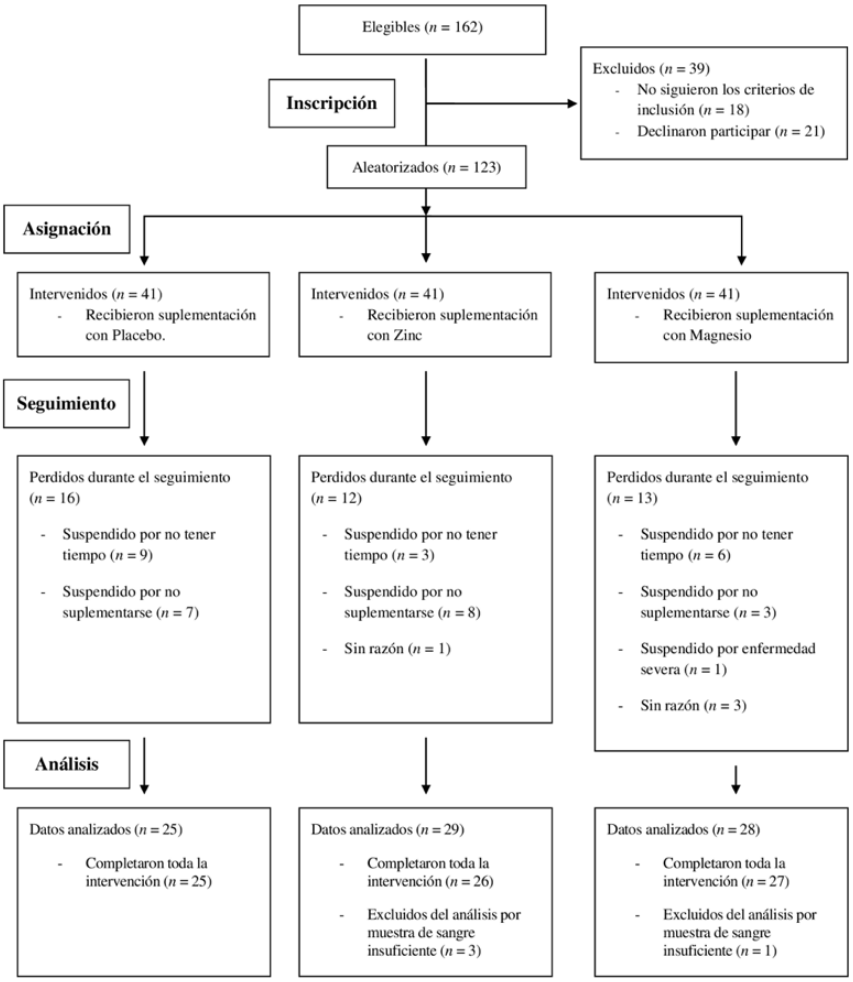

Figura 2. Diagrama de flujo de los participantes reclutados, inscritos e implicados en el estudio clínico.

11.4.2. Valoración de la Ingesta Dietética

La ingesta dietética de nutrientes fue evaluada cuantitativamente y administrada por un dietista profesional cualificado antes y después de la intervención mediante un R24H [2], teniendo en cuenta 1 día festivo y 2 días no festivos. Se empleó un conjunto de fotografías de alimentos preparados y platos que se consumen habitualmente en Granada (España) [3] con la finalidad de lograr una mayor precisión en el recordatorio. Se utilizó el programa informático Dietowin (versión 7.1., Barcelona, España) para convertir cuantitativamente la ingesta de alimentos en energía, macronutrientes y micronutrientes, determinando su adecuación según las IDR para la población menopáusica española dentro del rango de edad incluido en nuestro estudio [4].

11.4.3. Composición Corporal y Antropometría

Se realizaron mediciones de la CC de todos los participantes obteniendo el peso corporal (kg), la MM (kg), la MG (kg), y el porcentaje de MG (%) mediante un dispositivo de impedancia bioeléctrica (Tanita MC-980 Body Composition Analyzer MA Multifrequency Segmental, Barcelona, España). El dispositivo cumple con las normas europeas aplicables (93/42EEC, 90/384EEC) para su uso en investigación e industria médica. Todas las mediciones se realizaron simultáneamente por la mañana en ayunas. Se informó a las participantes de las condiciones requeridas antes de la medición: (I) no ingerir alcohol menos de 24 horas antes de la

medición, (II) no realizar ejercicio vigoroso menos de 12 horas antes de la medición, (III) no comer ni beber menos de 3 horas antes de la medición, y (IV) no orinar inmediatamente antes de la medición.

La talla (m) se midió de acuerdo con las normas internacionales para la evaluación antropométrica con un estadiómetro (SECA® Modelo 213, rango de 85 a 200 cm; precisión: 1 mm; Hamburgo, Alemania). El PC (cm) se calculó en el punto medio entre la parte superior de la cresta ilíaca y el margen inferior de la costilla menos palpable [5]. El PCC (cm) se midió en la parte más ancha de las nalgas, con la cinta paralela al suelo [6]. El PB (cm) se midió en el brazo derecho en el punto medio entre la punta del olécranon y el acromion, con el brazo colgando holgadamente [7]. Todos los perímetros anteriores se midieron utilizando un tallímetro con un margen de error de 0.01 cm (SECA® Modelo 201, Hamburgo, Alemania). El cálculo de la relación cintura/cadera se realizó dividiendo el PC (cm) por el PCC (cm). El IMC se obtuvo como peso corporal (kg)/talla (m^2).

11.4.4. Análisis Bioquímico

11.4.4.1. Procesamiento de las Muestras

Se realizó una extracción de sangre por la mañana en ayunas al inicio y al final de la intervención, centrifugándose a 4 °C durante 15 min a 3,000 rpm para extraer el plasma. Una vez extraído el plasma del tubo, se almacenó en un Vacutainer con anticoagulante y se lavaron los eritrocitos

para la obtención de las alícuotas eritrocitarias 4 veces con 3 mL de solución de cloruro sódico al 0.9%, centrifugando durante 15 min a 3,000 rpm después de cada lavado. A continuación, se retiró la solución salina sobrenadante del último lavado y se obtuvieron los eritrocitos. Las muestras se congelaron a –80 ºC hasta la determinación analítica de los distintos parámetros. Todas las muestras se midieron en una sola tanda, por duplicado, en el mismo lote de ensayo, y se incluyeron muestras ciegas de control de calidad en los lotes de ensayo para evaluar el error de laboratorio en las mediciones.

11.4.4.2. Determinación Analítica de Parámetros Bioquímicos Rutinarios

Todos los parámetros bioquímicos rutinarios se midieron en plasma. Los niveles circulantes de glucosa (mg/dL), creatinina (mg/dL), urea (mg/dL), ácido úrico (mg/dL), bilirrubina total (mg/dL), proteínas totales (g/dL), albúmina (mg/dL), prealbúmina (mg/dL), transferrina (mg/dL), Transaminasa Glutámico Oxalacética (TGO) (U/L), Transaminasa Glutámico Pirúvica (TGP) (U/L), Gamma-Glutamil Transferasa (GGT) (U/L), amilasa (U/L), Lactato Deshidrogenasa (LDH), TG, (mg/dL), LAD (mg/dL), LBD (mg/dL), CT (mg/dL), PCR (mg/dL), homocisteína (µmol/dL), osteocalcina (ng/mL), Parathormona (PTH) (pg/mL) y leptina (ng/mL) mediante Inmunoensayo Enzimático (IEE) (ECLIA, Elecsys 2010 y Modular Analytics E170, Roche Diagnostics, Mannheim, Alemania). Todos los parámetros se analizaron en el Hospital Virgen de

las Nieves (Unidad de Análisis), Granada (España) – los valores de referencia fueron proporcionados por la Unidad de Análisis –. El Folato (Fol) (ng/mL) y la Vitamina B_{12} (B_{12}) (pg/mL) se midieron utilizando un DxI® Autoanalyzer (Beckman Coulter, California, Estados Unidos de América) empleando un inmunoensayo por electro quimioluminiscencia competitivo para determinaciones cuantitativas en el Centro de Instrumentación Científica (CIC) de la Universidad de Granada, Granada, España. El CIC proporcionó los valores de referencia.

11.4.4.3. Análisis del Estatus Antioxidante

Los parámetros de estatus antioxidante se analizaron mediante métodos colorimétricos cinéticos comerciales. La CAT (µmol/L) se midió mediante el poder de reducción del Cu^{2+} a partir de la acción de los antioxidantes presentes en las muestras de plasma (kit CAT, Jaica, Shizuoka, Japón). La variabilidad se comprobó repetidamente evaluando 5 muestras y considerando una variabilidad inferior al 5% como válida [8]. La actividad de la SOD (U/mL) se analizó mediante el método colorimétrico basado en la reducción del citocromo c utilizando el kit Ransod (RANDOX Laboratories Ltd., Dublín, Irlanda, Reino Unido) [10]. Para determinar la actividad de la GPx (mU/mL) se utilizó un método inmunológico enzimático (kit Bioxytech GPx-340™, OxisResearch™, Portland, Oregón, Estados Unidos de América) basado en un ensayo colorimétrico indirecto de la actividad de la GPx [9]. Además, se calculó la relación SOD/GPx y la GAP antioxidante a partir

de la CAT, la albúmina y el ácido úrico [11]. Los parámetros de estatus antioxidante y sus valores de referencia fueron proporcionados por el CIC de la Universidad de Granada, Granada, España.

11.4.4.4. Medición de Minerales

Los niveles plasmáticos de Ca (mg/dL), Mg (Mg) (mg/dL), Fe (µg/dL), Zn (mg/dL) y Cu (µg/dL) se obtuvieron mediante EAAL (Perkin Elmer® modelo Analyst 300, Berlín, Alemania) previa mineralización húmeda. Adicionalmente se analizaron el Zn y Mg en eritrocito. La precisión del método se evaluó mediante el análisis de un material de referencia certificado (SeronormTM Trace Elements ref. MI0181 SERO AS, Billingstad, Noruega). Todos los minerales mencionados se analizaron a diferentes longitudes de onda óptimas para cada elemento (0.7 nm), utilizando un caudal (Aire/C_2H_2) de 10/1.9 $L \cdot min^{-1}$, y unas curvas de calibración de 5 puntos ($r^2 = 0.9997$). La fiabilidad se estableció fijando puntos de corte y considerando que una muestra era válida y fiable si mostraba un Coeficiente de Variación (CV) inferior al 5% y un Coeficiente de Correlación Interclase (CCI) superior al 0.90. El Fósforo (P) (mg/dL) se determinó mediante el método colorimétrico de Fiske-Subbarow con molibdato amónico $(NH_4)_2MoO_4$ (Thermo Scientific, Rockford, Illinois, Estados Unidos de América). Todas las mediciones de los minerales se realizaron en el CIC de la Universidad de Granada, Granda, España, el cual facilitó los valores de referencia.

11.4.4.5. Determinación Analítica de la Vitamina D

La vitamina D total (25(OH)D) (ng/mL) y sus formas (vitamina D_3 ($25(OH)D_3$ + vitamina D_2 ($25(OH)D_2$) se analizaron en la Facultad de Farmacia y el Instituto de Nutrición y Tecnología de los Alimentos "José Mataix" de la Universidad de Granada, Granada, España, mediante Cromatografía Líquida-Espectrometría de Masas en Tándem (CL–EMT) (Acquity UHPLC System I-Class Waters, Milford, Estados Unidos de América). Para la precipitación de proteínas, se tomaron 200 µL de plasma en un Eppendorf, a los que se añadieron 20 µL de Estándar Interno (EI) (Sigma Aldrich, St. Louis, Missouri, Estados Unidos de América) (0.5 µg/mL) y 500 µL de acetonitrilo. Se llevó al agitador de placas a una amplitud de 3 y forma 2 durante 1 min y se secó con N_2. A continuación, se centrifugó a 10,000× g durante 15 min a 4 °C. El sobrenadante se recogió en otro Eppendorf, desechando el resto. Para la fase de extracción, se mezcló el sobrenadante con 100 µL de agua y 200 µL de acetato de etilo, y se mezcló en un vórtex 30 s. Posteriormente, se centrifugó a 3,000× g durante 5 min. Se recogió el sobrenadante, y se repitió el paso anterior con el precipitado, recogiendo de nuevo el sobrenadante y combinándolo con el anterior. Finalmente, los sobrenadantes se secaron con N_2. Para el paso de derivatización, se preparó una solución de 4-fenil-3H-1,2,4-triazol-3,5(4H)-diona (Sigma Aldrich, St. Louis, Missouri, Estados Unidos de América) en acetonitrilo (0.5 mg/mL) y se mezcló en vórtex. A continuación, se añadieron 50 µL

de la solución anterior tanto a las muestras como a los estándares y se llevaron al agitador de placas durante 1 hora a temperatura ambiente cubiertos con papel de aluminio. Por último, las muestras se transfirieron a viales para cromatografía y se añadieron 50 µL de agua. Se cubrieron con papel de aluminio y se guardaron en el congelador a –20 °C para su punción en el cromatógrafo. Para preparar la línea de calibración, se añadieron a los viales concentraciones crecientes de 1 ppb, 2 ppb, 5 ppb, 10 ppb, 25 ppb, 50 ppb y 100 ppb de los estándares ($25(OH)D_3$) y ($25(OH)D_2$) (Sigma Aldrich, St. Louis, Missouri, Estados Unidos de América), así como 20 µL del EI. La mezcla se secó con N_2 y finalmente se derivatizó y almacenó a –20 °C para su posterior análisis.

Las muestras se analizaron utilizando el cromatógrafo Acquity UHPLC I-Class System de Waters (Waters, Londres, Reino Unido). La columna utilizada fue una columna Acquity UHPLC BEH C18 ™ de 2.1 × 50 mm, 1.7 µm a temperatura ambiente. El volumen de inyección de la muestra fue de 10 µL. La fase móvil del canal A fue agua con 50 mM de formiato de amonio y la del canal B fue metanol. El tiempo de análisis fue de 8 min y el caudal de 0.4 mL/min. El detector fue un espectrómetro de baja resolución de triple cuadrupolo XEVO-TQ-XS de Waters. La fuente de ionización fue por electrospray con ionización positiva. La temperatura de desolvatación fue de 600 °C y el caudal de gas de desolvatación fue de 500 L/hora. La temperatura de la fuente fue de 150 °C y el caudal de gas del cono de 150 L/hora. Los valores

bioquímicos de vitamina D obtenidos se clasificaron según los valores de referencia de 25(OH)D en plasma, siendo suficiencia > 30 ng/mL, insuficiencia 20-30 ng/mL y deficiencia < 20 ng/mL para la vitamina D total [12].

11.5. Cálculo del Tamaño Muestral

Se realizó el cálculo del tamaño muestral utilizando el software G*Power (versión 3.1.9.6, Kiel, Alemania) [13]. A cada estudio de intervención se le realizó un cálculo del tamaño muestral específico asociado a las variables estudiadas en este, el cual proviene de un cálculo de tamaño muestral más grande basado en la variable objetivo que más tamaño muestral generaba. Por tanto, el número de participantes a incluir en el estudio se calculó en función del cambio en el estatus de vitamina D tras la intervención con Zn y Mg en relación con el Pb. Hasta donde sabemos, no había información disponible sobre los cambios en las diferencias entre grupos sobre cambios en la vitamina D en mujeres posmenopáusicas que recibieron una intervención de Zn y Mg siguiendo protocolos similares a los realizados en nuestro estudio en comparación a un grupo Pb. Por lo tanto, asumimos un tamaño del efecto de 0.2 (tamaño del efecto pequeño) basándonos en observaciones previas en nuestro grupo. Se necesitó un total de 68 participantes para detectar una diferencia de medias entre grupos con un tamaño del efecto pequeño en la vitamina D con una potencia del 80% y un alfa de 0.05, asumiendo una pérdida máxima del 20% de los participantes (n = 82), para la prueba t de

Student para muestras independientes (dos grupos). El cálculo del tamaño de la muestra se realizó por separado (GP frente a GM y GP frente a GZ) debido a que el tratamiento estadístico principal fue la t de Student para comparar 2 muestras independientes en lugar del Análisis de la Varianza (ANOVA) unidireccional abarcando los 3 grupos de intervención.

11.6. Análisis Estadístico

La hipótesis de distribución normal se aceptó mediante la prueba de Kolmogórov-Smirnov como paso previo a la ejecución un modelo paramétrico o no. Las variables categóricas se representaron como Frecuencias (N) y Porcentajes (%). Las variables continuas se plasmaron como Media y Desviación Estándar (X ± DE). Para el análisis comparativo entre variables categóricas, se utilizó la prueba de chi-cuadrado. Respecto al análisis comparativo basado en la evolución de la intervención dentro de cada grupo, se utilizó la prueba t de Student para muestras relacionadas. Para el análisis comparativo entre grupos, se empleó la prueba t de Student para muestras no relacionadas. Respecto al análisis comparativo basado en más de 2 grupos, se empleó el ANOVA unidireccional ajustado por Bonferroni a pos-hoc. Los análisis de correlación y los coeficientes de correlación parcial se realizaron con la prueba correlación de Pearson. Se utilizó el análisis de regresión lineal para estimar el grado de asociación entre las variables continuas del estudio y se realizaron modelos de regresión lineal múltiple para

comprobar estas asociaciones tras ajustar por múltiples parámetros. Además, se realizó un análisis de mediación simple utilizando el macro-PROCESS desarrollado por Hayes [14]. Un *p* Valor inferior a 0.05 se consideró estadísticamente significativo. Todos los coeficientes obtenidos de los análisis anteriores se representaron tanto en tablas como en figuras. Los datos se trataron estadísticamente utilizando el software SPSS 22.0 para MAC (SPSS Inc. Chicago, Illinois, Estados Unidos de América). Para realizar los gráficos de las figuras se utilizó el programa GraphPad Prism 9 (GraphPad Software, San Diego, California, Estados Unidos de América).

11.7. Referencias de los Materiales y Metodología

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12. RESULTADOS Y DISCUSIÓN

12.1. CAPÍTULO I

El capítulo I de la presente Tesis Doctoral hace referencia a la relación existente a nivel trasversal al comienzo del estudio entre los diferentes parámetros evaluados. En concreto, con este capítulo se pretende evaluar posibles alteraciones en todos los parámetros del estudio tanto por déficit como por exceso y la relación existente entre cada uno de ellos previos a la intervención nutricional.

Se han incluido 3 artículos cuyos objetivos se describen a continuación:

Artículo 1: investigar la relación entre la edad y factores sociodemográficos, antropométricos, de composición corporal, nutricionales y bioquímicos (es decir, perfiles proteicos y lipídicos, metabolismo fosfocálcico y estado antioxidante) en mujeres posmenopáusicas.

Artículo 2: evaluar el estatus de la vitamina D en una población posmenopáusica y determinar su relación y la de sus metabolitos con parámetros antropométricos y de composición corporal.

Artículo 3: examinar la relación entre la composición corporal y los parámetros bioquímicos con el estado antioxidante en una cohorte sana de mujeres posmenopáusicas.

12.1.1. Manuscript 1

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Title: Sociodemographic, Anthropometric, Body Composition, Nutritional, and Biochemical Factors Influenced by Age in a Postmenopausal Population: A Cross-Sectional Study

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Abstract

Introduction: postmenopausal aging has become relevant for understanding health during the transition life stages – the aging process being involved in several disturbances of the human condition –. The present study aimed to investigate the relationship between postmenopausal aging and sociodemographic, anthropometric, body composition, nutritional, and biochemical (i.e., protein and lipid profiles, Phosphorous-Calcium (P-Ca) metabolism, and antioxidant status) factors in postmenopausal women. **Materials & Methods:** this cross-sectional study enrolled 78 healthy postmenopausal women (44–76 years). All parameters were assessed via routinary methods. **Results:** the anthropometrical data showed no differences by age. Biochemical parameters, especially those involved in the protein and P-Ca metabolism, were influenced by age in our cohort of postmenopausal women. In contrast, no associations were found when considering lipid and antioxidant parameters. **Conclusion:** height, fiber intake, blood glucose, protein profile and P-Ca metabolism markers seem to be the most affected nutritional-related factors by age in our cohort of healthy postmenopausal women. Primary prevention strategies focused on parameters at risk of disruption with postmenopausal aging are necessary to ensure the quality of life in older ages.

Keywords: menopause; aging; nutritional assessment; dietary intake; anthropometry; biochemical parameters

1. Introduction

Aging has become one of the major issues facing public health, as it promotes multiple diseases and socioeconomic problems [1]. Life expectancy is > 80 years for women in developed countries. A better comprehension of effective strategies for ameliorating the aging process will be needed because a third of women's lives will be spent in the menopausal period [2]. Menopause is considered as the cessation of menstruation permanently, with amenorrhea occurring during 12 successive months before natural menopause is detected, resulting from the loss of ovarian follicular activity directly related to several pathophysiologic conditions [3]. Thus, studies conducted on postmenopausal women have become important to understanding health during the transition stages of life, especially in older women [4].

As menopause advances, women may experience a variety of predictable disorders related to changes in sex hormone levels and aging [5]. In this regard, the decrease in estrogen production often leads to several menopausal symptoms and altered anthropometric and biochemical parameters, particularly those noted at the postmenopausal stage [6]. Similarly, the menopausal transition impacts a wide range of physical and sociodemographic conditions because of aging [7].

During post menopause, women may experience serum lipid changes [8] via the redistribution of the Fat Mass (FM) to the abdominal compartment, reducing activity, energy expenditure, and fat oxidation, thus modifying their Body Composition (BC) via the augmentation of FM, and diminishing muscle mass [9]. These disturbances in lipid me-

tabolism and anthropometric condition along with an imbalance in antioxidant status increase the risk of impaired cardiovascular and oxidative stress-related parameters as menopause advances [10]. Moreover, bone diseases have been proposed as one of the main factors in postmenopausal stages and are directly related to the aging process. In this regard, age has been inversely correlated with serum Calcium (Ca) levels in postmenopausal women, the latter of which are necessary to identify disturbances in Phosphorous-Calcium (P-Ca) metabolism factors involved in bone disorders, with the purpose of helping to minimize the morbidity/mortality rate and financial burden [11,12]. Physical activity and dietary restrictions have been proposed for menopausal women to help them to face the body composition-related problems mentioned above, these patterns being sometimes modified with aging [13]. As a result of all the previous-mentioned possible changes, postmenopausal women may be at risk of developing several diseases, such as osteopenia and osteoporosis, sexual dysfunction, Cardiovascular Diseases (CVDs), cancer, and obesity, among others [14].

As the aging process could affect several factors involved in the modulation of BC and nutritional factors, the present study aimed to investigate the relationship between postmenopausal aging and sociodemographic, anthropometric, BC, dietary, and biochemical factors.

2. Materials & Methods

2.1. Participants and Study Design

The present cross-sectional study was conducted in 78 healthy postmenopausal women aged between 44 and 76 years from the province of Granada (Spain). Women were divided by terciles of age (i.e., tercile 1 = < 54 years, tercile 2 = 54 – 62 years, and tercile 3 = 62 years). All participants signed a written informed consent form. The study was approved by the Ethics Committee of the University of Granada (149/CEIH/2016) and conducted according to the principles of the Declaration of Helsinki [15], in accordance with the International Conference on Harmonization/Good Clinical Practice Standards. Inclusion criteria were (I) to accept to participate in the study after being informed about it, (II) to present natural postmenopausal status (with at least 12 months of amenorrhea), and (III) to have healthy condition based on a previous routine hospital laboratory analysis (i.e., normal routinary biochemical parameters, namely glucose, protein, and lipid profiles). Exclusion criteria were (I) to refuse to participate in the study, (II) to take mineral and vitamin supplements, (III) to present any pathological condition that could affect their nutritional status (i.e., celiac disease, the main components of metabolic syndrome, bulimia or anorexia), (IV) to be treated with Hormone Replacement Therapy (HRT), (V) to have systemic inflammatory status (including C-Reactive Protein (CRP) as a reference biomarker to assess the inflammatory status of the patients), and (VI) to present menopause due to non-natural reasons (e.g., surgery or cancer).

2.2. Sociodemographic Data Collection

Data regarding physical activity was set as sedentary/non-sedentary, classifying as non-sedentary any participant who performed less than 30 min a day and less than 3 days/week of regular exercise. Regarding smoking habits (non-smoker/smoker), a smoker was a participant who smoked more than 1 cigarette a day. For educational level (basic educational level/secondary or high educational level), those who only finished primary studies were considered to have a basic educational level. All previous-mentioned data were retrieved using manual questionnaires administered by the researcher. Blood Pressure (BP) classified as (normal/high pressure) was measured with an electronic sphygmomanometer (HBP-9020, OMRON Co. Ltd., Kyoto, Japan) 3 times at 30 s intervals in seated participants after resting for 10 min – the mean value being used for further analysis –. Values above 80/120 mmHg, for diastolic and systolic BP, respectively, were considered high BP values.

2.3. Nutrient Intake

The assessment of dietary nutrient intake was performed by a qualified dietitian using a manual 24 h-recall, including 1 weekend day and 2 non-holidays. A set of photographs of prepared foods and dishes that are usually consumed in Granada (Spain) were employed for recall accuracy. Dietowin software (7.1. version, Barcelona, Spain) was used to quantitatively convert food intake to energy, carbohydrates, fats, proteins, and fiber content. Adequacy according to the Recommended Dietary Allowance (RDA) for postmenopausal women from Spain was also determined [16].

2.4. Anthropometric and Body Composition Analysis

The participants' body weight (Kg), FM (%), and Fat Free Mass (FFM) (%), were assessed by bioelectrical impedance (Tanita MC-980 Body Composition Analyzer MA Multifrequency Segmental, Barcelona, Spain). The device meets the applicable European standards (93/42EEC, 90/384EEC) for use in the medical research. Height (m) was measured with a stadiometer (Seca, model 213, range 85 to 200 cm; precision: 1 mm; Hamburg, Germany). Body Mass Index (BMI) was calculated as weight (kg)/height (m^2). The required conditions prior to the measurement were as follows: (I) no alcohol consumption the previous 24 h, (II) no food or drink intake less than 3 h before, (III) no vigorous exercise the previous 12 h, and (IV) no urination immediately before the analysis. Waist Perimeter (WP) was calculated at the midpoint between the top of the iliac crest and the lower margin of the least palpable rib [17]. Hip Perimeter (HP) was measured at the widest portion of the buttocks, with the tape parallel to the floor [18]. The calculation of Waist to Hip Ratio (WHR) was made by dividing WP (cm) by HP (cm).

2.5. Sample Processing

Blood samples were drawn in the morning in fasting conditions and centrifugated at 4 °C for 15 min at 3,000× *g* rpm, thus obtaining plasma samples, which were stored at −80 °C. The measurements were performed in the same assay batch with blinded quality control samples being included in the assay batches, in one run.

2.5.1. Measurement of Biochemical Parameters

All biochemical factors were measured in plasma. Glucose (mg/dL), creatinine (mg/dL), urea (mg/dL), uric acid (mg/dL), total bilirubin (mg/dL), total proteins (g/dL), albumin (mg/dL), prealbumin (mg/dL), transferrin (mg/dL), Triglycerides (TG) (mg/dL), CRP (mg/L), High Density Lipoprotein (HDL) (mg/dL), Low Density Lipoprotein (LDL) (mg/dL), Total Cholesterol (TC) (mg/dL), osteocalcin (ng/mL), Parathyroid Hormone (PTH) (pg/mL), and leptin (ng/mL) levels were analyzed at the Virgen de las Nieves Hospital (Analysis Unit), Granada (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany). The reference values were obtained from the Analysis Unit. Flame Atomic Absorption Spectrophotometry (FAAS, Perkin Elmer® Analyst 300 model, Berlin, Germany) was used to analyze Ca (mg/dL), Iron (Fe) (µg/dL), and Copper (Cu) (µg/dL), whereas the Fiske-Subbarow colorimetric method (Thermo Scientific, Rockford, Illinois, United States of America) was employed to determine phosphorous (P) (mg/dL). Vitamin D (ng/mL) was analyzed by Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) using the Waters Acquity UHPLC I-Class System chromatograph (Waters, London, United Kingdom). Total Antioxidant Capacity (TAC) (µmol/L) was measured via the reduction power of Cu^{2+} from the action of antioxidants present in plasma samples (TAC kit, Jaica, Shizuoka, Japan). An enzymatic immunological method (Bioxytech GPx-340™ kit, OxisResearch™, Portland, Oregon, United States of America) was used to determine Glutathione Peroxidase (GPx) (mU/mL) activity. Superoxide Dismutase (SOD) (U/mL) activity was analyzed by the colorimetric method based on cytochrome c reduction using the Ransod kit (RANDOX Laboratories Ltd., Dublin, Ireland, United

Kingdom). The rest of the previous-mentioned parameters and their reference values were provided by the Scientific Instrumentation Center (SIC) from the University of Granada.

2.6. Statistical Analysis

As a previous step to the execution of a parametric model or not, the Kolmogorov-Smirnov test was used to accept the hypothesis of normal distribution. Frequency analysis for categorical variables was shown as Frequencies (*n*) and Percentages (%). Descriptive analysis for continuous variables was shown as Mean ± Standard Deviation (X ± SD). For the comparative analysis based on inter-three groups, one-way Analysis of Variance (ANOVA) adjusted by Bonferroni post hoc analysis was employed. Simple linear regression models were used to evaluate the association of age with the significant biochemical parameters of the study. Multiple linear regression models were conducted to test these associations after adjusting for the significant sociodemographic, anthropometric, BC, and dietary parameters (i.e., smoking habits, educational level, height, and fiber intake). A p Value < 0.05 was considered significant. All analyses were carried out using the SPSS 22.0 software for Mac (SPSS Inc. Chicago, Illinois, United States of America).

3. Results

Table 1 shows the anthropometric, body composition, dietary, and sociodemographic variables of the study by age. Regarding anthropometric and BC variables, only height decreased in those women above 62 years of age compared to postmenopausal women below 54 years of age ($p < 0.05$). In contrast, the rest of the parameters showed no mean differences by age (all at $p > 0.05$). Regarding quantitative dietary intakes, fiber showed the highest values in women older than 62 years of age compared to those younger than 54 years of age ($p < 0.001$), whereas no mean differences by age were reflected for the rest of the studied intakes. In the case of sociodemographic characteristics, smoking habits decreased, and educational level was lower with higher ages ($p < 0.05$ and $p < 0.01$, respectively).

Table 2 represents the biochemical parameters of the study by age. Regarding glucose and protein metabolism parameters, glucose ($p < 0.001$), urea ($p < 0.05$), uric acid ($p < 0.01$) and total bilirubin ($p < 0.05$) increased significantly in women above 62 years of age compared to those below 54 years of age. Moreover, the lipid parameters of our study showed no mean differences by age (all $p > 0.05$). Regarding the parameters involved in P-Ca metabolism, P ($p < 0.01$), vitamin D ($p < 0.01$), and vitamin D_3 metabolite ($p < 0.05$) levels were significantly higher in women aged 54 – 62 years of age than in those aged < 54 years of age, whereas Cu levels were significantly lower in women above 62 years of age than in those < 54 years of age ($p < 0.001$). The rest of the parameters showed no mean differences by age group (all $p > 0.05$).

Table 1. Anthropometric, body composition, dietary, and sociodemographic variables by age.

Characteristics	Total Population (*n* = 78)	< 54 Years (*n* = 26)	54 – 62 Years (*n* = 26)	> 62 Years (*n* = 26)	*p* Value	Reference Values
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD		
Anthropometric and body composition parameters						
Weight (Kg)	68.7 ± 13.2	71.1 ± 15.1	68.5 ± 12.3	66.2 ± 11.8	0.414	–
Height (m)	159.3 ± 6.2	162.1 ± 6.3[a]	158.9 ± 5.7	156.5 ± 5.4[a]	**0.004**	–
BMI (Kg/m^2)	27.0 ± 4.6	26.9 ± 5.1	27.1 ± 4.5	27.0 ± 4.3	0.996	22.0 – 27.0
Waist perimeter (cm)	89.0 ± 12.6	87.4 ± 16.6	88.9 ± 12.4	91.0 ± 13.3	0.592	< 90.0
Hip perimeter (cm)	105.8 ± 10.5	105.9 ± 9.61	106.5 ± 10.7	104.9 ± 11.5	0.873	< 110.0
Waist to hip ratio	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.202	< 0.80
Fat mass (%)	37.6 ± 5.9	36.9 ± 5.7	38.6 ± 5.66	37.2 ± 6.5	0.556	23.0 – 31.0
Fat free mass (%)	62.4 ± 5.9	63.1 ± 5.7	61.4 ± 5.66	62.7 ± 6.5	0.556	> 69.0
Dietary intake						
Energy (kcal/day)	1378 ± 337	1361.8 ± 394.4	1326.4 ± 304.7	1457.8 ± 294.6	0.381	2000.0
Carbohydrates (g/day)	149.7 ± 42.5	144.9 ± 49.2	142.9 ± 40.1	162.7 ± 34.7	0.200	275.0
Fats (g/day)	59.1 ± 20.6	59.9 ± 25.4	56.1 ± 17.1	61.2 ± 18.1	0.658	70.0
Proteins (g/day)	61.6 ± 15.4	59.2 ± 14.3	60.3 ± 15.4	65.7 ± 16.3	0.281	50.0
Fiber (g/day)	15.9 ± 8.1	12.9 ± 5.2[a]	14.9 ± 5.83	20.7 ± 10.7[a]	**0.001**	> 25
Characteristics	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***p* Value**	**Reference Values**
Blood pressure	–	–	–	–	–	–
Normal blood pressure	43 (55)	18 (69)	14 (54)	11 (42)	0.165	–
High blood pressure	35 (45)	8 (31)	12 (46)	15 (58)		–
Physical exercise	–	–	–	–	–	–
Sedentary	20 (26)	9 (35)	5 (19)	6 (23)	0.310	–
Non-sedentary	58 (74)	17 (65)	21 (81)	20 (77)		–
Smoking habit	–	–	–	–	–	–
Non-smoker	62 (80)	17 (65)	23 (89)	22 (85)	**0.045**	–
Smoker	16 (20)	9 (35)	3 (11)	4 (15)		–

Educational level	–	–	–	–	–	–
Basic educational level	29 (37)	7 (27)	6 (23)	16 (62)	**0.008**	–
Secondary or high educational level	49 (63)	19 (73)	20 (77)	10 (38)		–

n = 78. Categorical variables are expressed as frequencies (*n*) and percentages (%). Continuous variables are indicated as mean ± Standard Deviation (SD). To compare inter-tercile groups' categorical variables, a Chi-square test was used. To compare inter-tercile groups' continuous variables, one-way ANOVA with adjustment by Bonferroni post hoc test analysis was used: a = < 54 years vs. > 62 years. Significance was set at a *p* Value < 0.05 and is highlighted in boldface. Abbreviations: BMI = Body Mass Index.

Table 2. Biochemical variables by age.

Characteristics	Total Population (*n* = 78)	< 54 Years (*n* = 26)	54 – 62 Years (*n* = 26)	> 62 Years (*n* = 26)	*p* Value	Reference Values
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD		
Glucose (mg/dL)	92.2 ± 15.9	85.0 ± 12.9[b]	91.6 ± 9.31	100.9 ± 20.2[b]	**0.001**	70.0 – 110.0
Creatinine (mg/dL)	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.085	0.5 – 0.9
Urea (mg/dL)	34.5 ± 9.1	32.9 ± 0.1	32.4 ± 6.9[c]	38.7 ± 10.5[c]	**0.024**	10.0 – 50.0
Uric acid (mg/dL)	4.4 ± 1.1	4.1 ± 0.9[b]	4.2 ± 0.9[c]	4.9 ± 1.2[b,c]	**0.005**	2.4 – 5.7
Total bilirubin (mg/dL)	0.5 ± 0.1	0.4 ± 0.1[b]	0.5 ± 0.1	0.5 ± 0.2[b]	**0.023**	0.1 – 1.2
Total proteins (g/dL)	7.1 ± 0.5	7.1 ± 0.6	7.1 ± 0.4	7.1 ± 0.5	0.883	6.6 – 8.7
Albumin (mg/dL)	4.4 ± 0.2	4.5 ± 0.2	4.5 ± 0.2	4.4 ± 0.2	0.120	3.5 – 5.2
Prealbumin (mg/dL)	25.2 ± 5.07	24.9 ± 4.1	25.8 ± 5.5	24.8 ± 5.9	0.805	20.0 – 40.0
Transferrin (mg/dL)	280.2 ± 45.9	287.5 ± 42.5	269.1 ± 45.9	283.4 ± 49.9	0.385	200.0 – 360.0
CRP (mg/L)	1.1 ± 6.9	2.3 ± 11.3	0.3 ± 0.4	0.2 ± 0.1	0.447	0.02 – 5.0
Triglycerides (mg/dL)	108.2 ± 67.9	102.5 ± 84.2	109.6 ± 62.8	113.4 ± 52.3	0.845	50.0 – 200.0
HDL (mg/dL)	66.6 ± 15.6	67.5 ± 13.1	65.2 ± 10.1	67.3 ± 22.3	0.845	40.0 – 60.0
LDL (mg/dL)	128.0 ± 31.3	122.2 ± 29.0	137.8 ± 31.2	124.4 ± 32.9	0.156	70.0 – 190.0
Total cholesterol (mg/dL)	220.5 ± 34.4	215.4 ± 31.0	227.2 ± 36.7	219.3 ± 35.8	0.453	110.0 – 200.0
Osteocalcin (ng/mL)	15.3 ± 9.8	13.1 ± 8.9	15.3 ± 8.4	18.0 ± 11.7	0.209	15.0 – 46.0

PTH (pg/mL)	56.2 ± 23.8	57.6 ± 31.0	51.7 ± 15.6	59.3 ± 20.7	0.516	20.0 – 70.0
Leptin (ng/mL)	13.9 ± 4.8	13.4 ± 5.0	14.9 ± 5.3	13.4 ± 4.07	0.400	3.60 – 11.1
25–OH–D (ng/mL)	23.5 ± 7.4	20.7 ± 5.5[a]	27.1 ± 8.3[a]	22.8 ± 7.0	**0.006**	> 30.0
25–OH–D_3 (ng/mL)	17.7 ± 7.1	15.1 ± 5.4[a]	20.9 ± 7.9[a]	17.5 ± 6.7	**0.012**	> 20
25–OH–D_2 (ng/mL)	5.7 ± 3.1	5.6 ± 2.3	6.2 ± 4.3	5.3 ± 2.4	0.585	> 10
Ca (mg/dL)	9.2 ± 0.4	9.1 ± 0.3	9.3 ± 0.4	9.2 ± 0.6	0.531	8.6 – 10.2
P (mg/dL)	3.49 ± 0.5	3.2 ± 0.6[a]	3.7 ± 0.4[a]	3.5 ± 0.4	**0.003**	2.7 – 4.5
Fe (µg/dL)	92.6 ± 30.7	83.1 ± 31.8	100.2 ± 29.0	95.7 ± 29.5	0.109	60.0 – 170.0
Cu (µg/dL)	101.4 ± 23.0	111.8 ± 24.3[b]	101.4 ± 20.6	85.3 ± 15.4[b]	**0.001**	85.0 – 180.0
TAC (µmol/L)	1539.3 ± 483.1	1585.0 ± 658.3	1405.5 ± 388.1	1634.9 ± 264.1	0.209	1500.0
GPX (mU/mL)	118.2 ± 47.7	117.4 ± 43.9	116.9 ± 37.6	120.7 ± 62.1	0.957	120.0
SOD (U/mL)	184.4 ± 34.2	181.6 ± 33.1	184.3 ± 35.2	187.8 ± 35.5	0.813	164.0 – 240.0

n = 78. All variables are expressed as mean ± Standard Deviation (SD). To compare inter-tercile groups' continuous variables, one-way ANOVA with adjustment by Bonferroni post hoc test analysis was used: a = < 54 years vs. 54–62 years, b = < 54 years vs. > 62 years, and c = 54 – 62 years vs. > 62 years. Significance was set at a *p* Value < 0.05. Abbreviations: Ca = Calcium; CRP = C-Reactive Protein; Cu = Copper; Fe = Iron; GPx = Glutathione Peroxidase; HDL = High Density Lipoprotein; LDL = Low Density Lipoprotein; P = Phosphorous; PTH = Parathyroid Hormone; SOD = Superoxide Dismutase; TAC = Total Antioxidant Capacity; 25(OH)D = 25-Hydroxyvitamin D; 25(OH)D_2 = 25-Hydroxyvitamin D_2; 25(OH)D_3 = 25-Hydroxyvitamin D_3.

Table 3 shows the significant associations between age and the biochemical parameters of the study (Model 0), adjusted for the sociodemographic, anthropometric, BC, and dietary parameters showing differences by age in ***Table 1***. Age showed a direct correlation with glucose, uric acid, and osteocalcin (all $p \leq 0.032$; Model 0), and a negative relationship with albumin and Cu (all $p \leq 0.038$; Model 0), which persisted after including smoking habits, educational level, height, and fiber intake as covariates (all $p \leq 0.042$; Model 1). Age additionally showed a direct relationship with creatinine, urea, and total bilirubin (all $p \leq 0.011$; Model 0), which lost their significance after adjusting by the covariates mentioned above (all $p \geq 0.072$; Model 1).

Table 3. Significant associations between age and the biochemical parameters of the study.

Characteristics	Model 0			Model 1		
	ß	R^2	*p* Value	ß	R^2	*p* Value
Glucose (mg/dL)	0.425	0.181	**0.001**	0.377	0.237	**0.007**
Creatinine (mg/dL)	0.298	0.089	**0.009**	0.273	0.100	0.072
Urea (mg/dL)	0.307	0.094	**0.007**	0.169	0.133	0.252
Uric acid (mg/dL)	0.404	0.163	**0.001**	0.391	0.167	**0.008**
Total bilirubin (mg/dL)	0.292	0.085	**0.011**	0.162	0.186	0.260
Albumin (mg/dL)	−0.243	0.059	**0.038**	−0.398	0.121	**0.011**
Cu (μg/dL)	−0.379	0.144	**0.002**	−0.347	0.160	**0.042**
Osteocalcin (ng/mL)	0.247	0.061	**0.032**	0.436	0.130	**0.004**

ß = standardized regression coefficient. R^2 and *p* are from simple and multiple regression analyses between age and the significant biochemical parameters: Model 0 = simple regression analysis; Model 1 = Multiple regression analysis adjusted by significant sociodemographic, anthropometric, body composition and dietary parameters which showed significant differences by age (i.e., smoking habits, educational level, height, and fiber intake). Significance was set at *p* Value < 0.05 and is highlighted in boldface. Abbreviations: Cu = Copper.

4. Discussion

This study tried to elucidate the influence of aging upon several socio-demographic, anthropometric, BC, dietary, and biochemical factors in a cohort of postmenopausal women. Both educational level and smoking habits tended to be lower with aging, and contrary to our primary hypothesis, the analyzed anthropometric and BC data showed no differences by age. In contrast, biochemical parameters, especially those involved in the protein and P-Ca metabolism, were influenced by age, whereas such associations were not found when including lipid and antioxidant parameters.

Regarding anthropometric and BC parameters, no differences by age were found except for height, which was significantly lower in the group with greater age compared to the youngest group, with BMI showing no intergroup differences, which was considerably high (around 27 kg/m^2) in the 3 groups. Some authors have reported that height decreases with age, whereas BMI and weight significantly increase at the same time [19]. This could be due to the loss in both lean mass and height with aging [20], and to the lower physical condition at older ages [21]. Aging is independently correlated with increasing BMI [22], and significantly higher WP values were found with higher ages [23]. Postmenopausal women tend to present impaired BC, energy expenditure, or insulin sensitivity compared to similarly overweight premenopausal women [24].

In the present study, we observed a lower educational level with advancing age. Educational level is considered one of the best socioeconomic indicators, and its relation to menopause has been considered by some authors [25]. Older people, having less access to education than

their younger counterparts, present lower educational attainment, which could affect their health [26]. In addition, smoking habits decreased significantly, and physical activity showed no differences with aging in our population of postmenopausal women. It has been observed that low physical activity and smoking habits are less prevalent in postmenopausal women than in premenopausal and climacteric women [27]. Concerning BP, no statistically significant differences by age were found. Counterintuitively, menopause is the major determinant of BP increase in women [28].

The biochemical variables analyzed in the present study showed differences by age in several parameters. Plasma glucose levels were higher at greater ages, but lipid parameters showed no significant differences. Aging has been proposed to be a risk factor in developing insulin resistance, thus increasing circulating glucose levels [29]. Among women, menopause is associated with a more atherogenic lipid profile [30]. TC, TG, and the TC/HDL ratio increase from premenopausal to postmenopausal status [31]. However, in middle-aged women, age does not seem to modulate such changes in most of the studied metabolic health indicators during the menopausal transition (i.e., glucose, TG, TC, HDL, and LDL) [32]. With aging, we found a significant increase in protein metabolism (i.e., urea, uric acid, and bilirubin). The influence of age on urea levels has been previously addressed in a population of 1000 middle-aged (40 – 60 years) women (50% postmenopausal) – circulating urea levels being higher in postmenopausal women than in women in the menopausal transition and premenopausal stages – [33]. Other authors have reported decreases in circulating urea levels, whereas circulating total proteins and albumin are higher in postmenopausal women at 60 years of age than in women at premenopausal stages at 42 years of age

[34]. Age is one of the main contributors to increased circulating uric acid levels [35]. Blood urea nitrogen, creatinine, and uric acid are circulating markers of renal function whose values are elevated when renal function is reduced, especially in aging [36]. In this regard, we have observed a significant association between age and urea, uric acid, and creatinine, with uric acid maintaining its significance after the adjustment of the above-mentioned covariates, which could indicate a deterioration in the renal function at greater ages.

The homeostasis of Ca and P is maintained by a concerted interplay of absorption and resorption and by storage and mobilization from the bone, regulated mainly by PTH, vitamin D, and calcitonin [37]. In this regard, we found higher mean vitamin D levels in women between 54 – 62 years than in those below 54 years, observing the same trend for P levels. However, no statistically significant mean differences were found for osteocalcin, PTH, and Ca, which is in contrast with the decreased serum Ca levels with increasing age in 252 Nepalese postmenopausal women reported by Pardhe et al. [11]. Age appears to be an important independent predictor of serum Vitamin D in this population [38]. A study conducted on 99 women aged ≥ 50 years showed higher vitamin D levels at higher ages in postmenopausal women [39]. Other authors have found low vitamin D levels in women whose age was below 48 and over 60 [40]. It must be noted that the low levels of vitamin D that we obtained are mainly due to low levels of the 25(OH)D_3 metabolite. The supplementation with Ca and vitamin D_3 may normalize 25(OH)D_3 levels in postmenopausal women with inadequate circulating levels of 25(OH)D_3 and could reduce circulating PTH levels [41]. Furthermore, we observed a significant direct association of age with osteocalcin, which persisted after adjusting for smoking habits, educational level,

height, and fiber intake. Osteocalcin is a small bone-specific non-collagen protein produced by osteoblasts, being a sensitive marker of bone formation [42]. Osteocalcin is also involved in energy metabolism and preventing age-related muscle loss [43]. Age and years since menopause have been reported to be significantly associated with serum osteocalcin levels and bone mineral density [44], age being one of the most important predictors of osteocalcin status in postmenopausal women [45]. Interestingly, higher plasma osteocalcin levels have been found in postmenopausal compared to perimenopausal women [46].

Healthy aging is particularly important in women, as their lifespan is generally longer than that of men, leaving women at higher risk for age-related diseases [47]. Our findings may contribute to the understanding of processes of womens' healthy aging and the development of interventions targeting these indicators, which could have significant relevance for public health.

The present study suffers from some limitations, including that (I) we had no information about time living with menopause, which could have helped to enrich the way to interpret age-related results, (II) it has a cross-sectional design, which means that no causal relationships can be established, (III) we recruited fewer participants than desired and therefore the sample size of the study may not be big enough to obtain more statistically significant results, (IV) the study population was limited to postmenopausal women aged between (44 – 76 years old) from a specific area of southern Spain, and hence these results may not be generalizable to postmenopausal women of different regions or with ages not included in our range of age, and (V) it would have been interesting to include women in the premenopausal period to compare the effect of aging upon

the studied parameters in women of different menopausal stages. Moreover, further studies including larger sample sizes and more parameters should additionally be addressed.

5. Conclusions

In conclusion, height, fiber intake, blood glucose, protein and P-Ca metabolism parameters were the main variables that seem to be altered with the aging process. In contrast, anthropometric, lipid, and antioxidant parameter values showed no changes with aging. Further studies are needed to confirm our results in other postmenopausal populations to establish primary prevention protocols to maintain the variables that tend to be altered with aging in postmenopausal populations.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Title: Association between Body Fatness and Vitamin D_3 Status in a Postmenopausal Population

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Abstract

Introduction: Vitamin D is a micronutrient that plays a key role in phosphocalcic metabolism. The postmenopausal population presents a risk of deficiency in this vitamin due to hormonal alterations which, in the case of obesity, would be exacerbated. The objective was to assess the status of vitamin D in a postmenopausal population and determine the relationship of 25-Hydroxyvitamin D (25(OH)D) and its metabolites with anthropometric and Body Composition (BC) parameters. **Materials & Methods:** The study included 78 healthy postmenopausal women aged from 44 to 76. The nutrient intake assessment was carried out using the 24 hours Recall (24HR). 25(OH)D was analyzed using Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS). Anthropometric and BC parameters were determined using a measuring tape and a bioelectrical impedance device, respectively. **Results:** A total of 80% and 68% of the women studied did not reach sufficient values of 25(OH)D and 25-Hydroxivitamin D_3 (25(OH)D_3), respectively, which was inversely correlated with Body Mass Index ($r = -0.25$, $p = 0.04$), Hip Perimeter ($r = -0.26$ and r = −0.24, all $p < 0.05$), arm circumference ($r = -0.29$, $p = 0.01$) and Fat Mass (FM) ($r = -0.28$ and $r = -0.26$, all $p < 0.05$). 25(OH)D_3 is the metabolite that contributed most to this association. **Conclusion:** In conclusion, 25(OH)D_3 levels are related to anthropometric and BC parameters in the postmenopausal women of this study, confirming insufficient status in most of the population. Approach strategies are necessary to correct and avoid this risk of deficiency to ensure future quality of life.

Keywords: vitamin D; vitamin D_3; vitamin D_2; menopause; LC–MS/MS; BMI.

1. Introduction

Vitamin D is a fat-soluble vitamin that enters the body through exposing the skin to sunlight and through food and dietary supplement intake. Vitamin D is formed by the sum of the metabolites vitamin D_2 and vitamin D_3, with vitamin D_3 being most important and plentiful in humans because it comes from more food sources than vitamin D_2; in addition, it is the only metabolite that is synthesized in the epidermis. For vitamin D to become biologically active, it must first be hydroxylated in the liver to 25-Hydroxyvitamin D (25(OH)D), which is the metabolite used to assess a subject's vitamin D status, and then in the kidneys to 1,25-Dihydroxyvitamin D ($1,25(OH)_2D$), which is its active form [1]. Vitamin D deficiency is a worldwide public health problem related to skeletal and non-skeletal problems [2] due to nutritional deficits, liver and/or kidney failure, resistance to the action of vitamin D [3], and a low exposure to sunlight and the use of sunscreen [4]. Likewise, the theory that genetics has an impact on vitamin D deficiency is gaining strength [5]. On the other hand, vitamin D is well known for its role in regulating phosphocalcic metabolism [6]. Vitamin D deficiency causes a decrease in the intestinal absorption of Calcium (Ca), reducing its status and triggering the release of Parathyroid Hormone (PTH), levels of which are inversely proportional to the levels of 25(OH)D [7]. Therefore, the optimal serum concentration of 25(OH)D is defined as the concentration that suppresses the maximum release of PTH, being a measure of vitamin D deficiency and vitamin D toxicity [8].

Vitamin D deficiency is a widespread problem in the overweight population [9]. Obese subjects usually have lower sun exposure, reduced skin biosynthesis, or some intrinsic factor related to obesity, such as the

volumetric dilution of vitamin D in adipose tissue [10]. Therefore, obesity can also be a factor to consider when establishing vitamin D recommendations [1]. Coupled with an increased low-grade inflammatory state [11] (which seems to be more pronounced in women), this makes imposes higher vitamin D requirements in obesity [12]. Although the increase in body weight may have a protective effect on bone fractures, the scientific rationale showed some potential explanations that link obesity with increased fracture risk with reduced 25(OH)D concentrations [13]. In addition, the extent to which serum concentrations are sensitive to change because of weight loss has not been determined [9]. The decrease in the concentrations of 25(OH)D may be due to its contribution to muscle metabolism, recommending special attention to supplementation in overweight postmenopausal women who do physical activity [14].

The problem of a lack of vitamin D is also very common in menopause, which is the transitioning phase of a woman's life from the reproductive to the non-reproductive period. In this stage, there will be endocrine changes due to decreased ovarian activity, biological changes due to decreased fertility, and clinical changes resulting from changes in the menstrual cycle [15], with a wide variety of symptoms [16]. During menopause, women will have thinner skin and a lower capacity to produce vitamin D, in addition to a decrease of intestinal absorption of vitamin D and a decrease in vitamin D hydroxylation in the liver and kidneys. These metabolic problems will be accompanied by a tendency towards limited outdoor activity and a lower dietary intake of vitamin D [17]. On the other hand, an association has been found between vitamin D and sex hormones in postmenopausal women, where the reduction of estrogen levels, as well as other hormonal changes, causes a tendency to develop

low levels of vitamin D [18]. These hormonal alterations can cause musculoskeletal, metabolic, and cardiovascular conditions and can affect mental health, all of which being related to vitamin D deficiency [19,20]. In studies carried out of menopause, low levels of vitamin D were associated with a higher frequency of clinical fractures and low bone mass [21], in addition to the percentage of postmenopausal women with osteoporosis being higher in those with vitamin D deficiency [22]. During menopause, it has been shown that women may be particularly susceptible to the consequences of vitamin D deficiency, since a decrease in Bone Mineral Density (BMD) and lean mass, as well as an increase in Fat Mass (FM), occurs [23] in this period of life as a result of the decrease in estrogen levels.

The scientific literature reflects that the levels of 25(OH)D are inversely associated with BMI in postmenopausal women, though the role of different vitamin D metabolites in BMI and other anthropometric parameters is not yet clarified [1,24]. Therefore, our study aimed to assess the status of vitamin D in a postmenopausal population and determine the relationship between 25(OH)D and its metabolites with anthropometric and BC parameters in a postmenopausal population from Granada.

2. Materials and Methods

2.1. Subjects and Study Design

The present study is a cross-sectional design in which the study population was 78 postmenopausal women from Granada, aged between 44 and 76 years. The inclusion criteria were based on acceptance to participate in the study after being informed about it and presenting amenorrhea for at least 12 months. The exclusion criteria were being a perimenopausal woman, undergoing Hormone Replacement Therapy (HRT), refusal to participate in the study for various reasons, the presence of pathologies that may affect the absorption of nutrients, as well as being in a situation of disease that could alter the biochemical parameters analyzed. The confidentiality of all the data used and collected has been guaranteed at all times, complying with the principles of the Declaration of Helsinki and the approval by the Ethics Committee of the University of Granada (149/CEIH/2016).

2.2. Data Collection

All recorded data were obtained through the use of questionnaires that reflected information on personal data, sociodemographic aspects, nutrient intake and anthropometric measurements, as well as other aspects, such as an adequate diagnosis of the postmenopausal situation, smoking habits and physical activity.

2.3. Intake Rating

Dietary nutrient intake was quantitatively assessed using a R24h, considering 1 holiday and 2 non-holidays. Recall accuracy was recorded with a

set of photographs of prepared foods and dishes that are frequently consumed in Spain. The food intake assessment was converted to both energy and nutrients, determining the adequacy of the macronutrient and micronutrient intake according to the Recommended Daily Allowances (RDA) of that nutrient for the Spanish population of women within the age range included in our study using the using the Dietowin software (7.1. version, Barcelona, Spain).

2.4. Anthropometric and Body Composition Assessment

Height (m) and total body weight (kg) were measured according to the international standards for anthropometric assessment. Height was assessed with a stadiometer (Seca, model 213, range 85 to 200 cm; precision: 1 mm; Hamburg, Germany). BC measurements were taken for all participants, obtaining muscle mass (kg), FM (kg), percentage of body fat (%) and Body Mass Index (BMI) with bioelectrical impedance (Tanita MC-980 Body Composition Analyzer MA Multifrequency Segmental, Barcelona, Spain). In addition, the Arm (AP), Waist (WP), and Hip (HP) Perimeters (all in cm) were measured using a height rod with a 0.01 cm range of error. BMI was calculated as weight (kg) divided by the square of height (m^2). The study group was classified according to their BMI in the following groups: Normal weight < 27 kg/m^2; overweight-obesity > 27 kg/m^2 [25,26], corresponding to the median of the data. WP (cm) was measured at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest [27]. HP (cm) was measured at the widest portion of the buttocks, with the tape parallel to the floor [28]. Middle AP (cm) was measured in the right arm at the midpoint between the tip of the olecranon and the acromion, with the arm hanging loosely [29]. A

global adiposity factor for further analysis was calculated through the mean z-score values for BMI, AP, and HP.

2.5. Measurement of Biochemical Parameters

A blood extraction was performed and was centrifuged at 4 °C for 15 min at 3,000 rpm, extracting the plasma. Ca (mg/dL) was determined through Flame Atomic Absorption Spectrophotometry (FAAS, Perkin Elmer® Analyst 300 model, Berlin, Germany), and P (mg/dL) was determined using the Fiske-Subbarow colorimetric method (Thermo Scientific, Rockford, Illinois, United States). Osteocalcin (ng/mL) and PTH (pg/mL) were measured using EIA by colorimetric methods (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany). The remaining biochemical parameters, such as glucose (mg/dL), transferrin (mg/dL), prealbumin (mg/dL), albumin (mg/dL), Homocysteine (Hcy) (µmol/L), creatinine (mg/dL), LDH (U/L), urea (mg/dL), uric acid (mg/dL), Triglycerides (TG) (mg/dL), High Density Lipoprotein (HDL) (mg/dL) and Low Density Lipoprotein (LDL) (mg/dL), TC (mg/dL), transaminases (mg/dL), CRP (mg/L), total bilirubin (mg/dL), and total proteins (g/dL) levels were determined in the analysis unit at the Virgen de las Nieves Hospital, Granada, Spain.

2.5.1. Analytical Determination of Vitamin D

Vitamin D and its metabolites were analyzed by Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS). For protein precipitation, 200 µl of plasma was taken in an Eppendorf, to which 20 µL of internal standard (IS) (Sigma Aldrich, St. Louis, Missouri, USA) (0.5 µg/mL) and 500 µL of acetonitrile were added. This was taken to the plate shaker at an amplitude of 3, form 2 for 1 min and dried with N_2. It

was then centrifuged at 10,000× *g* for 15 min at 4 °C. The supernatant was collected in another Eppendorf, discarding the rest. For extraction phase, the supernatant was mixed with 100 µL of water and 200 µL of ethyl acetate, then mixed in vortex 30 s. Subsequently, it was centrifuged at 3,000× *g* for 5 min. The supernatant was collected, and the previous step was repeated with the precipitate, collecting the supernatant again and combining it with the previous one. Finally, the supernatants were dried with N_2. For derivatization step, a solution of 4-Phenyl-3H-1,2,4-Triazole-3,5(4H)-Dione (Sigma Aldrich, St. Louis, Missouri, United States of America) in acetonitrile (0.5 mg/mL) was prepared and mixed in vortex. Then, 50 µL of PTAD was added to both samples and standards and was taken to the plate shaker for 1 h at room temperature covered with aluminum foil. Finally, the samples were transferred to vials for chromatography and 50 µL of water was added. They were covered with aluminum foil and stored in the freezer at −20 °C to puncture in the chromatograph. For preparing the calibration line, increasing concentrations of 1 ppb, 2 ppb, 5 ppb, 10 ppb, 25 ppb, 50 ppb and 100 ppb of the standards (25(OH)D_3) and (25(OH)D_2) (Sigma Aldrich, St. Louis, Missouri, United States of America) were added to the vials, as well as 20 µL of the IS. The mixture was dried with N_2 and finally derivatized and stored at −20 °C for further analysis.

Samples were analyzed using the Waters Acquity UHPLC I-Class System chromatograph (Waters, London, United Kingdom). The column used was an Acquity UHPLC BEH C18 ™ column 2.1 × 50 mm, 1.7 µm at room temperature. The injection volume of the sample was 10 µL. The mobile phase of channel A was water with 50 mM of ammonium formate and that of channel B was methanol. The analysis time was 8 min and the flow rate 0.4 mL/min. The detector was a Waters XEVO-TQ-XS Triple

Quadrupole Low Resolution Spectrometer. The source of ionization was electrospray ionization with positive ionization. The desolvation temperature was 600 °C and the desolvation gas flow rate was 500 L/hour. The source temperature was 150 °C and the gas flow rate of the cone 150 L/hour. The biochemical values of 25(OH)D obtained were classified according to the reference values of 25(OH)D and 25(OH)D_3 in plasma. These are sufficiency > 30 ng/mL, insufficiency 20 – 30 ng/mL, and deficiency < 20 ng/mL for 25(OH)D [30] and sufficiency > 20 ng/mL and deficiency < 20 ng/mL for 25(OH)D_3 [31,32].

2.6. Statistical Analysis

Data were obtained using SPSS 22.0 Software for MAC (SPSS Inc. Chicago, Illinois, United States of America). GraphPad Prism 9 software (GraphPad Software, San Diego, California, United States of America) was used for plotting the graphs. Descriptive statistics were used for data expression, indicating the results of numerical variables as the arithmetic mean ± Standard Deviation (X ± SD), and the results of the categorical variables were expressed in Frequencies (N) and Percentages (%). As a previous step to the execution (or not) of a parametric model, the hypothesis of the normal distribution was accepted using the Kolmogorov–Smirnov test. For the comparative analysis based on the BMI categories (BMI < 27 kg/m^2 and BMI > 27 kg/m^2), the t-test for parametric samples was used. Likewise, Student's t-test and the Analysis of Variance test (ANOVA) were used to compare the status of both vitamins according to the BMI. Linear regression analysis was used to estimate the degree of association between the vitamin D and D_3 status, and the anthropometrical and BC parameters.

3. Results

Table 4 shows the anthropometric, BC, and nutrient intake values of the participants of the study by BMI categorized by BMI < 27 kg/m^2 and BMI > 27 kg/m^2. With reference to anthropometrical and BC parameters, it should be noted that the percentage of body fat, especially in the group with the highest BMI, was much higher than its reference value (32%). The other anthropometric parameters are approximately within the reference values, although they were more suitable in the group with the lowest BMI. Regarding energy intake, the diet was hypocaloric in both groups, but the group with the highest BMI had a higher hypocaloric tendency. In general, the population followed a slightly hyperproteic diet, whereas the rest of the variables were within the reference values, except for vitamin D levels which were below the reference values in both groups (< 40% RDA). The average carbohydrates intake was lower in the group with the highest BMI ($p < 0.05$).

Table 5 reflects the biochemical parameters of the participants of the study classified by BMI categories < 27 kg/m^2 and > 27 kg/m^2. The data obtained were framed within the reference values, reflecting that the biochemical parameters of the postmenopausal population of the study showed no differences by BMI (all $p \geq 0.061$)

Table 6 shows the average values for the most relevant parameters of phosphocalcic metabolism in the postmenopausal population classified by BMI categories. From a general view, 80% of the general population did not reach sufficient vitamin D status, and about half had deficiency levels of < 20 ng/mL. In the case of vitamin D_3, 80% of the pop-

ulation was deficient and 32% had sufficient status. In addition, the decrease in 25(OH)D and 25(OH)D_3 as the BMI increased was statistically significant ($p < 0.05$), an association that 25(OH)D_2 and the rest of the parameters did not experience (all $p \geq 0.2$).

Table 4. Anthropometric, body composition, and nutrient intake values of the participants of the study by BMI.

Characteristics	BMI < 27 (kg/m^2) (n = 39)	BMI > 27 (kg/m^2) (n = 39)	p Value	Reference Values
	Mean ± SD	Mean ± SD		
Age (years)	57.7 ± 8.2	58.5 ± 8.5	0.708	–
BMI (kg/m^2)	23.3 ± 2.6	30.5 ± 3.1	**0.001**	22 – 27
Blood pressure[1]	1.5 ± 1.4	1.9 ± 1.4	0.230	–
Physical exercise[2]	1.2 ± 0.9	1.2 ± 0.8	0.934	–
Anthropometry and body composition				
Arm perimeter (cm)	27.9 ± 1.9	31.7 ± 2.7	**0.001**	< 30
WP (cm)	80.0 ± 8.9	97.5 ± 9.5	**0.001**	< 90
Hip perimeter (cm)	98.3 ± 6.2	113 ± 8.7	**0.001**	< 110
Waist/hip ratio	0.8 ± 0.1	0.8 ± 0.1	**0.005**	< 0.80
Body fat (%)	33 ± 5.6	41 ± 3.7	**0.001**	23 – 31
Fat mass (kg)	20.1 ± 5.1	32.1 ± 6.7	**0.001**	–
Muscle mass (kg)	37.9 ± 7.7	45.8 ± 4.7	**0.001**	–
Dietary intake				
Energy intake (Kcal)	1500 ± 300	1300 ± 400	0.062	2000
CHO intake (g/day	160 ± 37.5	140 ± 45.0	**0.037**	275
Protein intake (g/day)	63.6 ± 14.1	59.5 ± 16.3	0.242	50
Fat intake (g/day)	61.6 ± 17.1	56.6 ± 23.3	0.293	70
Cholesterol intake (mg/day)	170 ± 73.8	163 ± 63.5	0.733	< 300
Fiber intake (g/day)	16.8 ± 6.7	15.1 ± 9.3	0.382	> 25
Ca intake (mg/day)	841 ± 262.0	800 ± 255	0.707	800 – 1000
P intake (mg/day)	1045 ± 321	1031 ± 292	0.842	800
Vitamin D intake (µg/day)	3.7 ± 3.7	3.3 ± 2.7	0.643	< 10

[1]Blood pressure shows the values 0 = optimal, 1 = normal, 2 = normal – high, 3 = grade 1 hypertension; [2]Physical exercise covers the values 0 = non-sedentary exercise, 1 = < 1 h/day, 2 = 1 – 2 h/day, 3 = < 2 h/day. Values are expressed as mean ± standard deviation. The fourth column shows the statistical significance after applying the comparison of means for not related samples. Significance was set at p Value < 0.05 and is highlighted in boldface. Abbreviations: BMI = Body Mass Index; CHO = Carbohidrates; P = Phosporous; WP = Waist Perimeter.

Table 5. Biochemical parameters of the participants of the study by BMI.

Characteristics	BMI < 27 (kg/m^2) (*n* = 39) (Mean ± SD)	BMI > 27 (kg/m^2) (*n* = 39) (Mean ± SD)	*p* Value	Reference Values
Glucose (mg/dL)	88.2 ± 17.5	100 ± 13.5	0.064	70 – 110
Transferrin (mg/dL)	300 ± 53.3	300 ± 37.4	0.760	200 – 360
Prealbumin (mg/dL)	24.1 ± 5.8	26.2 ± 4.1	0.098	20 – 40
Albumin (mg/dL)	4.5 ± 0.3	4.4 ± 0.2	0.452	3.5 – 5.2
Homocysteine (µmol/L)	11.9 ± 4.7	11.4 ± 4.9	0.642	< 13
Creatinine (mg/dl)	0.7 ± 0.1	0.7 ± 0.1	0.478	0.5 – 0.9
LDH (U/L)	200 ± 26.4	192 ± 58.6	0.277	110 – 295
Urea (mg/dL)	34.0 ± 9.3	35.0 ± 8.9	0.624	10 – 50
Uric acid (mg/dL)	4.2 ± 1.2	4.6 ± 0.9	0.162	2.4 – 5.7
Triglycerides (mg/dL)	106 ± 63.4	100 ± 72.6	0.823	50 – 200
HDL cholesterol (mg/dL)	70.2 ± 18.3	63.3 ± 11.5	0.061	40 – 60
LDL cholesterol (mg/dL)	129 ± 32.7	127 ± 30.3	0.749	70 – 190
Total cholesterol (mg/dL)	223 ± 34.8	218 ± 34.2	0.562	110 – 200
GOT (U/L)	22.0 ± 5.0	22.5 ± 7.7	0.746	< 37
GPT (U/L)	18.0 ± 7.5	21.2 ± 12.6	0.184	< 41
GGT (U/L)	18.0 ± 9.9	21.8 ± 18.2	0.264	11 – 50
CRP (mg/L)	1.9 ± 9.9	0.3 ± 0.2	0.306	0.02 – 5
Total bilirubin (mg/dL)	0.5 ± 0.2	0.5 ± 0.12	0.243	0.10 – 1.2
Total proteins (g/dL)	7.1 ± 0.5	7.1 ± 0.5	0.773	6.6 – 8.7

Values are expressed as mean ± Standard Deviation (SD). The fourth column shows the statistical significance after applying the comparison of means for not related samples. Significance was set at *p* Value < 0.05. Abbreviations: CRP: C-Reactive Protein; LDH: Lactate Dehydrogenase; GOT: Glutamic oxaloacetic transaminase; GPT: Glutamic pyruvic transaminase; GGT: Gamma-glutamyltransferase;.

Table 6. Phosphocalcic metabolism parameters classified according to BMI.

Parameter	BMI < 27 (kg/m²) (*n* = 39)	BMI > 27 (kg/m²) (*n* = 39)	*p* Value	Reference Values
	Mean ± SD	Mean ± SD		
25(OH)D (ng/mL)	26.1 ± 7.3	21.9 ± 6.6	**0.01**	30 – 100
25(OH)D_3 (ng/mL)	19.5 ± 7.4	16.1 ± 6.5	**0.04**	> 20
25(OH)D_2 (ng/mL)	5.8 ± 3.9	5.7 ± 2.3	0.9	> 10
Ca (mg/dL)	9.3 ± 0.5	9.1 ± 0.4	0.2	8.6 – 10.2
P (mg/dL)	3.5 ± 0.5	3.4 ± 0.5	0.4	2.7 – 4.5
Osteocalcin (ng/mL)	14.2 ± 10.6	16.2 ± 9.1	0.4	15 – 46
PTH (pg/mL)	53.2 ± 17.5	58.9 ± 28.2	0.3	20 – 70

Values are expressed as mean ± Standard Deviation (SD). The fourth column shows the statistical significance after applying the comparison of means for not related samples. Significance was set at *p* Value < 0.05 and is highlighted in boldface. Abbreviation: Ca = Calcium; P = Phosporous; PTH = Parathyroid hormone; 25(OH)D = 25-Hydroxyvitamin D; 25(OH)D_2 = 25-Hydroxyvitamin D_2; 25(OH)D_3 = 25-Hydroxyvitamin D_3.

Table 7 shows Pearson's bivariate correlations of BMI, 25(OH)D and, 25(OH)D_3 levels with the anthropometric and BC parameters analyzed in our study. A statistically significant inverse association was found between 25(OH)D and anthropometric and BC parameters in general, such as BMI, arm, and HP, as well as FM (all $p \leq 0.04$). This correlation was maintained when performing the bivariate analysis with the 25(OH)D_3 metabolite. In addition, there was a statistically significant inverse correlation between 25(OH)D_3 and categorized BMI, with this difference being more marked in the group with the highest BMI, HP, and FM (all $p < 0.05$).

Additionally, ***Figure 3*** shows the average levels of 25(OH)D and 25(OH)D_3 metabolite classified by BMI and vitamin D_3 and D status.

The intragroup analysis revealed that postmenopausal women with a normal vitamin D_3 status had significantly lower levels for this metabolite when the BMI was greater than 27 ($p = 0.001$).

Table 7. Matrix for correlation coefficients (r) showing the simple linear relationship between anthropometrical and body composition characteristics, vitamin D and vitamin D_3.

	Categorized BMI (kg/m^2)	**25(OH)D (ng/mL)**	**25(OH)D_3 (ng/mL)**
Uncategorized BMI (kg/m^2)	$r = 0.8$ **$p < 0.001$***	$r = -0.25$ **$p = 0.04$***	$r = -0.2$ $p = 0.09$
Categorized BMI (kg/m^2)	-	$r = -0.29$ **$p = 0.01$***	$r = -0.24$ **$p = 0.04$***
Arm circumference (cm)	$r = 0.7$ **$p < 0.001$***	$r = -0.24$ **$p = 0.04$***	$r = -0.2$ $p = 0.1$
Waist circumference (cm)	$r = 0.6$ **$p < 0.001$***	$r = -0.14$ $p = 0.2$	$r = -0.11$ $p = 0.3$
Hip perimeter (cm)	$r = 0.7$ **$p < 0.001$***	$r = -0.26$ **$p = 0.03$***	$r = -0.24$ **$p = 0.04$***
Waist/hip ratio	$r = 0.3$ **$p = 0.005$***	$r = 0.06$ $p = 0.6$	$r = 0.06$ $p = 0.5$
Body fat (%)	$r = 0.6$ **$p < 0.001$***	$r = -0.2$ $p = 0.1$	$r = -0.2$ $p = 0.1$
Fat mass (kg)	$r = 0.7$ **$p < 0.001$***	$r = -0.28$ **$p = 0.02$***	$r = -0.26$ **$p = 0.03$***
Muscle mass (kg)	$r = 0.5$ **$p < 0.001$***	$r = -0.2$ $p = 0.1$	$r = -0.15$ $p = 0.2$
25(OH)D (ng/mL)	$r = -0.29$ **$p = 0.01$***	-	$r = 0.90$ **$p < 0.001$***
25(OH)D_3 (ng/mL)	$r = -0.24$ **$p = 0.04$***	$r = 0.90$ **$p < 0.001$***	-

Matrix correlations are presented as correlation coefficients (r). *Significance was set at p Value < 0.05 and is highlighted in boldface. Abbreviations: BMI = Body Mass Index; 25(OH)D = 25-Hydroxyvitamin D; 25(OH)D_3 = 25-Hydroxyvitamin D_3

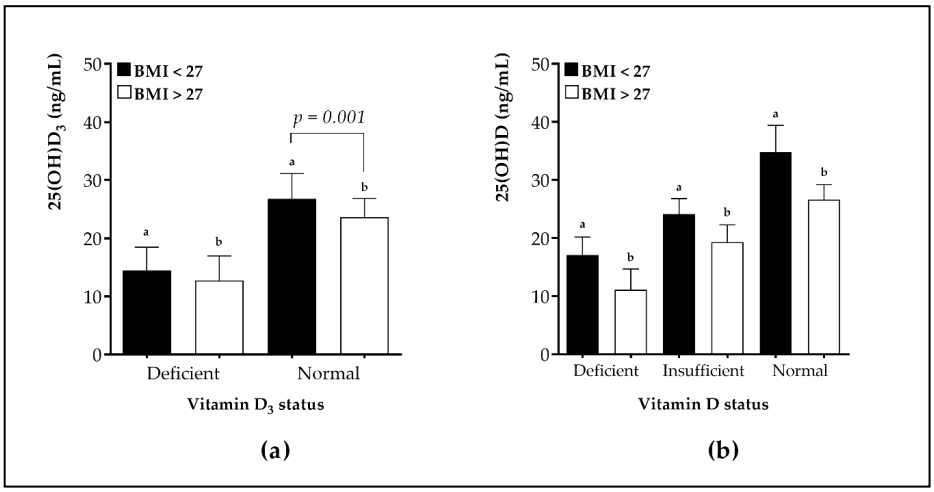

Figure 3. Average levels of 25(OH)D and 25(OH)D_3 metabolite classified by BMI and vitamin D_3 and D status (**a**) Mean levels of 25(OH)D_3 metabolite in BMI > 27 and BMI < 27 (expressed in kg/m^2**)** groups, each divided into subgroups based on vitamin D_3 status (Subgroup I: sufficiency > 20 ng/mL; subgroup II: deficiency < 20 ng/mL). **(b)** Mean levels of 25(OH)D metabolite in BMI > 27 and BMI < 27 (expressed in kg/m^2) groups, each divided into subgroups based on the vitamin D status (Subgroup I: sufficiency > 30 ng/mL; subgroup II: insufficiency 20–30 ng/mL; subgroup III: deficiency < 20 ng/mL). [a]statistically significant differences ($p < 0.05$) in vitamin D or D_3 status when BMI < 27 kg/m^2. [b]statistically significant differences ($p < 0.05$) in vitamin D or D_3 status when BMI < 27 kg/m^2. Abbreviations: BMI = Body Mass Index; 25(OH)D = 25-Hydroxyvitamin D; 25(OH)D_3 = 25-Hydroxyvitamin D_3.

Finally, ***Figure 4*** shows the bivariate correlation analysis between anthropometric parameters expressed as fatness z-score and 25(OH)D and 25(OH)D_3 metabolite levels. The results showed an inverse relationship between the predominance of fat and the levels of 25(OH)D and 25(OH)D_3 ($p \leq 0.049$).

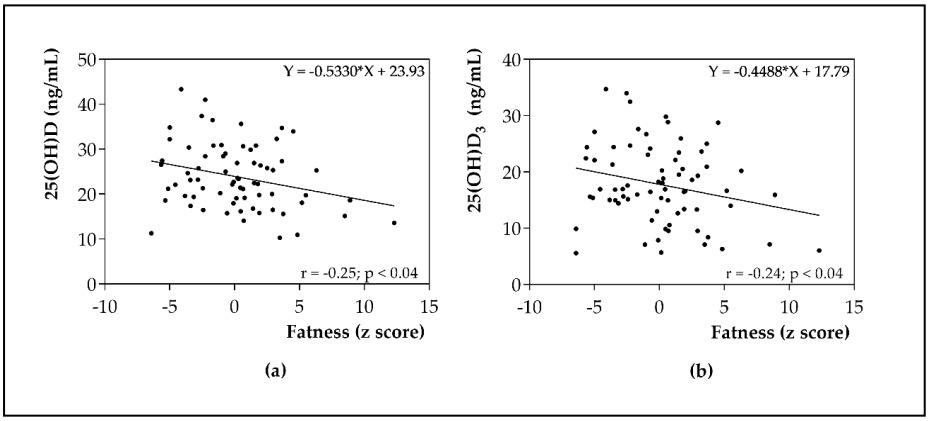

Figure 4. Pearson's bivariate correlation of 25(OH)D and 25(OH)D_3 with fatness as z score **(a)** Pearson's bivariate correlation of 25(OH)D with fatness as z score; **(b)** Pearson's bivariate correlation of 25(OH)D_3 metabolite with fatness as z score. Abbreviations: 25(OH)D = 25-Hydroxyvitamin D; 25(OH)D_3 = 25-Hydroxyvitamin D_3.

4. Discussion

The main findings of the present study showed that 80% of the general population did not have a sufficient vitamin D status (< 30 ng/mL). Similarly, 68% of the population was deficient for 25(OH)D_3 metabolite. The relationship between BC through BMI and 25(OH)D levels revealed that those women with higher BMI (BMI > 27 kg/m^2) presented lower status of that vitamin. The same results were observed for 25(OH)D_3 levels ($p < 0.05$), with higher levels being observed in those with a lower BMI and FM. However, when the comparative analysis was carried out according to BMI for 25(OH)D_2 metabolite, this statistically significant difference was not observed. On the other hand, no statistically significant correlation was observed between either 25(OH)D or its metabolites and age or the other parameters of phosphocalcic metabolism when categorized in the groups obtained according to BMI.

One of the most important changes during the menopausal stage refers to the changes in anthropometric and BC parameters mainly due to hormonal alterations during this stage. Previous evidence in the postmenopausal population showed an inverse association between vitamin D status and BMI. The role of anthropometric and BC parameters on the status of vitamin D_3 in this population is not clarified enough, and results demonstrate that a BMI cut-point of 30 kg/m^2 does not appear to be an appropriate indicator of true obesity status in postmenopausal women [26]. In this line, Banack et al. [26] indicated that BMI cut points should potentially be replaced by 26.5 kg/m^2 or 27.1 kg/m^2; therefore, this study established it as 27 kg/m^2, also based on the recommendations for an aged postmenopausal population [25].

In our study, vitamin D intake was slightly low, not considering the role of the sun on vitamin D input. This trend coincides with another Spanish postmenopausal population, in which 96% of the women studied had Ca and vitamin D intakes lower than the RDA, highlighting the need to take measures aimed at protecting the bone health of the Spanish female population [33]. This pattern of intake deficit has already been repeated in other parts of the world, such as in the study by Macdonald et al. [34] conducted in a population of 3113 women in the United Kingdom, in which vitamin D intake was 4.2 μg/day; therefore, vitamin D intakes are far below what is required in latitudes that are different to those of Spain as well. Vitamin D intake should be emphasized for all latitudes, especially for those women living in latitudes with limited sun exposure [35] where it has become very common to supplement with vitamin D in the postmenopausal population [36]. In a study of vitamin D supplementation in the postmenopausal population, Zhao et al. [37] observed that subjects who began a vitamin D supplementation trial with a low serum vitamin D status during a cold season were more sensitive to vitamin D supplementation compared to subjects who started during a hot season and had elevated baseline levels of serum vitamin D.

In relation to the levels of 25(OH)D and its metabolites, a high prevalence of vitamin D deficiency was found in our population, which corresponds to a greater or lesser extent with other populations, such as that in the study performed by Arévalo et al. [38], in which the Argentine postmenopausal population showed a 27% vitamin D deficiency and 29% vitamin D insufficiency, finding a negative association with age. Stewart et al. [39] assessed a large-scale sample of postmenopausal women that included 18 countries worldwide. They found that there was a high prevalence of vitamin D deficiency in all countries studied, placing

a special emphasis on the fact that 64% of the postmenopausal women with osteoporosis had insufficient levels of 25(OH)D. In the study by Li et al. [40] performed on Chinese postmenopausal women, approximately 72% of the women had a vitamin D deficiency. In this study, serum vitamin D levels were not correlated with BMI or FM, contradicting the results of our study. On the other hand, other authors, such as Shirazi et al. [41], found a positive association between 25(OH)D_3 serum levels and age, phosphorus (P), Ca, and a high intake of vitamin D. One possible reason for the observed results that contradict our own is that in the present study, the older women in this cohort consume relatively more vitamin D.

In terms of the rest of the phosphocalcic metabolism parameters, authors such as Zhang et al. [42] state that serum P levels decrease progressively with age in postmenopausal women. In our study, P levels were within normal values, possibly due to a diet adequate in P. On the other hand, we observed that osteocalcin levels were higher as age increased ($r = 0.28$, $p = 0.01$). In the study by Alissa et al. [17], vitamin D intake and BMI were associated with low levels of osteocalcin; however, the same association with vitamin D and BMI was not found in our study. Other studies found an association between phosphocalcic metabolism parameters, such as P and PTH, and anthropometric and BC parameters, like the study performed by Billington et al. [43], in which the authors maintain that serum P from the postmenopausal population was inversely correlated with weight, BMI and FM. Bolland et al. [44] also found that PTH was positively correlated with body weight, regional, and total FM and body fat percentage, but was negatively correlated with vitamin D. Similarly, we observed the same positive association between FM and PTH ($r = 0.32$, $p = 0.005$), although we did not find the direct relationship

of this hormone with vitamin D status. Another study by Macdonald et al. [34] reflects that obese subjects had a lower vitamin D status and higher concentrations of PTH compared to non-obese subjects. Finally, Khadka et al. [45] found that vitamin D and Ca were negatively correlated with the year of menopause onset, suggesting medical supervision of hormonal changes and periodic dosing of vitamin D and Ca in postmenopausal women to reduce the bone health problem.

In our study, an inverse association was found between vitamin D status and FM. However, the weakness of this association might be explained by the presence of numerous factors which modulate FM, such as ethnicity, age [46], sun exposure habit, diet, and the season of the year in which the samples were taken, which can mean that even though a woman has a high FM, its influence on vitamin D status can lose strength [47,48]. In our study, due to the limited sample size, we could not control for some of these confounding factors. Authors such as Lucas et al. [49] maintain that 25(OH)D levels are inversely related to FM and positively related to physical activity. However, we have not been able to demonstrate the positive relationship of vitamin D status with physical activity, probably due to low physical activity patterns in our postmenopausal women. Authors like Vuksanovic et al. [50] stated that visceral fat is more harmful than subcutaneous fat, emphasizing that women with high amounts of visceral fat have low serum 25(OH)D levels. Therefore, it would be necessary to assess the location of body fat and its influence on vitamin D status for future studies. Similarly, we observed how the HP was negatively correlated with both 25(OH)D and 25(OH)D_3 levels, confirming the relationship of those vitamins with parameters that are usually used to determine the central obesity. Abboud et al. [51] found lower vitamin D status in those subjects with a high HP; nevertheless, they did

not reflect the role of 25(OH)D_3 metabolite in this association. According to our results, a decrease in body fat is accompanied by an increase in the status of vitamin D_3. However, Holecki et al. [52] performed an intervention to reduce body weight in menopause, achieving a significant decrease in body fat and concluding that in obese subjects the serum concentration of 25(OH)D_3 was significantly lower before and after intervention. In addition, in our study we found an inverse relationship between 25(OH)D and 25(OH)D_3 levels with BMI. Sousa-Santos et al. [53] found this correlation between BMI and vitamin D status, although they also failed to discern between the different metabolites of vitamin D. Other authors, such as Liu et al. and Kocot et al. [54,55], support the role that BMI has on vitamin D_3 status ($r = 0.09$, $p = 0.01$), although they did not assess whether this association was influenced by total body fat as we demonstrated in our results. The prevalence of obesity, associated with a reduced quality of life, morbidity, and mortality, underscores the need for a food reeducation program during the postmenopausal period [56]. Therefore, to improve the status of vitamin D, regular use of low doses of supplemental vitamin D_3 [57,58] in case of deficiency, compliance with vitamin D and Ca RDAs, and maintaining an adequate weight is recommended in postmenopausal population [59].

5. Conclusions

Our data reflect that the high prevalence of vitamin D and vitamin D_3 deficit observed in our postmenopausal population is generally correlated with BMI, in addition to anthropometric and BC parameters such as hip and arm circumference and FM defined as fatness. According to our data, it seems that $25(OH)D_3$ is the vitamin D form that is most closely related to the anthropometric and BC parameters studied. Therefore, nutritional assessment and vitamin D_3 supplementation policies are proposed, as well as related healthy habits to improve the status of vitamin D in at-risk groups, such as postmenopausal women, to optimize their quality of life.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (protocol code 149/CEIH/2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Title: Relationship between Body Composition and Biochemical Parameters with Antioxidant Status in a Healthy Cohort of Postmenopausal Women

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Abstract

Introduction: An adequate prooxidant-antioxidant balance – which may be influenced by Body Composition (BC) and biochemical status – is essential to maintain human health, especially in circumstances under which the antioxidant defense decreases, such as menopause. The present study aimed to examine the relationship between BC and biochemical parameters with antioxidant status in a healthy cohort of postmenopausal women. **Materials & Methods:** This cross-sectional study was carried out in a cohort of 78 postmenopausal women aged 44–76 years. The BC profile was assessed through bioelectrical impedance. The determination of the Total Antioxidant Capacity (TAC) and Superoxide Dismutase (SOD) activity was conducted by colorimetric methods. Glutathione Peroxidase (GPx) activity was determined by enzymatic immunological methods. The vitamin D levels were measured by liquid Chromatography–tandem mass spectrometry. The mineral status was assessed through flame atomic absorption spectrophotometry. The rest of the biochemical parameters were assessed through an immunoassay. **Results:** TAC and antioxidant gap were negatively influenced by BC (all $p \leq 0.049$) and positively related to protein metabolism parameters (all $p \leq 0.048$), whereas circulating levels of different micronutrients (all $p \leq 0.048$) and enzymes (all $p \leq 0.047$) appeared to play an important role in the glutathione peroxidase and superoxide dismutase activities. **Conclusion:** In conclusion, the menopause-related antioxidant status changes may be influenced by key BC and biochemical profiles. To confirm this statement, further trials aiming to evaluate the BC and biochemical intervention-induced changes upon antioxidant defense are needed.

Keywords: total antioxidant capacity; superoxide dismutase; glutathione peroxidase; body composition; postmenopausal women; menopause

1. Introduction

Menopause occurs progressively between the ages of 45 and 55 years during the life of women and is characterized by permanent loss of menstrual cycles and a significant decrease in the estrogen and progesterone levels, influencing many organs, systems, and processes [1]. The estrogen levels are closely associated with the circulating redox status, thus controlling, in part, circulating antioxidant levels [2]. In this regard, estrogen's capacities to prevent oxidative stress processes and to produce antioxidant molecules decrease in menopause [3]. Furthermore, the mechanisms involved in antioxidant protection deteriorate with age [4], with an adequate prooxidant–antioxidant balance being essential to maintaining healthy conditions for physiological activities [5].

The Total Antioxidant Capacity (TAC) gathers the synergic and redox interactions between the different antioxidant molecules present in foods and biological fluids [6]. The TAC has been observed to be reduced in postmenopausal women compared with premenopausal women [7]. In order to assess and understand the antioxidant status, antioxidant enzymes, such as Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx), need to be evaluated [8]. SOD faces oxidative stress by catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide [9], whereas GPx is involved in preventing the harmful accumulation of intracellular hydrogen peroxide, thus reducing oxidative stress [10]. In this regard, SOD has been suggested to be one of the most significant antioxidants in the human body, and its status may be compromised in menopausal stages [11]. Moreover, the deprivation of estrogens decreases the gene expression of GPx, thus decreasing its circulating levels [12]. Altogether, the SOD and GPx enzyme activities in the ovaries of

postmenopausal women have been observed to be significantly lower than those in the premenopausal stage [13]. Regretfully, there is a lack of information available on the association of plasma TAC with individual endogenous and exogenous antioxidant components in humans, especially in postmenopausal women [8]. Changes in Body Composition (BC), with gains in Fat Mass (FM) and losses of Fat-Free Mass (FFM), are menopausal transition-related processes [14]. The previously mentioned changes in the BC, together with anthropometrical disturbances, have been shown to decrease antioxidant defense in menopausal women [15]. In this regard, although the TAC levels and SOD and GPx activities may be influenced by several factors (e.g., aging, Body Mass Index (BMI), and gender), evidence in this field to date has been conflicting [16].

Therefore, research on the relationship between the antioxidant status and menopausal status has not yet been completely elucidated [17]. In this regard, knowledge of the mechanisms based on the biochemical and BC parameters that could influence antioxidant defense is needed and could help to understand the behavior of antioxidant defense in humans at risk of antioxidant disturbances (e.g., postmenopausal women) [11]. Therefore, the present study aimed to examine the relationship between BC and biochemical parameters with antioxidant status in a healthy cohort of postmenopausal women.

2. Materials and Methods

2.1. Study Design and Participants

A cohort of 78 healthy postmenopausal women volunteers from the province of Granada, Spain, aged 44–76 years was included in the present cross-sectional study. All individuals signed written informed consent. The study was performed following the principles of the Declaration of Helsinki [18] and approved by the Ethics Committee of the University of Granada (149/CEIH/2016), in accordance with the International Conference on Harmonization/Good Clinical Practice Standards. The inclusion criteria were: (I) presenting postmenopausal status (with at least 12 months of amenorrhea), (II) accepting participation in the study after being informed about it, and (III) presenting normal parameters from a previous routine hospital laboratory analysis. The exclusion criteria were: (I) refusing to participate in the study, (II) taking vitamin and mineral supplements, (III) presenting any pathology that could affect their nutritional status (i.e., the main components of metabolic syndrome, celiac disease, bulimia, and anorexia), (IV) undergoing hormone-replacement therapy, and (V) presenting systemic inflammatory status (C-Reactive Protein (CRP) was included as a reference biomarker to assess the inflammation status of the participants) – the cutoff value being below 5.00 mg/L –.

2.2. Sociodemographic Data Collection

Blood Pressure (BP) (mmHg) classified as normal pressure/high pressure was measured 3 times at 30 s intervals in seated participants with an electronic sphygmomanometer (HBP-9020, OMRON Co. Ltd., Kyoto, Japan) after the participants had rested for 10 min, and the mean value was

used for analysis. BP values above 120 mmHg/80 mmHg, for systolic and diastolic BP, respectively, were considered high pressure values. The following data were obtained through manual questionnaires administered by the researcher: (I) Physical exercise was defined as sedentary/non-sedentary, classifying as non-sedentary a woman who reported less than 30 min/day of regular exercise, less than 3 days/week. (II) Smoking habits were classified as non-smoker/smoker; a smoker was the subject who smoked more than 1 cigarette/day. (III) Educational level was divided into basic educational level/secondary or high educational level; individuals presenting the basic educational level were those who only finished primary studies.

2.3. Body Composition and Anthropometry Analysis

Height (m) was determined using a stadiometer (Seca, model 213, range 85 to 200 cm; precision: 1 mm; Hamburg, Germany). The individuals' body weight (kg), FM (kg and %), and FFM (%) were obtained through bioelectrical impedance (Tanita MC-980 Body Composition Analyzer MA Multifrequency Segmental, Barcelona, Spain). The analyzer complies with the applicable European Standards (93/42EEC, 90/384EEC) for use in the medical industry. The subjects of the study were informed in advance of the required conditions prior to measurement: (I) no alcohol intake in the previous 24 hours, (II) no vigorous exercise in the previous 12 hours, (III) no food or drink intake in the previous 3 hours, and (IV) no urination immediately before measurement. The BMI was calculated as weight (kg)/height (m^2). The Waist Perimeter (WP) (cm) was measured at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest [19]. The Hip Perimeter (HP) (cm) was determined with the tape parallel to the floor, at the widest portion of the

buttocks [20]. The waist/hip ratio was calculated as the WP divided by the HP.

2.4. Samples Treatment and Analysis

All samples were obtained in the morning under fasting conditions through blood extraction in the antecubital vein. Plasma was separated by centrifugation at 3,000 rpm for 4 min at 4 °C, and the aliquots were frozen at −80 °C until analysis.

The circulating glucose (mg/dL), creatinine (mg/dL), urea (mg/dL), uric acid (mg/dL), total bilirubin (mg/dL), total proteins (g/dL), albumin (g/dL), prealbumin (mg/dL), transferrin (mg/dL), CRP (mg/L), Homocysteine (Hcy) (µmol/L), Glutamic Oxaloacetic Transaminase (GOT) (U/L), Glutamic Pyruvic Transaminase (GPT) (U/L), Gamma-Glutamyl Transferase (GGT) (U/L), amylase (U/L), Lactate Dehydrogenase (LDH), Triglycerides (TG) (mg/dL), High-Density lipoprotein (HDL) (mg/dL), Low-Density Lipoprotein (LDL) (mg/dL), Total Cholesterol (TC) (mg/dL), osteocalcin (ng/mL), Parathyroid Hormone (PTH) (pg/mL), and leptin (ng/mL) levels were analyzed in the Analysis Unit – which provided reference values – at the Virgen de las Nieves Hospital, Granada (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany). The plasma Calcium (Ca) (mg/dL), Magnesium (Mg) (mg/dL), Iron (Fe) (µg/dL), and Copper (Cu) (µg/dL) levels were obtained through Flame Atomic Absorption Spectrophotometry (FAAS, Perkin Elmer® Analyst 300 model, Berlin, Germany), and the plasma Phosphorous (P) (mg/dL) levels were determined through the Fiske–Subbarow colorimetric method (Thermo Scientific, Rockford, Illinois, United States of America). Folate (Fol) (ng/mL) and Vitamin B_{12}

(B_{12}) (pg/mL) were measured using a DxI® Autoanalyzer (Beckman Coulter, California, United States of America) employing a competitive electrochemiluminescence immunoassay for quantitative determinations. Vitamin D_3 (ng/mL) and vitamin D_2 (ng/mL) were measured by Liquid Chromatography–tandem Mass Spectrometry (LC–MS/MS) using a Waters Acquity UHPLC I-Class System Chromatograph (Waters, London, United Kingdom). Total vitamin D (ng/mL) was calculated as vitamin D_3 + vitamin D_2. TAC determination was conducted by evaluating the reduction power of Cu^{2+} from the action of antioxidants present in samples (TAC kit, Jaica, Shizuoka, Japan). The GPx activity was determined by the enzymatic immunological method using the Bioxytech GPx-340™ kit (OxisResearch™, Portland, Oregon, United States of America). The SOD activity was determined by the colorimetric method based on cytochrome c reduction using a Randox Ransod kit (RANDOX Laboratories Ltd., Dublin, Ireland, United Kingdom). In addition, we calculated the SOD/GPx ratio and antioxidant gap (GAP) as previously reported [21]:

The remainder of the above-mentioned parameters were analyzed in the Scientific Instrumentation Center (SIC) at the University of Granada, where reference values were provided. All biochemical parameters were analyzed in the plasma. All samples were measured in one run, in duplicate, in the same assay batch, and blinded quality control samples were included in the assay batches.

2.5. Statistical Analysis

Data were obtained using SPSS 22.0 Software for MAC (SPSS Inc. Chicago, Illinois, USA). GraphPad Prism 9 software (GraphPad Software,

San Diego, California, USA) was used for plotting the graphs. As a previous step to the execution of a parametric model or not, the hypothesis of normal distribution was accepted using the Kolmogorov–Smirnov test. Categorical variables are shown as Frequencies (N) and Percentages (%). Continuous variables are presented as the Mean and Standard Deviation (X ± SD). We performed sample size calculation for our primary aim of the cross-sectional study based on the relationship between the BC and biochemical parameters with antioxidant status in a healthy cohort of postmenopausal women by using G*Power software (version 3.1.9.6, Kiel, Germany). The number of participants to be included in the study was calculated based on the main statistical method used (unpaired t-test). An a priori power analysis indicated that a total of at least 70 participants were required. This calculation was based on a moderate effect size (effect size d = 0.70), an alpha level of 0.05, and a beta value of 0.90 for an unpaired t-test calculating the difference between 2 independent means (2 groups) [22]. Student's unpaired-samples test was conducted to study differences between early and late menopause. Pearson's correlation models were used to evaluate the significant associations of BC and biochemical parameters with TAC, GPx, and SOD. Simple linear regression models were used to evaluate the significant associations of the BC and biochemical parameters with the SOD/GPx ratio and GAP, (Model 0) and adjusted by age, BMI, physical activity, and early vs. late menopause (Model 1). β (standardized regression coefficient), R^2, and *p* values from simple linear regression analyses were obtained. A *p* Value of less than 0.05 was considered significant.

3. Results

1 in 4 participants had a sedentary status, and almost half presented high BP. Moreover, a fifth of the subjects smoked. Finally, a third of the participants presented a basic educational level (***Table 8***).

Table 8. Sociodemographic variables of the study.

Characteristics	Total population (n = 78)	
	N	(%)
Sociodemographic		
Blood pressure	–	–
Normal blood pressure	43	(55)
High blood pressure	35	(45)
Physical exercise	–	–
Sedentary	20	(26)
Non-sedentary	58	(74)
Smoking habit	–	–
Non-smoker	62	(80)
Smoker	16	(20)
Educational level	–	–
Basic educational level	29	(37)
Secondary or high educational level	49	(63)

N = 78. All variables are expressed as Frequencies (N) and Percentages (%).

Table 9 shows the anthropometrical, body composition, and biochemical variables of the study. The BC variables that showed the lowest percentages below the reference values were FM in % (almost the whole population) and waist/hip ratio (one in ten subjects). Regarding the biochemical variables, 25(OH)D (with 4 in 5 participants) was the biochemical variable that showed the highest percentage of participants below the reference values. The height, FFM, and albumin were higher in early vs. late menopause (all $p \leq 0.048$), whereas glucose, urea, uric acid, total bilirubin, and osteocalcin were lower in early vs. late menopause (all $p \leq 0.017$).

Table 9. Anthropometrical, body composition, and biochemical variables of the study.

Characteristics	Total Population (*n* = 78)	Below Reference	Early Menopause (*n* = 39)	Late Menopause (*n* = 39)	Reference Values
	Mean ± SD	%	Mean ± SD	Mean ± SD	Range
Body Composition					
Weight (kg)	68.7 ± 13.2	–	70.3 ± 13.9	66.9 ± 12.3	–
Height (m)	159.3 ± 6.2	–	161.5 ± 6.2	156.8 ± 5.3*	–
BMI (kg/m^2)	27.0 ± 4.6	14.1	26.9 ± 4.8	27.2 ± 4.4	22.0 – 27.0
WP (cm)	89.0 ± 12.6	50.0	87.8 ± 12.4	90.5 ± 13.0	< 90.0
HP (cm)	105.8 ± 10.5	65.4	105.7 ± 9.5	105.9 ± 11.6	< 110.0
Waist/hip ratio	0.8 ± 0.1	12.8	0.8 ± 0.1	0.8 ± 0.1	< 0.80
FM (%)	37.6 ± 5.92	1.30	37.4 ± 5.5	37.8 ± 6.4	23.0 – 31.0
FM (kg)	26.3 ± 8.5	–	26.8 ± 8.5	25.7 ± 8.6	–
FFM (%)	62.4 ± 5.9	100.0	43.5 ± 6.3	40.2 ± 8.3*	> 69.0
Biochemical parameters					
Glucose (mg/dL)	92.2 ± 15.9	3.90	87.4 ± 12.4	97.4 ± 17.9*	70.0 – 110.0
Creatinine (mg/dL)	0.7 ± 0.1	2.60	0.7 ± 0.1	0.7 ± 0.2	0.5 – 0.9
Urea (mg/dL)	34.5 ± 9.1	0.00	32.2 ± 8.0	37.2 ± 9.6*	10.0 – 50.0
Uric acid (mg/dL)	4.4 ± 1.1	0.00	4.1 ± 0.9	4.7 ± 1.2*	2.4 – 5.7
Total bilirubin (mg/dL)	0.5 ± 0.1	0.00	0.4 ± 0.1	0.5 ± 0.1*	0.1 – 1.2
Total proteins (g/dL)	7.1 ± 0.5	14.7	7.20 ± 0.5	7.0 ± 0.5	6.6 – 8.7
Albumin (g/dL)	4.4 ± 0.2	0.00	4.50 ± 0.2	4.4 ± 0.2*	3.5 – 5.2
Prealbumin (mg/dL)	25.2 ± 5.1	11.1	25.6 ± 4.5	24.6 ± 5.8	20.0 – 40.0
Transferrin (mg/dL)	280.2 ± 45.9	3.20	279.0 ± 43.1	281.8 ± 50.0	200.0 – 360.0
CRP (mg/L)	1.04 ± 6.9	0.00	1.7 ± 9.3	0.2 ± 0.2	0.02 – 5.0
Hcy (µmol/L)	11.7 ± 4.7	73.3	11.6 ± 4.5	11.8 ± 5.2	< 13.0
GOT (U/L)	22.3 ± 6.5	97.4	22.1 ± 4.8	22.5 ± 8.0	< 37.0
GPT (U/L)	19.7 ± 10.5	96.1	19.0 ± 7.2	20.6 ± 13.4	< 41.0
GGT (U/L)	20.0 ± 14.8	19.7	19.9 ± 17.3	20.1 ± 11.5	11.0 – 50.0
Amylase (U/L)	69.8 ± 25.5	9.50	66.0 ± 23.6	74.1 ± 27.1	40.0 – 140.0
LDH (U/L)	186.4 ± 46.3	1.30	183.9 ± 53.2	189.3 ± 37.3	110.0 – 295.0
TG (mg/dL)	108.2 ± 67.9	3.90	108.2 ± 82.0	108.2 ± 48.4	50.0 – 200.0
HDL (mg/dL)	66.6 ± 15.6	1.30	66.9 ± 12.1	66.4 ± 19.0	40.0 – 60.0
LDL (mg/dL)	128.0 ± 31.3	3.90	126.4 ± 30.3	130.0 ± 32.8	70.0 – 190.0
TC (mg/dL)	220.5 ± 34.4	0.00	219.1 ± 33.7	222.1 ± 35.6	110.0 – 200.0
Osteocalcin (ng/mL)	15.3 ± 9.8	48.0	12.8 ± 8.6	18.3 ± 10.5*	15.0 – 46.0
PTH (pg/mL)	56.2 ± 23.8	0.00	54.9 ± 27.0	57.8 ± 19.4	20.0 – 70.0
Leptin (ng/mL)	13.9 ± 4.83	0.00	14.0 ± 5.1	13.8 ± 4.6	3.6 – 11.1
Folic acid (ng/mL)	11.2 ± 4.1	0.00	10.8 ± 4.3	11.6 ± 3.8	2.70 – 17.0
Vitamin B_{12} (pg/mL)	527.1 ± 271.9	1.40	527.5 ± 220.9	526.5 ± 330.7	190.0 – 900.0
25(OH)D (ng/mL)	23.5 ± 7.4	79.2	23.7 ± 7.8	23.3 ± 7.1	30.0 – 100.0
25(OH)D_3 (ng/mL)	17.7 ± 7.1	62.5	17.7 ± 7.1	17.8 ± 7.1	> 20
25(OH)D_2 (ng/mL)	5.7 ± 3.1	93.1	6.0 ± 3.3	5.5 ± 2.9	> 10
Ca (mg/dL)	9.2 ± 0.4	6.50	9.2 ± 0.4	9.3 ± 0.5	8.6 – 10.2

P (mg/dL)	3.5 ± 0.5	3.90	3.4 ± 0.5	3.6 ± 0.4	2.7 – 4.5
Mg (mg/dL)	1.9 ± 0.2	23.1	1.9 ± 0.2	1.9 ± 0.3	1.7 – 2.2
Fe (µg/dL)	92.6 ± 30.7	13.0	88.3 ± 32.8	97.5 ± 27.8	60.0 –170.0
Cu (µg/dL)	101.4 ± 23.0	27.0	107.3 ± 23.0	92.9 ± 20.8*	85.0 –180.0

n = 78. All variables are expressed as the mean ± Standard Deviation (SD). % represents the percentage of subjects below the reference values. Early and late menopause women are those below and above the median age (i.e., 57 years), respectively. T-Student's unpaired-samples test was used for comparing the mean differences between early and late menopause. *Significant mean differences with *p* Values less than 0.05. Abbreviations: BMI = Body Mass Index; B_{12} = Vitamin B_{12}; Ca = Calcium; CRP = C-Reactive Protein; Cu = Copper; Fe = Iron; FFM = Fat-Free Mass; FM = Fat Mass; Fol = Folic Acid; GGT = Gamma–Glutamyl Transferase; GOT = Glutamic Oxaloacetic Transaminase; GPT = Glutamic Pyruvic Transaminase; Hcy = Homocysteine; HDL = High-Density Lipoprotein; HP = Hip Perimeter; LDH = Lactate Dehydrogenase; LDL = Low-Density Lipoprotein; Mg = Magnesium; P = Phosphorous; PTH = Parathyroid Hormone; TC = Total Cholesterol; TG = Triglycerides; WP = Waist Perimeter; 25(OH)D = 25-Hydroxyvitamin D; 25(OH)D_2 = 25-Hydroxyvitamin D_2; 25(OH)D_3 = 25-Hydroxyvitamin D_3.

Table 10 shows the antioxidant status variables of the study. TAC and GPx – with half of the subjects – were the biochemical variables that showed the highest percentage of participants below the reference values. All antioxidant parameters showed no significant differences between groups (all $p > 0.05$).

Figure 5 shows the significant associations of the anthropometrical, BC, and biochemical parameters of the study with TAC. Regarding anthropometry and BC, the weight, HP, and FM expressed in kg showed a significant indirect association with TAC (all $p \leq 0.049$; ***Figure* 5A–C**), whereas BMI showed no significant relationship with TAC ($p = 0.062$) (data not shown). In the case of biochemical parameters, urea, uric acid, total bilirubin, total proteins, and albumin, were seen to be directly related to TAC (all $p \leq 0.047$; ***Figure* 5D–H**).

Table 10. Antioxidant status variables of the study.

Characteristics	Total Population (*n* = 78)	Below Reference	Early Menopause (*n* = 39)	Late Menopause (*n* = 39)	Reference Values
	Mean ± SD	%	Mean ± SD	Mean ± SD	Range
Antioxidant Parameters					
TAC (µmol/L)	1539.3 ± 483.1	51.9	1534.0 ± 594.8	1545.7 ± 308.1	1500.0
GPX (U/mL)	118.2 ± 47.7	50.6	117.3 ± 40.9	119.4 ± 55.4	120.0
SOD (U/mL)	184.4 ± 34.2	31.2	184.5 ± 34.5	184.2 ± 34.4	164.0 – 240.0
SOD/GPx ratio	2.42 ± 4.9	0.00	1.9 ± 1.4	3.0 ± 7.2	–
GAP (µmol/L)	823.9 ± 473.3	–	845.6 ± 593.2	799.8 ± 293.9	–

n = 78. All variables are expressed as the mean ± Standard Deviation (SD). % represents the percentage of subjects below the reference values. Early and late menopause women are those below and above median age (i.e., 57 years), respectively. T-Student's unpaired-samples test was used for comparing the mean differences between early and late menopause. Significant was set for *p* Values less than 0.05. Abbreviations: GAP = Antioxidant GAP; GPx = Glutathione Peroxidase; SOD = Superoxide Dismutase; TAC = Total Antioxidant Capacity.

Figure 6 represents the significant relationships of the BC and biochemical parameters of the study with GPx. Regarding BC, no significant associations (data not shown) were observed with GPx (all $p > 0.05$). When referring to biochemical parameters, glucose, GPT, and GGT were seen to be inversely associated with GPx (all $p \leq 0.024$; ***Figure 6A–C***), whereas a direct association of Mg with GPx was observed ($p \leq 0.006$, ***Figure 6D***).

Figure 7 shows the significant associations of the BC and biochemical parameters of the study with SOD. No significant associations (data not shown) were observed (all $p > 0.05$) for the BC variables and SOD activity. In the case of biochemical parameters, $25(OH)D_3$ and amylase were seen to be directly related to SOD (all $p \leq 0.039$, ***Figures 7A,C***), whereas an inverse association was seen between $25(OH)D_2$ and LDH (all $p \leq 0.048$, ***Figures 7B,D***).

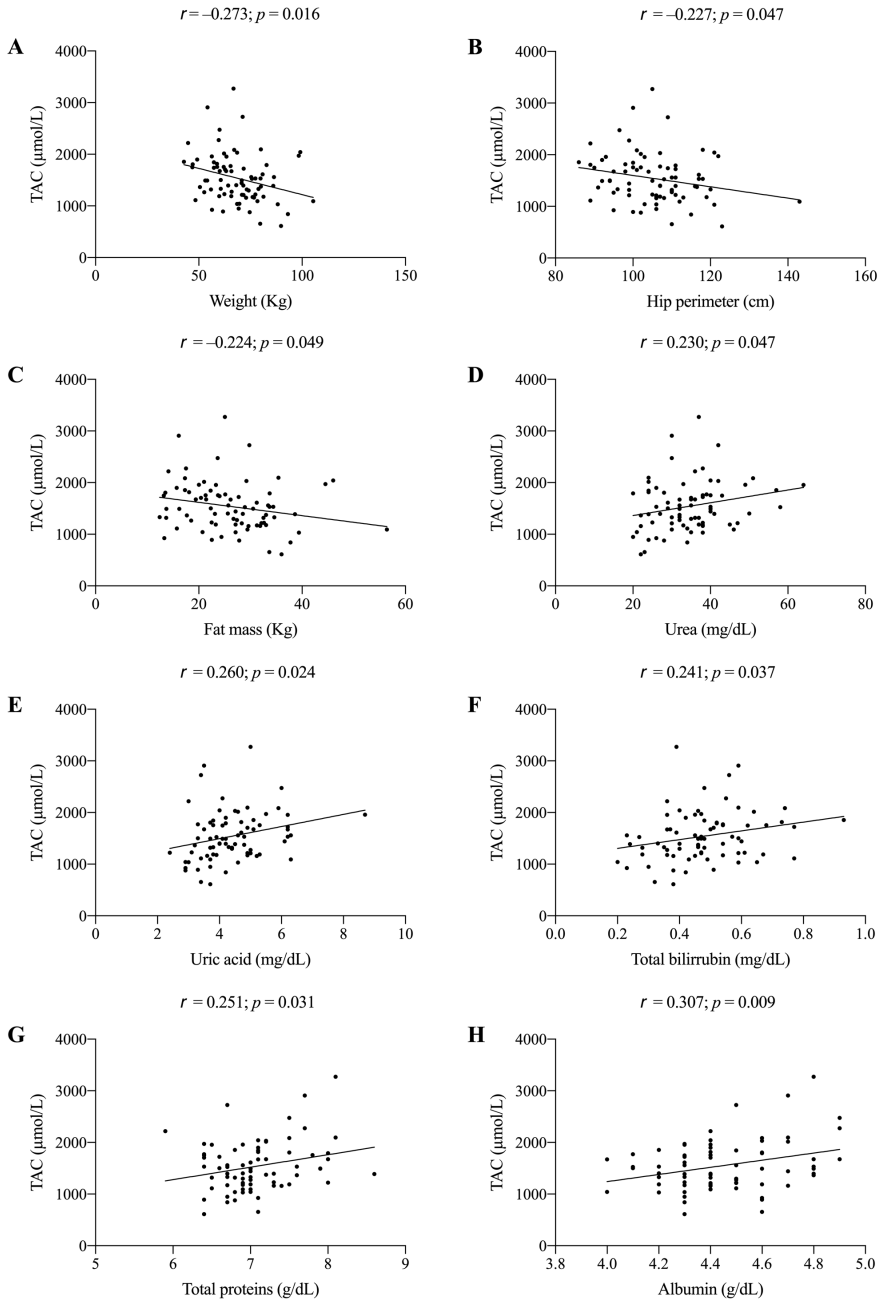

Figure 5. Significant associations of the anthropometrical, body composition, and biochemical parameters of the study with TAC. (A) weight and TAC, (B) hip perimeter and TAC, (C) fat mass and TAC, (D) urea and TAC, (E) uric acid and TAC, (F) total bilirubin and TAC, (G) total proteins and TAC, and (H) albumin and TAC. r, Pearson's correlation coefficient. A *p* Value less than 0.05 was considered significant. Abbreviations: TAC = Total Antioxidant Capacity.

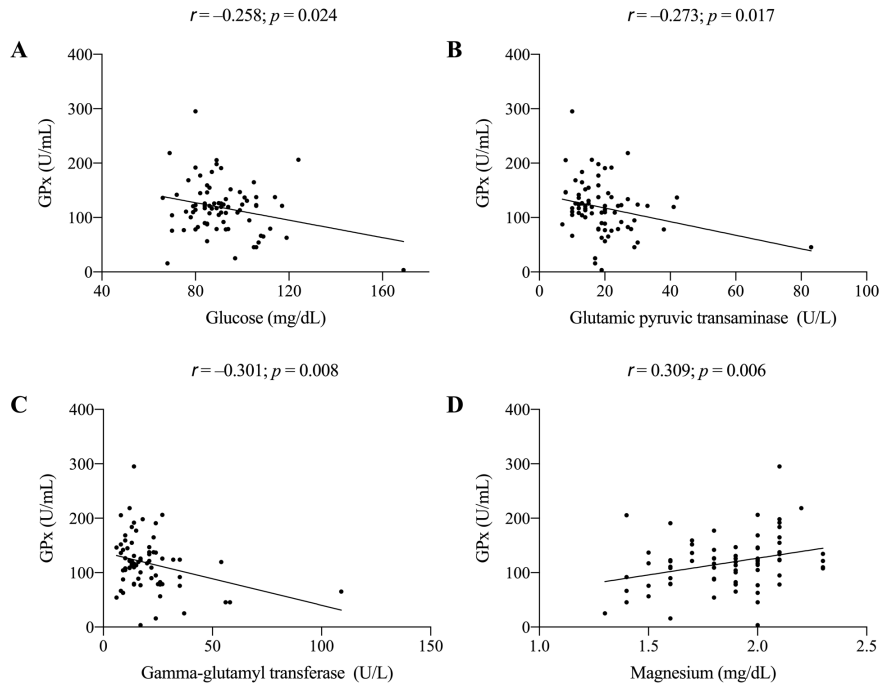

Figure 6. Significant associations of the anthropometrical, body composition, and biochemical parameters of the study with GPx. (A) glucose and GPx, (B) glutamic pyruvic transaminase and GPx, (C) gamma-glutamyl transferase and GPx, and (D) magnesium and GPx. r, Pearson's correlation coefficient. A *p* Value less than 0.05 was considered significant. Abbreviations: GPx = Glutathione Peroxidase.

Table 11 shows the results of regression analysis between the anthropometrical, BC, and biochemical parameters of the study and the SOD/GPx ratio and GAP. No significant associations (data not shown) were observed (all $p > 0.05$) between the SOD/GPx ratio and BC variables. In the case of the biochemical parameters, the SOD/GPx ratio showed a direct relationship with the circulating glucose levels, which persisted after adjusting by confounders (all $p \leq 0.001$, Model 0 and Model 1). Regarding the relationship between GAP and the BC, GAP showed an inverse association with weight, HP, FM, and FFM (all $p \leq 0.021$, Model 0), which lost their significance after adjusting by covariates (all $p > 0.078$, Model 1). Additionally, GAP was negatively associated with BMI, which

persisted after adjusting by confounders (all $p \leq 0.020$, Model 0 and Model 1). In the case of the biochemical variables of the study, GAP was directly related to albumin, which persisted after including covariates (all $p \leq 0.034$, Model 0 and Model 1). Moreover, GAP, which was directly related to the total proteins ($p = 0.048$, Model 0), lost its significance after including covariates ($p = 0.095$, Model 1).

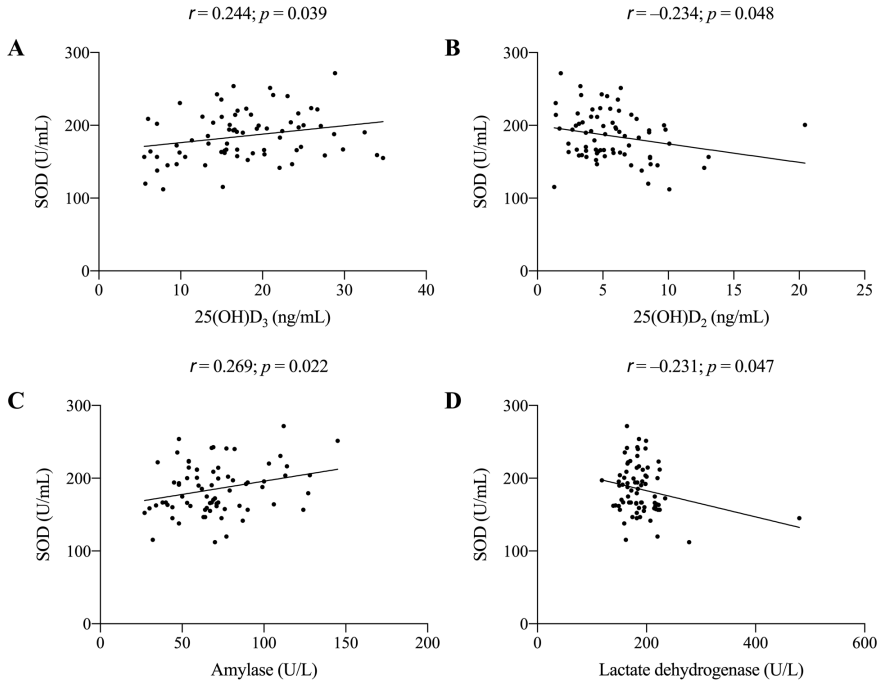

Figure 7. Significant associations of the anthropometrical, body composition, and biochemical parameters of the study with SOD. (A) $25(OH)D_3$ and SOD, (B) $25(OH)D_2$ and SOD, (C) amylase and SOD, and (D) lactate dehydrogenase and SOD. r, Pearson's correlation coefficient. A p Value less than 0.05 was considered significant. Abbreviations: SOD = Superoxide dismutase.

Table 11. Regression analysis between the anthropometrical, body composition, and biochemical parameters of the study and the SOD/GPx ratio and GAP.

Characteristics	Model 0			Model 1		
	ß	R^2	P	ß	R^2	P
SOD/GPx Ratio						
Glucose (mg/dL)	0.425	0.309	**0.001**	0.582	0.312	**0.001**
GAP						
Weight (kg)	−0.310	0.096	**0.008**	−0.309	0.096	0.250
BMI (kg/m^2)	−0.274	0.075	**0.020**	−0.278	0.080	**0.012**
HP (cm)	−0.274	0.075	**0.020**	−0.271	0.076	0.854
FM (kg)	−0.272	0.075	**0.021**	−0.270	0.075	0.994
FFM (kg)	−0.285	0.081	**0.015**	−0.283	0.081	0.078
Total proteins (g/dL)	0.235	0.055	**0.048**	0.246	0.062	0.095
Albumin (g/dL)	0.267	0.071	**0.023**	0.295	0.085	**0.034**

ß, standardized regression coefficient. R^2 and P are from simple and multiple regression analyses between age and the significant biochemical parameters of the study: Model 0, simple regression analysis; Model 1, multiple regression analysis adjusted by age, BMI, physical activity, and early vs. late menopause. Bold numbers indicate a statistically significant association. Significance was set at p Value < 0.05. Abbreviations: BMI = Body Mass Index; GAP = Antioxidant GAP; HP = Hip Perimeter; FM = Fat Mass; FFM = Fat-Free Mass.

4. Discussion

The main results of the present study show that the activity of TAC and GAP were influenced by BC, along with protein metabolism parameters. Moreover, the circulating levels of different micronutrients and enzymes may play an important role in GPx and SOD. These findings shed light on the idea that both the BC and biochemical parameters are decisive for maintaining an adequate antioxidant status in postmenopausal women.

Various metabolic factors may influence TAC, which tends to increase at an early stage of overweightness and obesity, possibly as a compensatory response to oxidative stress [23]. However, TAC is usually significantly lower in obese subjects compared with non-obese subjects [15,24]. Along this line, our results showed a significant inverse association of weight, HP, and FM (expressed in kg) with TAC, whereas no such significant relationships were observed when considering BMI. In the literature, the WP and HP, weight, BMI, and FM percentage, among others, have been negatively correlated with TAC [15,25]. On the other hand, urea, uric acid, total bilirubin, total proteins, and albumin were seen to be directly related to TAC. The main determinants of plasma TAC are albumin, bilirubin, and uric acid [26], and positive correlations have been widely reported between them in the literature [27–30]. In fact, it has been suggested that uric acid plays a specific role in the antioxidant response due to the different kinetics of TAC on the plasma and serum uric acid levels [31]. Furthermore, the antioxidant activity of uric acid has been estimated to account for approximately 50–65% of the TAC of biological fluids in humans [32,33]. These relationships have been studied in different pathologies, such as renal disease, with high TAC probably caused by the accumulation of urea in serum [34], as well as a decrease

in plasma TAC after dialysis, which was equivalent to a diminution in creatinine, urea, and uric acid levels [35].

Regarding GPx activity, it was positively influenced by Mg. This relationship has been reported previously in subjects with diabetes, supporting the hypothesis that hypomagnesemia may influence the development of oxidative stress in these subjects [36]. Moreover, the glucose, GPT, and GGT levels were inversely related to GPx activity. In diabetic patients, fasting glucose has been negatively correlated with GPx, which may be due to the role of hyperglycemia upon increasing oxidative stress through several pathways [37]. Likewise, fasting serum glucose has been correlated with GGT, with higher GGT levels and lower GPx activity in patients with type 2 diabetes compared with a control group [38]. On the other hand, the elevation of GPT has been associated with significant changes in GPx activity in animal models [39], and the use of a methanolic extract has shown a preventive effect on the increase in GPT, enhancing GPx activity [40].

The results of SOD activity showed negative influences of vitamin D_2 and LDH, and positive influences of vitamin D_3 and amylase. Vitamin D_3 has been demonstrated to diminish the formation of free radicals by enhancing antioxidative defense systems, including SOD [41]. Meanwhile, an enriched diet combined with vitamin D_2 appears to increase SOD in animal models [42]. However, the role of vitamin D is not clear – its supplementation not producing significant changes in erythrocyte SOD in patients with hearing loss – [43]. Regarding LDH, increased SOD levels along with decreased LDH levels, and vice versa, have been observed in critical patients, with lower SOD levels and abnormal activity of LDH, being a possible indicator of worse prognosis or infection

severity [44]. The same tendency has been observed after bariatric surgeries, with increased amylase and SOD levels, and decreased LDH [45]. However, in diabetic patients, amylase activity was increased, whereas SOD activity was decreased [46]. Moreover, higher blood amylase levels have been positively related to higher insulin sensitivity in pig models after bariatric surgery [47]. All of this suggests that any disturbance in biochemical metabolism may increase oxidative stress, especially those parameters related to glycemia.

SOD and GPx are important antioxidant enzymes in humans – SOD converts superoxide anion radicals into oxygen and hydrogen peroxide, which is then converted by GPx to water and oxygen – and the SOD/GPx ratio is more relevant than the absolute activities of the individual enzymes [48]. Indeed, it has been suggested that an imbalance in this ratio results in the accumulation of hydrogen peroxide and may be an important factor of cellular aging [49]. In our cohort, the SOD/GPx ratio was directly associated with glucose, which is probably due to the observed negative correlation between the glucose levels and GPx activity.

Finally, the total proteins and albumin were directly associated, and weight, BMI, HP, FM, and FFM were inversely related with GAP. These associations persisted after adjusting by confounders (i.e., age, BMI, physical activity, and early vs. late menopause). The principal antioxidants (by mass and activity) of human plasma are albumin and uric acid, with GAP reflecting the combined activity of other extracellular antioxidants [50]. In our study, the main determinant of GAP appears to be weight since both FM and FFM would have similar effects on GAP.

However, more evidence is needed to understand the role of BC in antioxidant defense.

The present study suffers from some limitations, including: (I) its cross-sectional design, which means that no causal relationships can be established, (II) the lack of dietary assessment, which could have helped to enrich the study, (III) the lack of information about other minor antioxidant parameters, which could have helped to enrich the way to interpret and confirm our results, (IV) fewer participants than desired being recruited, and (V) the study population being limited to postmenopausal women aged from 44–76 years old from a specific area of southern Spain; hence these results may not be generalizable to postmenopausal women of different regions or with ages not included in our range.

5. Conclusions

In conclusion, the menopause-related antioxidant status changes may be influenced by BC and biochemical profile. To confirm this statement, further trials aimed at evaluating BC and biochemical intervention-induced changes upon antioxidant defense are needed.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (protocol code 149/CEIH/2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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12.2. CAPÍTULO II

El capítulo II de la Tesis Doctoral comprende evaluar el efecto de la suplementación con Zn sobre una de las variables principales del estudio, la cual es el estatus plasmático y eritrocitario de Zn, así como una posible reducción del porcentaje de deficiencia en este mineral. Adicionalmente, se valora el efecto que tiene dicha intervención sobre las variables secundarias del estudio como el estatus nutricional de la vitamina D y la defensa antioxidante y otros parámetros rutinarios asociados, así como la relación existente entre estos parámetros con el estatus bioquímico del Zn.

Se han incluido 3 artículos experimentales cuyos objetivos se describen a continuación:

Artículo 4: evaluar el efecto de la suplementación con Zn durante 8 semanas sobre las concentraciones circulantes de Hcy, B_{12} y Fol en una población de mujeres posmenopáusicas.

Artículo 5: investigar la influencia de la suplementación con Zn sobre el estado de la vitamina D_3 y otros parámetros lipídicos en mujeres posmenopáusicas.

Artículo 6: evaluar el efecto de 8 semanas de suplementación con Zn sobre el estado antioxidante y los parámetros clínicos nutricionales de una población posmenopáusica.

12.2.1. Manuscript 4

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Title: Effect of zinc supplementation on circulating concentrations of homocysteine, vitamin B_{12}, and folate in a postmenopausal population

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Abstract

Introduction: The decrease in estrogen levels associated with menopause increases the risk of deficiencies of key micronutrients such as Zinc (Zn) and of disturbances in methylation cycle-related markers. The present study assesses the effect of 8-week Zn supplementation upon circulating concentrations of Homocysteine (Hcy), Vitamin B_{12} (B_{12}), and Folate (Fol) levels in a population of postmenopausal women. **Materials & Methods:** A total of 51 postmenopausal women aged between 44-76 years took part in the study. Two randomized groups (Placebo (Pb) and Zn [50 mg/day]) were treated for 8 weeks. Nutrient intake was assessed based on the 24-hour recall method. Zn was analyzed by flame atomic absorption spectrophotometry. Clinical-nutritional parameters were determined by enzyme immunoassay techniques. **Results:** Fol levels increased significantly ($p < 0.05$) in the Zn group on comparing the baseline versus follow-up values. Hcy decreased in the inter-group analysis ($p < 0.05$) after the intervention. Furthermore, higher Fol ($r = –0.632$; $p = 0.005$) and B_{12} ($r = -0.512$; $p = 0.030$) levels were correlated to low Hcy levels in the Zn group after the intervention, although the Zn intervention had the same effect on B_{12} levels in both groups. **Conclusion:** Zn supplementation enhanced circulating Fol and Hcy by improving the Fol values in the Zn-supplemented group and decreasing Hcy levels inter-groups. Further studies involving larger

samples and optimizing the doses and intervention period are needed to reinforce our main findings.

Keywords: Menopause; zinc supplementation; homocysteine; folate; vitamin B_{12}

1. Introduction

Menopause is the time in life when menstrual cycles cease due to a diminished secretion of ovarian hormones [1]. The decrease in estrogen production could lead to changes in lipid profile, which together with certain menopausal physiological disorders, could disturb clinical-nutritional cardiovascular health related parameters, deriving in Cardiovascular Diseases (CVDs) [2,3]. Moreover, if this menopausal situation is not monitored, the risk of deficiencies in the nutritional status of numerous key micronutrients, such as minerals, is seen to increase [4].

Zinc (Zn) is one of the most important trace elements, and its deficiency is a major health problem worldwide [5]. Zn is a cofactor of more than 300 enzymes, carrying out its cardiovascular protective functions by combating oxidative stress and inflammation, which are risk factors for CVDs [6]. Abnormalities in Zn homeostasis have been reported in patients with metabolic disorders [7,8], since Zn deficiency may cause multiple systemic disturbances, CVD, and dyslipidemia [9].

Supplementation of trace elements such as Zn with the aim of improving cardiovascular health is becoming increasingly popular [10]. Although several trials support the beneficial effects of Zn supplementation upon regulating the cardiovascular profile, there is a lack of clarity in the data reported on the impact of Zn supplementation upon human health, especially in postmenopausal women [11]. In this regard, Zn status may also influence the circulating amounts of clinically relevant car-

diovascular biomarkers such as Homocysteine (Hcy), by increasing Folate (Fol) and Vitamin B_{12} (B_{12}), which are inversely related to Hcy leveles [12]. It is well documented that Fol and B_{12} deficiencies result in increased Hcy levels in aged populations such as postmenopausal women [13,14]. Recently, this relationship has been explained, albeit in a Zn-supplemented younger population, by the fact that Zn, Hcy, Fol and B_{12} play an important role in the methylation cycle. In the case of Zn deficiency, Hcy concentrations seem to be increased due to disturbances in the methylation cycle [15]. In this line, two methionine synthase and betaine Hcy methyltransferase, which are enzymes in Hcy metabolism, are Zn-dependent, being Zn deficiency an important factor in the increase in Hcy levels [16]. It therefore would be interesting to assess the possible influence of Zn upon B_{12} and Fol levels in order to elucidate possible alterations in Hcy metabolism in the postmenopausal population.

To our knowledge, few data are available on the relationship between Zn supplementation and Hcy cycle-related parameters in postmenopausal women. The present study assesses the effect of 8-week Zn supplementation upon circulating concentrations of Hcy, B_{12}, and Fol levels, in a population of postmenopausal women.

2. Materials and methodology

2.1. Study Design and Intervention

This is an 8-week, double-blinded, Placebo (Pb)-controlled, randomized intervention trial. Participants were randomly assigned to one of two treatment groups: Zn Group (ZG) – 50 mg/day of Zn – (ZG: 26 women) and Pb group (PG: 25 women). Zn capsules were provided by SM Natural Solutions, Sabadell, Spain (Number 0B62713821). Pb capsules were made of the same color and size as Zn supplements for identical taste and appearance. The manufacturer recommended a period of 8 weeks in order to ensure the supplementation effects. The supplementation period took place in winter from January 15th to March 15th.

Adherence to both Zn and Pb interventions was checked as the percentage of capsules ingested during the intervention period. To verify the adverse effects, the safety, and the efficacy of the product, 2 biochemical analytics were performed at baseline and follow-up. Written informed consent was obtained from all patients. The intervention was conducted according to the principles of the Declaration of Helsinki and the approval by the Ethics Committee of the University of Granada (149/CEIH/2016), in accordance with the International Conference on Harmonization/Good Clinical Practice Standards. The study was registered at the US National Institutes of Health (ClinicalTrials.gov) NCT03672513.

2.2. Study Participants

A total of 51 healthy postmenopausal women volunteers from the province of Granada, Spain, aged between 44 and 76 years, were recruited once informed consent was signed. All participants are derived from a larger cohort of postmenopausal women [17]. Inclusion criteria were (I) to present postmenopausal status (with at least 12 months of amenorrhea), (II) to present baseline plasma Zn (1 to 5 days before the intervention) determined by Flame Atomic Absorption Spectrophotometry (FAAS), (III) to be non-sedentary, (IV) to smoke and drink alcohol in occasional and moderate quantities, (V) to present normal parameters of a routine hospital laboratory analysis. Exclusion criteria were (I) not to accept the randomization procedure, (II) to take vitamin and mineral supplements, (III) to present any pathology that could affect their nutritional status (i.e., the main components of metabolic syndrome, celiac disease, bulimia and anorexia), (IV) to be subjected to Hormone Replacement Therapy (HRT), (V) not to present systemic inflammatory status (C-Reactive Protein (CRP) was included as a reference biomarker to assess inflammation status of the participants at baseline).

Randomization was performed in a 1:1 ratio using a table of random numbers, prepared by a researcher who did not participate in the data collection. Allocation concealment was ensured by not releasing the randomization code until the participants were recruited into the trial

after all baseline measurements were completed. Women were randomly assigned (simple randomization) to study groups (parallel design). Both study participants and investigators were blinded to the group allocation.

2.3. Body Composition Analysis

We measured the participants height (m) with a stadiometer (Seca, model 213, range 85 to 200 cm; precision: 1 mm; Hamburg, Germany). Body weight (kg) was assessed with bioelectrical impedance (Tanita MC-980 Body Composition Analyzer MA Multifrequency Segmental, Barcelona, Spain). The analyzer complies with the applicable European standards (93/42EEC, 90/384EEC) for use in the medical industry. BMI was obtained as body weight (kg)/ height (m^2). Participants were informed in advance of the required conditions prior to the measurement: (I) no alcohol intake the previous 24 hours, (II) no vigorous exercise 12 hours before the measurement, (III) no food or drink intake less than 3 hours prior to the measurement, and (IV) no urination immediately before the measurement.

2.4. Nutrient Intake

Dietary nutrient intake was assessed and administered by a professional dietitian before and after intervention using a manual 24h-recall, including one weekend day. Recall accuracy was recorded with a set of photographs of prepared foods and dishes that are frequently consumed in the region where the study was performed. Dietowin software (7.1.

version, Barcelona, Spain) was employed to quantitatively convert food intake to both energy and micronutrients, determining their adequacy according to the Recommended Dietary Allowance (RDA) for the menopausal Spanish population [18].

2.5. Sample Treatment

Plasma samples were obtained through a blood extraction which was centrifuged at 4 °C during 15 minutes at 3,000 rpm in the morning in fasting conditions before and after intervention. To obtain erythrocyte aliquots, 3 mL of 0.9% sodium chloride solution was employed during 4 erythrocyte washes, centrifuging for 15 minutes at 3,000 rpm after each wash. The samples were stored at –80 ºC for further analysis. All samples were measured in one run, in the same assay batch and blinded quality control samples were included in the assay batches.

2.5.1. Measurement of Biochemical Parameters

Biochemical parameters such as glucose (mg/dL), urea (mg/dL), uric acid (mg/dL), albumin (mg/dL), prealbumin (mg/dL), total bilirubin (mg/dL), transferrin (mg/dL), total proteins (g/dL), Triglycerides (TG) (mg/dL), Hcy (µmol/L), CRP (mg/L), Lactate Dehydrogenase (LDH) (U/L), High-Density Lipoprotein (HDL) (mg/dL), Low-Density Lipoprotein (LDL) (mg/dL) and Total Cholesterol (TC) (mg/dL), were determined in the Analysis Unit at the Virgen de las Nieves Hospital, Granada (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche

Diagnostics, Mannheim, Germany). All reference values were provided by the Analysis Unit. Zn content was analyzed by FAAS (Perkin Elmer A. Analyst'300 Norwalk, Connecticut, United States of America), previous wet-mineralised way, in the Scientific Instrumentation Center (SIC) from the University of Granada. Accuracy of the method was evaluated by analysis of a Certified Reference Material (SeronormTM Trace Elements ref. MI0181 SERO AS, Billingstad, Norway), at different optimal wavelengths (slit 0.7 nm), using a flow rate (Air/C_2H_2) of 10/1.9 $L \cdot min^{-1}$, and a 5-point calibration curves (r^2 = 0.9997). The reliability was established by setting cut-off scores and considering a sample to be valid and reliable if it showed a Coefficient of Variation (CV) of less than 5% and an Inter-class Correlation Coefficient (ICC) above 0.90. B_{12} (pg/mL) and Fol (ng/mL) were measured in SIC using a DxI® Autoanalyzer (Beckman Coulter, California, United States of America) employing a competitive electrochemiluminescence immunoassay for quantitative determinations. The reference values of Zn, B_{12} and Fol were provided by the SIC.

2.6. Statistical Analysis

All calculations were performed using the SPSS 22.0 Software for MAC (SPSS Inc. Chicago, Illinois, United States of America). GraphPad Prism 9 software (GraphPad Software, San Diego, California, United States of America) was used for plotting the graphs. Previously, a sample size calculation was estimated to determine the effect of a 50 mg/day Zn

intervention upon Hcy levels in menopausal women. To our knowledge, no information is available on differences in Means and Standard Deviations (X ± SD) in Hcy response to Zn treatment compared to a PG. The sample size calculation was determined by applying a two-factor repeated measures Analysis of Variance (ANOVA) test being considered an effect size of 0.20 (small effect size), an alpha error of 0.05 and a power of 90%. Based on sample size calculations it was determined that a total sample size of 46 participants would be needed (G*Power, version 3.1.9.6; Universität Kiel, Germany). Descriptive analysis was presented as (X ± SD). The hypothesis of normal distribution was accepted using the Kolmogorov-Smirnov test as a previous step to the execution of a parametric model or not. For the comparative analysis based intra-group and inter-group, the paired and unpaired *t*-tests for parametric samples were used, respectively. Correlation analyses and partial correlation coefficients were performed with Pearson test. Significance was set at *p* Value < 0.05.

3. Results

All participants self-reported 100% adherence to the intervention in both PG and ZG. The baseline characteristics of the postmenopausal population by groups are presented in ***Table 12***. In relation to the anthropometric parameters, the total population presented type I overweight on average. In terms of nutrient intake, energy consumption was seen to be below the reference values. Regarding the intake of micronutrients, Zn intake was below 50% of RDA, and reached over 100% of RDA after the Zn intervention in ZG. Moreover, two-thirds of Fol intake were not covered by 66% of the total population, and B_{12} intake was above the reference values in both groups. The biochemical parameters did not show mean differences at baseline (all $p > 0.05$).

The supplementation protocol has been published elsewhere. In summary, Zn status was assessed prior to the intervention, evidencing a deficient plasma and erythrocyte Zn status in 58.3% and 54.2% of all postmenopausal women, respectively. After Zn supplementation in ZG, the deficient plasma Zn levels were corrected by 50% of baseline Zn deficient intervened women ($p < 0.001$), and erythrocyte Zn deficiency was almost completely corrected ($p < 0.001$) [19]. ***Figure 8*** shows the effect of Placebo and Zn interventions upon main parameters of the study by groups. Fol values were within the reference ranges, increasing significantly (percentage change: 14.9% in ZG versus 6.5% in PG) after Zn supplementation in ZG ($p < 0.05$). In relation to B_{12}, although the

levels were within normal limits in both PG and ZG, this parameter increased significantly after the intervention in both groups ($p < 0.05$). The Hcy levels in turn were above the reference values in PG, and significantly higher than in ZG after the intervention (percentage change: –5.4% in ZG versus 9.1% in PG) ($p < 0.05$).

Figure 9 shows the bivariate Pearson correlation coefficients between Hcy-related parameters of the study by groups. With regard to the relationship between erythrocyte Zn and Hcy (***Figure 9A and 9B***), no significant associations were observed by groups (all $p > 0.05$). When relating B_{12} and Hcy (***Figure 9C and 9D***), a significant inverse correlation was found for ZG ($p = 0.030$). In case of the Fol and Hcy (***Figure 9E and 9F***), the indirect significant correlation only was observed too for ZG ($p = 0.005$).

Table 12. Baseline characteristics of the study population by groups.

Features	Reference Values	Total population (*n* = 51)	ZG (*n* = 26)	PG (*n* = 25)	*p* Value
		Baseline (X ± SD)	Baseline (X ± SD)	Baseline (X ± SD)	
Age (Years)	–	58.3 ± 8.67	57.1 ± 8.34	59.7 ± 9.15	0.167
Weight (Kg)	–	68.1 ± 12.8	67.7 ± 14.4	69.2 ± 11.0	0.687
Height (cm)	–	158.8 ± 6.24	160.3 ± 6.31	157.2 ± 6.01	0.086
BMI (Kg/m^2)	22.0 – 25.0	26.9 ± 4.54	26.2 ± 4.56	28.0 ± 4.31	0.150
Energy intake (Kcal)	2000.0	1414.9 ± 341.8	1487.9 ± 385.5	1339.5 ± 283.1	0.130
CHO intake (g/day)	275.0	149.8 ± 39.7	154.1 ± 39.8	146.6 ± 40.3	0.520
Protein intake (g/day)	50.0	61.8 ± 14.2	63.8 ± 14.4	59.7 ± 14.2	0.316
Fat intake (g/day)	70.0	62.2 ± 22.6	67.7 ± 26.1	56.1 ± 17.2	0.073
Zn intake (mg/day)	12.0	6.24 ± 3.67	5.82 ± 1.39	6.74 ± 5.16	0.383
Folic acid intake (µg/day)	400.0	240.8 ± 85.4	246.1 ± 80.8	238.2 ± 92.2	0.750
Vitamin B_{12} intake (µg/day)	2.40	5.29 ± 4.79	5.75 ± 5.45	4.80 ± 4.14	0.496
Glucose (mg/dL)	70.0 – 110.0	93.2 ± 17.9	90.6 ± 16.0	96.0 ± 19.8	0.295
Urea (mg/dL)	10.0 – 50.0	34.7 ± 9.23	33.3 ± 8.20	36.3 ± 10.2	0.262
Uric acid (mg/dL)	2.40 – 5.70	4.39 ± 0.99	4.28 ± 1.00	4.50 ± 0.98	0.436
Albumin (mg/dL)	3.50 – 5.20	4.42 ± 0.22	4.37 ± 0.22	4.48 ± 0.21	0.070
Prealbumin (mg/dL)	20.0 – 40.0	25.4 ± 4.23	24.6 ± 3.50	26.6 ± 5.02	0.133
Total bilirubin (mg/dL)	0.10 – 1.20	0.48 ± 0.15	0.47 ± 0.17	0.49 ± 0.13	0.533
Transferrin (mg/dL)	200.0 – 360.0	278.0 ± 45.3	273.0 ± 48.6	285.8 ± 39.6	0.381
Total proteins (g/dL)	6.60 – 8.80	7.08 ± 0.53	7.05 ± 0.62	7.11 ± 0.43	0.705

CRP (mg/L)	< 5.00	0.29 ± 0.33	0.29 ± 0.39	0.29 ± 0.24	0.994
LDH (U/L)	110.0 – 295.0	183.0 ± 54.2	183.2 ± 69.8	182.8 ± 29.3	0.975
Triglycerides (mg/dL)	50.0 200.0	106.6 ± 76.1	98.2 ± 82.7	115.8 ± 68.9	0.419
HDL (mg/dL)	40.0 – 60.0	66.7 ± 16.2	70.4 ± 19.3	62.6 ± 11.2	0.089
LDL (mg/dL)	70.0 –190.0	126.8 ± 33.8	119.6 ± 31.3	134.4 ± 35.3	0.123
Total cholesterol (mg/dL	110.0 – 200.0	218.3 ± 36.5	212.8 ± 33.1	224.1 ± 39.7	0.279

n = 51. Baseline values are expressed as Mean (X) ± Standard Deviation (SD). For inter-groups *p* Value, unpaired *t*-student test was used. Intake reference values were obtained from the Reference Dietary Intakes (RDI) for the Spanish Population [18]. Reference values from biochemical parameters were provided from the Virgen de las Nieves Hospital and the Scientific Instrumental Center, Granada. Abbreviations: BMI = Body Mass Index; CHO = Carbohydrates; CRP = C-reactive protein; HDL = High density lipoprotein; LDH = Lactate dehydrogenase; LDL = Low density lipoprotein; PG = Placebo Group; ZG = Zinc Group; Zn = Zinc.

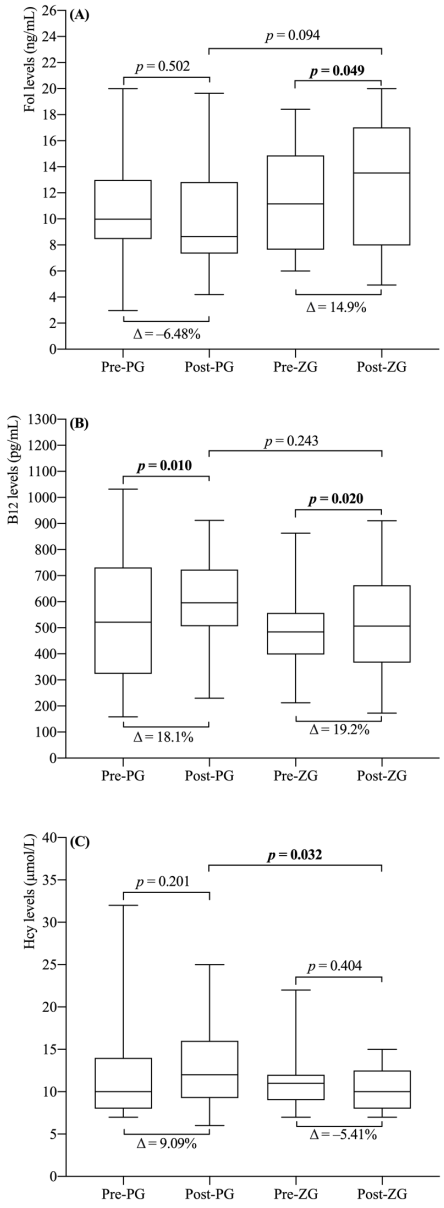

Figure 8. Effect of placebo and zinc interventions upon Fol, B_{12}, and Hcy by groups. (A) Fol, (B) B_{12}, and (C) Hcy. n = 51. Baseline (Pre) and follow-up (Post) values are expressed as Mean (X) ± Standard Deviation (SD). Mean changes intra-group are expressed as mean change (%). Both for intra-group and inter-groups p Value, paired and unpaired t-student test was used. Significance was set at p Values < 0.05 and is

highlighted in boldface. Reference values were provided from the Virgen de las Nieves Hospital and the Scientific Instrumental Center, Granada: Fol = 2.70–17.0 ng/mL; B_{12} = 190.0–900.0 pg/mL; Hcy = < 13 µmol/L. Abbreviations: B_{12} = Vitamin B_{12}; Fol = Folate; Hcy = Homocysteine; PG = Placebo Group; ZG = Zinc Group; Zn = Zinc.

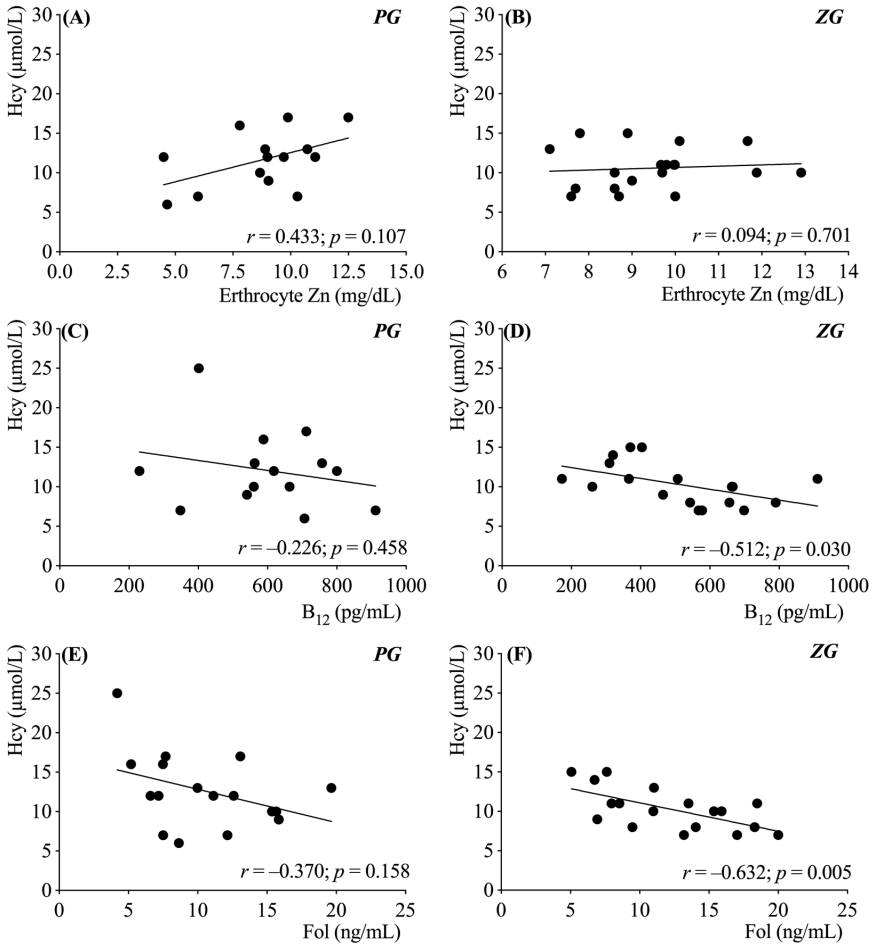

Figure 9. Bivariate Pearson's correlation coefficients between homocysteine-related parameters of the study by groups. (A) Erythrocyte Zn and Hcy in PG. (B). Erythrocyte Zn and Hcy in ZG. (C) B_{12} and Hcy in PG. (D) B_{12} and Hcy in ZG. (E) Fol and Hcy in PG. (F) Fol and Hcy in ZG. Abbreviations: B_{12} = Vitamin B_{12}; Fol = Folate; Hcy = Homocysteine; PG = Placebo Group; ZG = Zinc Group.

4. Discussion

The current study, which to our knowledge is the first of its kind in a postmenopausal population, was designed to investigate the effectiveness of an 8-week Zn intervention upon circulating concentrations of Hcy, B_{12}, and Fol levels in a population of postmenopausal women. The results of our study showed a decrease of Hcy levels at the end of the intervention inter-groups. Moreover, the postmenopausal women supplemented with Zn were seen to increase their Fol levels to within the reference ranges, compared to the baseline values. Interestingly, this improvement in Fol levels was correlated to a decrease in plasma Hcy in this group. Likewise, B_{12} was inversely related with Hcy levels, but, regretfully, the intervention had the same effect upon B_{12} in both groups.

Zn status in humans depends on multiple factors (e.g., gender, age, physiological condition, and diet), with Zn intake being the main contributor to Zn levels [20]. In our study, low Zn intake (below 50% of RDAs) before the intervention was reported. The main cause of Zn deficiency is inadequate dietary intake, which is common in many parts of the world and is further increased in populations at risk of deficiency, such as postmenopausal women [21]. Folic acid intake was found to be below two-thirds of RDA in 66% of our postmenopausal population. Some authors have found Fol intake to decrease with ageing, with inadequate nutrient intakes being most notorious in postmenopausal women, with the RDAs for Fol not being covered in 75% of the cases [22]. Some

studies have reported lower rates of inadequate Fol intake in other European countries, where deficiency was found to range from 17.6% to 45.9% in women older than 64 years [23]. Regarding B_{12} intake, our results evidenced sufficient intake, which is not consistent with the above-mentioned study conducted in postmenopausal women, where lower B_{12} intakes were recorded [22].

Approximately 60% of the postmenopausal population in our study presented Zn deficiency in both the plasma and erythrocyte compartments. In this line, recent studies have found high rates of hypozincemia in elderly women [24]. This tendency seems to be less manifest in women of childbearing age, where lower deficiency rates (30%) have been found compared with the present study [25]. Moreover, our results showed a significant increase in both erythrocyte and plasma Zn levels in ZG after the Zn intervention, reversing most of the initial deficiency seen in both compartments. In this sense, a previous study reported that plasma Zn levels were significantly lower than when postmenopausal subjects were intervened with Zn [26]. Humans respond better to a Zn intervention when Zn status is low, as seen in a study performed in a cohort of Zn-intervened postmenopausal women who were adapted to a Zn intervention within 8 weeks [27]. It should be noted that plasma Zn is characterized by rapid dynamics, increasing the risk of suffering several pathophysiological alterations in response to numerous conditions [28]. In this line, the regulation of cellular Zn is complex, and

may involve several mechanisms to take into account for regulating cellular Zn turnover besides plasma and erythrocyte Zn [29]. Thus, cellular biomarkers such as erythrocyte Zn may not be a sensitive and a reliable marker of Zn status as previously described [30]. Accordingly, the observed increase in plasma and erythrocyte Zn levels, that could reflect genuine improvement of Zn status after Zn supplementation, should be interpreted with caution.

In our study, the plasma Fol values increased significantly in the Zn-supplemented group after the intervention. Published information on the effect of Zn supplementation upon Fol levels is scarce and controversial. The proposed mechanism underlying this increase was described by Manger et al. [31], who suggested that a Zn-deficient state reduces the activity of Fol Zn-dependent conjugase, which is necessary for the absorption of Fol; accordingly, an adequate Zn status could improve the Fol levels. To the best of our knowledge, there have been no Zn supplementation studies in postmenopausal populations assessing changes in Fol levels. A study carried out in an elderly Australian population (65–85 years of age) observed the same significant increase in plasma Fol levels after a 12-week Zn intervention [32]. However, in another study carried out in an elderly population supplemented with Zn during 6 months, the increase in plasma Fol was not accompanied by an increase in erythrocyte Fol. This would suggest that a Zn intervention would only be effective in correcting low plasma Fol levels, which reflect short-term Fol deficiency [33]. On the other hand, it is known that Zn

could affect B_{12} through its effect upon Zn-dependent methionine synthase and therefore on the Hcy cycle also mediated by Fol [31]. In this line, we found the B_{12} levels to be within the reference ranges, but, regretfully, they increased significantly over follow-up in both ZG and PG in our postmenopausal population. Unfortunately, the limitations of 24-hour recall did not allow us to determine whether the regular diet was responsible for the increase observed in PG [34].

Studies that jointly examine Zn, Hcy, Fol and B_{12} status in humans are limited. In our study, a negative correlation was found between Hcy and both Fol and B_{12} levels after the intervention in our Zn-supplemented women. Previous studies involving animal models have found Zn-deficient status in rats to be related to increased Hcy levels and decreased Fol values [35]. Other studies in children and adolescents, involving a 15 mg/day Zn-intervention for 3 months, found the Hcy and B_{12} levels to decrease in the Zn-supplemented group, while the Fol values showed no significant changes [15]. Similar findings have been reported by other authors in diabetic Zn-supplemented individuals, with Zn supplementation leading to a significant rise in serum Fol and B_{12} levels, associated to a decrease in Hcy concentrations [36]. These findings moreover are in agreement with a previous study that evidenced no effect upon the plasma Hcy, B_{12}, and Fol levels after 6 months of Zn intervention (15 mg/day and 30 mg/day) in a population of French healthy elderly individuals [37]. Since we observed these associations in postmenopausal women, we believe that the lack of significance in the

abovementioned studies could be due to the administration of lower Zn supplementation doses in the general older population.

Some limitations must be considered in the interpretation of our findings. Firstly, the sample size, while appropriate for the present study, was small. Future studies involving larger samples are needed to confirm the validity of our results. Secondly, other biomarkers for identifying Zn homeostatic mechanisms triggered by Zn supplementation, such as Zn transporters, should be considered to elucidate the underlying processes. Thirdly, more 24-hour recalls should have been performed during the intervention, as this could have helped to elucidate the observed B_{12} increase in PG. Fourth, the present study included postmenopausal women with a wide age range, which makes it necessary to interpret the results with caution. Lastly, the Zn intervention period may have been insufficient to evidence more significant changes in cardiovascular risk factors. We thus suggest the need for Zn supplementation studies involving longer follow-up periods among postmenopausal women.

5. Conclusions

An 8-week Zn intervention reduced the initial high prevalence of Zn deficiency and improved Fol in the postmenopausal Zn-intervened group, together with a decrease in Hcy levels when comparing inter-both intervened groups. The previous mentioned findings, and in accordance to the inverse correlations between the Hcy and Fol levels, could reflect an enhancement of some Hcy cycle-related parameters in the studied population after the Zn intervention, improving circulating Fol and Hcy. Regretfully, the intervention had the same effect in B_{12} levels for both intervened groups. Further studies involving larger sample sizes and long-term Zn interventions are needed to clarify the possible influence of a prolonged Zn supplementation upon circulating Hcy, B_{12} and Fol levels in a population at risk of Hcy cycle-related parameters disturbances, such as postmenopausal women.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (protocol code 149/CEIH/2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Title: Effectiveness of eight-week zinc supplementation on vitamin D_3 status and leptin levels in a population of postmenopausal women: A double-blind randomized trial.

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Abstract

Introduction: The menopausal period is characterized by hormonal imbalance related to the alteration of parameters involved in lipid metabolism. In addition, menopause increases the risk of deficiencies of key vitamins and minerals such as vitamin D and Zinc (Zn) in such women. The present study was aimed to investigate the influence of Zn supplementation on the status of vitamin D_3 and other lipid parameters in postmenopausal women. **Materials & Methods:** A total of 51 healthy postmenopausal women aged 44-76 years from the province of Granada (Spain) were divided into 2 groups (Placebo (Pb) and Zn) of 25 and 26 women, respectively. The Zn group was supplemented with 50 mg/day of Zn for 8 weeks. Nutrient intake assessment was performed by means of a 24-hour reminder. Zn was determined by flame atomic absorption spectrophotometry. Vitamin D was analyzed by liquid chromatography–tandem mass spectrometry. Leptin was analyzed by Enzyme Immuno-Assay (EIA). **Results:** Zn supplementation improved the initial vitamin D_3 status of the postmenopausal population ($p = 0.049$). Plasma levels of 25(OH)D_3 increased significantly after Zn supplementation in women with lower age at menopause ($p = 0.045$). Both intake and plasma Zn levels were inversely correlated to serum leptin levels ($p = 0.044$ and $p = 0.033$, respectively), being significantly lower in lower age at menopause ($p < 0.001$). **Conclusions:** Zn supplementation improved vitamin D_3

status and was associated to low leptin levels in the postmenopausal women of the study.

Keywords: zinc; vitamin D_3; leptin; liquid chromatography - tandem mass spectrometry; menopause.

1. Introduction

Menopause is defined as the time when menstruation stops after 12 months of amenorrhea due to altered hormonal status [1]. At this stage, there will be endocrine changes due to decreased ovarian activity, biological changes due to decreased fertility, and clinical changes resulting from changes in the menstrual cycle [2]. Consequently, there is an increased risk of suffering alterations in lipid metabolism and deficiencies in the nutritional status of numerous nutrients, as well as of multiple symptoms [3].

Zinc (Zn) is a mineral that is widely deficient in the general population, representing a major global public health problem [4]. This micronutrient is necessary for the correct activity of approximately 300 enzymes and is also a key mineral in the immune and reproductive systems, and in antioxidant capacity and growth [5–7]. During menopause, the appearance of bone disease is common [8], and Zn is a trace element which is involved in bone formation together with key minerals such as Calcium (Ca) and Phosphorous (P) [8–10]. Studies suggest that given the widespread deficiency of Zn in postmenopausal women, and the increased risk of bone disease, Zn supplementation should be advised to maintain bone health in menopause [11].

Vitamin D is a fat-soluble vitamin. 25-Hydroxyvitamin D (25(OH)D) is the metabolite used to assess vitamin D status, due to its

long half-life in plasma or serum (approximately 1 month) [12], and is characterized by synergic action of its 2 main metabolites: 25-Hydroxyvitamin D_2 ($25(OH)D_2$), which is obtained from plant sources, and 25-Hydroxyvitamin D_3 ($25(OH)D_3$), which comes from animal products and endogenous synthesis in skin through exposure to sunlight [13]. $25(OH)D_3$ is more appropriate than $25(OH)D_2$ to sustain adequate levels of 25(OH)D [14]. Vitamin D is involved in P-Ca metabolism, preserving bone mineralization through close regulation of serum Ca and P. These minerals, thanks to the action of vitamin D, are easily absorbed in the intestine and reabsorbed at renal level, avoiding major body losses and the risk of demineralization [15]. Thus, it would be advisable to prescribe vitamin D supplements in postmenopausal women [16]. The prevalence of vitamin D deficiency in postmenopausal population has increased considerably. There is controversy regarding the optimization of analytical data since no standard method has yet been adopted in clinical practice. Nevertheless, Liquid Chromatography– Tandem Mass Spectrometry (LC–MS/MS) has been suggested as the gold standard for vitamin D assays [17–19]. Zn is a mineral that influences the metabolism of vitamin D [20]. However, the role of Zn in vitamin D_3 metabolism has not been studied in detail, though it has been suggested that the genes involved in Zn regulation are more intensely expressed following increased vitamin D_3 supplementation [21].

Leptin is one of the most abundant and important adipokines, and actively participates in the regulation of body weight and energy balance [22]. Both in vitro and in vivo studies demonstrated that leptin could act on bone metabolism by systemic and central ways [23]. Leptin may impact bone growth through the activation of fibroblast growth factor 23 [24]. Moreover, leptin receptor intervenes in osteoblasts growth and bone development [25]. It has been observed that leptin levels are increased due to the hormonal alterations of menopause [26]. In this line, it has been demonstrated that leptin may play an important physiological role in maintaining the quality of bone mass in postmenopausal women with osteoporosis [27]. Individuals with a deficiency of Zn tend to gain FM, because it plays a major role in energy metabolism and satiety control [28]. Zn regulates leptin levels, and consequently controls FM [29]. However, the influence of Zn supplementation upon leptin levels has not been fully clarified in the scientific literature [28].

The postmenopausal period could be associated with a genuine risk of deficiency of several micronutrients [30,31]. Thus, due to the lack of evidence on the importance of monitoring Zn status and its possible influence on key biochemical parameters in postmenopausal women, the aim of the present study was to assess the effectiveness of Zn supplementation upon vitamin D and vitamin D_3 levels and other lipid parameters such as leptin, in a population of postmenopausal women.

2. Materials and methodology

2.1. Study Design and Intervention

This is an 8-week, double-blinded, Placebo (Pb)-controlled, randomized intervention trial. **(*Figure 10*)**. Participants were randomly assigned to 1 of 2 treatment groups: Pb Group (PG: 25 women) and Zn Group – 50 mg/day of Zn – (ZG: 26 women). Randomization was performed in a 1:1 ratio using a table of random numbers, prepared by a researcher who did not participate in the data collection. Allocation concealment was ensured, as the referred researcher did not release the randomization code until the participants were recruited into the trial after all baseline measurements were completed. Zn supplements were supplied by SM Natural Solutions, Sabadell, Spain (Number 0B62713821), following the period of 8 weeks recommended. Pb capsules were made of the same size and colour as Zn supplements for identical appearance and taste. The intervention was carried out in winter from January 15th to March 15th. The study was registered at the US National Institutes of Health (ClinicalTrials.gov) NCT03672513.

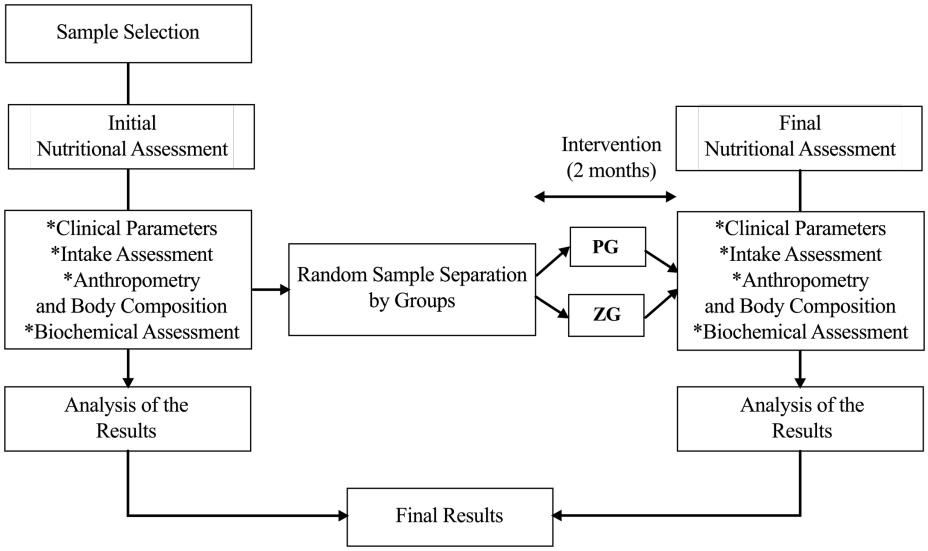

Figure 10. Study design. Abbreviations: PG = Placebo Group. ZG = Zinc Group.

2.2. Study Participants

A total of 51 healthy postmenopausal women volunteers from the province of Granada, Spain aged between 44 and 76 years were recruited once they had been informed about the protocol. Inclusion criteria were (I) to present postmenopausal status (with at least 12 months of amenorrhea), (II) to present baseline plasma Zn (1 to 5 before the intervention) determined by Flame Atomic Absorption Spectrophotometry (FAAS). Exclusion criteria were (I) not to accept the randomization procedure (II) to present any pathology that could affect their nutritional status, (III) to be subjected to Hormone Replacement Therapy (HRT), (IV) to take vitamin and mineral supplements. Written informed consent was obtained from all patients taking into account the approval of the Ethics Committee and the Research Committee of the

Centre. The present study was conducted according to the principles of the Declaration of Helsinki and the approval by the Ethics Committee of the University of Granada (149/CEIH/2016), in accordance with the International Conference on Harmonization/Good Clinical Practice Standards.

Eligible participants of this study were 121 participants. Of these, 39 menopausal women were excluded because 18 women did not meet the inclusion criteria and 21 women declined to participle in the study after the initial interview, and so, 82 menopausal women were enrolled in the study and randomly assigned to the 2 arms (***Figure 11***). Of the 41 postmenopausal women that were allocated to intervention in the PG, a total of 16 women withdrew the study due time and supplementation commitment. Finally, in reference to the 41 postmenopausal women allocated in ZG, a total of 12 women were excluded during the follow-up in ZG due to time and supplementation commitment and not giving any reason. There were 3 women excluded from the data analysis in ZG due to insufficient blood sample collection. Thus, 25 women in PG, and 26 women in ZG were enrolled in the present study.

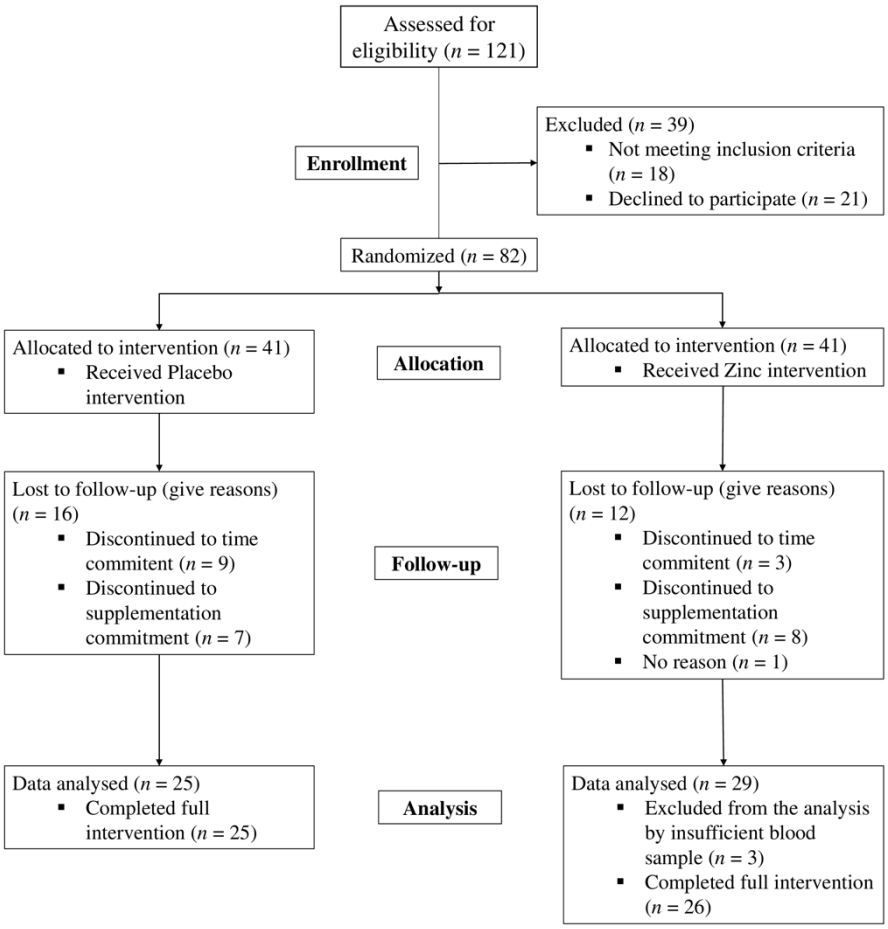

Figure 11. Flowchart of participants recruited, enrolled, and involved in the clinical study.

2.3. Randomization and Blinding

Women were randomly assigned (simple randomization) to study groups (parallel design). In order to ensure comparable distribution across the treatment arms, women were stratified to balance baseline covariates. Both study participants and investigators were blinded to the group allocation. Initial and follow-up visits for evaluating dietary intake, Body

Composition (BC), biochemical and hormonal parameters were performed at baseline and follow-up.

2.4. Sample Size

We performed sample size calculation for our primary aim of a randomized controlled trial based on the influence of a Zn supplementation on vitamin D status. The number of participants to be included in the study was calculated based on the change in vitamin D status after Zn intervention. To the best of our knowledge there were no available information regarding group difference changes on vitamin D in Zn intervened postmenopausal women. Therefore, we assumed a difference of 2.63 ng/mL as clinically meaningful based on previous observations in our group (unpublished data). A total of 68 participants were needed to detect a mean group difference of 2.63 ng/mL and a Standard Deviation (SD) of 3.85 ng/mL in vitamin D with a power of 80% and an alpha of 0.05, and assuming a maximum loss of 20% of participants (n = 82).

2.5. Compliance Evaluation

Adherence/compliance to nutritional intervention was determined as the percentage of all the supplement capsules ingested throughout the study period. In addition, subjects were asked to keep daily records about side effects or other problems related to the supplements. Moreover, biochemical, and clinical-nutritional parameters were taken at baseline and follow-up to evaluate the safety of the product and to verify the adverse effects.

2.6. Data Collection

All recorded data were obtained through the use of manual questionnaires administered by the interviewer that reflected information on personal data, sociodemographic aspects, an adequate diagnosis of the postmenopausal situation, smoking habits and physical activity [32].

2.7. Body Composition Analysis

Anthropometric recorded data were height (m) (SECA® model 213, Hamburg, Germany), Waist Perimeter (WP) (cm) (SECA® Model 201, Hamburg, Germany), and BC by bioelectrical impedance (Tanita MC-980 Body Composition Analyzer MA Multifrequency Segmental, Barcelona, Spain). The analyzer complies with the applicable European standards (93/42EEC, 90/384EEC) for use in the medical industry. Participants were informed in advance of the required conditions prior to the measurement: no alcohol less than 24 hours before the measurement, no vigorous exercise less than 12 hours prior to the measurement, no food or drink less than 3 hours prior to the measurement, and no urination immediately before the measurement. All measurements were taken simultaneously during the morning in fasting conditions. Weight was obtained and BMI was calculated as body weight (kg)/ height (m^2).

2.8. Intake Rating

Dietary nutrient intake was assessed using a manual 24h-recall, taking into account 1 holiday and 2 non-holidays days, both at baseline and follow-up, which was administered by the interviewer. Recall accuracy was recorded with a set of photographs of prepared foods and dishes that are frequently consumed in Spain. The food intake assessment was converted to both energy and nutrients, determining the adequacy of the macronutrient and micronutrient intake according to the Recommended Dietary Allowance (RDA) for the female Spanish population within the age range included in our study [33] using the Dietowin software (7.1. version, Barcelona, Spain).

2.9. Sample Treatment

A blood extraction in the morning in fasting conditions was performed at baseline and follow-up, being centrifuged at 4 °C for 15 minutes at 3,000 rpm to extract the plasma. Once the plasma was removed from the tube, the erythrocytes were washed 4 times with 3 mL of 0.9% sodium chloride solution, centrifuging for 15 minutes at 3,000 rpm after each wash. Then, the supernatant saline solution was removed from the last wash and the erythrocytes were obtained with a Pasteur Pipette. The samples were frozen at –80 ºC until the analytical determination of the different parameters. All samples were measured in one run, in the same assay

batch and blinded quality control samples were included in the assay batches to assess laboratory error in the measurements.

2.9.1. Measurement of Biochemical Parameters

Vitamin D levels (ng/mL) were measured by LC–MS/MS (Acquity UHPLC System I-Class Waters, Milford, United States of America). The biochemical values of vitamin D obtained were classified according to the reference values of 25(OH)D in plasma, being sufficiency > 30 ng/mL, insufficiency 20–30 ng/mL and deficiency < 20 ng/mL for total vitamin D [32]. Parathyroid Hormone (PTH) (pg/mL) and osteocalcin (ng/mL) levels were measured using EIA by colorimetric method (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany). The remaining biochemical parameters such as glucose (mg/dL), transferrin (mg/dL), prealbumin (mg/dL), urea (mg/dL), uric acid (mg/dL), creatinine (mg/dL), Triglycerides (TG) (mg/dL), Total Cholesterol (TC) (mg/dL), High-Density Lipoprotein (HDL) (mg/dL), Low Density Lipoprotein (mg/dL), albumin (mg/dL), Homocysteine (Hcy) (µmol/L), bilirubin (mg/dL), and leptin (ng/mL) levels, were determined in the Analysis Unit at the Virgen de las Nieves Hospital, Granada (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany). All reference values were provided by the Analysis Unit.

2.9.2. Measurement of Trace Elements

Ca (mg/dL) and Zn (mg/dL) content were analyzed by FAAS (Perkin Elmer A. Analyst'300 Norwalk, Connecticut, United States of America), previous wet-mineralised way, in the Scientific Instrumentation Center (SIC) from the University of Granada. Accuracy of the method was evaluated by analysis of a Certified Reference Material (SeronormTM Trace Elements ref. MI0181 SERO AS, Billingstad, Norway). Levels of Ca and Zn were analyzed at different optimal wavelengths for each element (slit 0.7 nm), using a flow rate (Air/C_2H_2) of 10/1.9 $L \cdot min^{-1}$, and using a 5-point calibration curves (r^2 = 0.9997). P (mg/dL) was determined with the colorimetric method of Fiske-Subbarow with ammonium molybdate $(NH_4)_2MoO_4$ (Thermo Scientific, Rockford, Illinois, United States of America) in the SIC. The reference values of Ca, Zn, and P, were provided by the SIC.

2.10. Statistical Analysis

All calculations were performed using the SPSS 22.0 Software for MAC (SPSS Inc. Chicago, Illinois, USA). GraphPad Prism 9 software (GraphPad Software, San Diego, California, United States of America) was used for plotting the graphs. Descriptive analysis has been used for data expression, indicating the results of the numerical variables as Mean ± Standard Deviation (X ± SD) and the results of the categorical variables were expressed in Frequencies (%). As a previous step to the execution

of a parametric model or not, the hypothesis of normal distribution was accepted using the Kolmogorov-Smirnov test. For the comparative analysis based on baseline and follow-up, the paired *t*-test for parametric samples was used. For the comparative analysis based on groups, the impaired *t*-test for parametric samples was employed. Correlation analyses and partial correlation coefficients were performed with Pearson test. A *p* Value less than 0.05 was considered statistically significant. Finally, simple mediation analysis was performed using the macro-PROCESS developed by Hayes [34] with a bootstrap threshold of 10,000. We explored whether the Zn content in plasma mediated the relationship between the supplementation and the 25(OH)D and $25(OH)D_3$ levels. The variable supplementation was converted into a dummy (0 = PG / 1 = ZG). The variables included in the mediation analysis were adjusted for age, BMI, and leptin levels. If zero was not included in the 95% confidence interval of the estimate, we concluded that the indirect effect was statistically significant. Two factors were obtained: Δ 25(OH)D and Δ $25(OH)D_3$ levels were created by adjusting the values of both vitamins after the intervention with respect to the baseline values (Δ 25(OH)D = 25(OH)D follow-up minus 25(OH)D baseline; Δ $25(OH)D_3$ = $25(OH)D_3$ follow-up minus $25(OH)D_3$ baseline).

3. Results

Table 13 shows the general characteristics of the studied postmenopausal population by groups. Energy intake was found to be within the reference ranges, though a slight significant increase was noted at the end of the study in the ZG with respect to PG – with protein and fat being the main contributors ($p < 0.05$) –. On the other hand, it should be noted that the study population presented mean initial intake values below the recommendations for both Zn (below 50% of RDA) and vitamin D (below 35% of RDA). After the intervention, Zn intake reached 100% of RDA, while that of vitamin D remained at 35% of RDA. Thus, at baseline, 87% and 92.3% of the postmenopausal population did not cover 2/3 of RDA for Zn in PG and ZG, respectively. After the intervention, 94.7% (PG) and 0% (ZG) of the women did not reach 2/3 of RDA. Regarding vitamin D intake, at baseline, 87% and 88.5% of the population did not cover 2/3 of RDA for vitamin D in PG and ZG, respectively. Similarly, after the intervention, 73.7% (PG) and 88.5% (ZG) of the women in the study did not reach 2/3 of RDA for vitamin D.

Table 14 shows the biochemical parameters of the studied postmenopausal population by groups – the values being within the normal range –. Plasma and erythrocyte values of Zn at baseline of the study in both PG and ZG were below the reference values and were seen to increase significantly after the intervention (58% and 57%, respectively) in ZG. Creatinine decreased significantly (10%) after

intervention comparing ZG with PG. On the other hand, the plasma levels of 25(OH)D and 25(OH)D_3 were below the reference values both at baseline and follow-up. Moreover, when analyzing the status of vitamin D and vitamin D_3 by groups in the postmenopausal population, approximately 80% of the women in the study had vitamin D deficiency both before and after the intervention. For total vitamin D, no significant change was observed after the intervention. However, in reference to vitamin D_3, while 75% of the women were initially deficient, this variable decreased significantly to 55% after the Zn intervention.

Table 15 shows the parameters of phosphorous-calcium metabolism of the postmenopausal population divided by groups and median age of each group. With the exception of the plasma levels of 25(OH)D, all other parameters were within normal ranges. Osteocalcin increased significantly a 90% after Zn supplementation in ZG among the younger menopausal women. Ca levels decreased in the older women in ZG ($p < 0.05$). On the other hand, leptin decreased significantly in ZG in both age groups (39% for younger and 13% for older postmenopausal women) after intervention. In addition, plasma levels of 25(OH)D_3 increased significantly a 22% after Zn supplementation in younger menopausal women.

Table 13. General characteristics of the study population by groups.

Features	Reference values	PG (*n* = 25)		ZG (*n* = 26)		*p* Value PG Follow-up	*p* Value ZG Follow-up	*p* Value inter-groups
		Baseline (Mean ± SD)	Follow-up (Mean ± SD)	Baseline (Mean ± SD)	Follow-up (Mean ± SD)			
Age (Years)	–	60.1 ± 9.16	60.1 ± 9.16	57.1 ± 8.34	57.1 ± 8.34	–	–	–
Anthropometry and Body Composition								
Weight (Kg)	–	69.0 ± 11.2	68.2 ± 11.4	67.7 ± 14.4	67.8 ± 14.6	0.48	0.81	0.91
Height (cm)	–	156.8 ± 5.83	156.8 ± 5.83	160.3 ± 6.31	160.3 ± 6.31	–	–	–
BMI (Kg / m^2)	22.0 – 27.0	28.1 ± 4.38	28.0 ± 4.31	26.2 ± 4.56	26.3 ± 4.71	0.45	0.51	0.22
Blood pressure *n* (%)								
Normal blood pressure	–	10 (40)	–	18 (70)	–	–	–	–
High blood pressure	–	15 (60)	–	8 (30)	–	–	–	–
Physical exercise *n* (%)								
Sedentary	–	8 (30)	–	6 (25)	–	–	–	–
Non-sedentary	–	17 (70)	–	16 (75)	–	–	–	–
Smoking habit *n* (%)								
Non-smoker	–	19 (78)	–	21 (80)	–	–	–	–
Smoker	–	6 (22)	–	5 (20)	–	–	–	–
Educational level *n* (%)								
Basic educational level	–	11 (44)	–	8 (31)	–	–	–	–

Secondary or high educational level	–	14 (56)	–	18 (69)	–	–	–	–
Dietary Intake								
Energy intake (Kcal)	2000.0	1329.5 ± 285.1	1218.2 ± 285.5	1487.9 ± 385.5	1501.5 ± 300.4	0.18	0.85	**0.003**
CHO intake (g/day)	275.0	143.9 ± 38.8	146.5 ± 34.3	154.0 ± 39.9	156.2 ± 45.2	0.56	0.82	0.44
Protein intake (g/day)	50.0	59.3 ± 14.3	56.7 ± 10.5	63.9 ± 14.4	70.1 ± 17.7	0.53	0.08	**0.005**
Fat intake (g/day)	70.0	56.1 ± 17.6	46.3 ± 17.5	67.7 ± 26.2	64.7 ± 17.8	0.09	0.58	**0.001**
Cholesterol intake (mg/day)	< 300.0	152.3 ± 62.5	149.4 ± 65.3	194.3 ± 72.6	207.2 ± 93.3	0.97	0.45	**0.03**
Fiber intake (g/day)	> 25.0	17.0 ± 10.9	16.3 ± 4.13	15.5 ± 5.77	19.6 ± 11.0	0.29	**0.02**	0.22
P intake (mg/day)	800.0	1002.2 ± 261.7	989.8 ± 224.4	1122.9 ± 327.9	1172.9 ± 376.7	0.98	0.51	0.06
Ca intake (mg/day)	800.0 – 1000.0	744.5 ± 212.9	682.7 ± 172.7	875.6 ± 276.0	903.0 ± 324.5	0.47	0.69	**0.01**
Zn intake (mg/day)	12.0	6.76 ± 5.27	5.24 ± 1.82	5.82 ± 1.39	56.4 ± 1.76	0.23	**0.001**	**0.001**
Vitamin D intake (µg/day)	10.0 – 15.0	3.39 ± 3.05	4.39 ± 2.98	3.26 ± 3.04	3.49 ± 2.34	0.37	0.72	0.26

n = 51. Baseline and follow-up values are expressed as mean ± Standard Deviation (SD). Both for intra-group and inter-groups *p* Value, paired and unpaired *t*-student test was used. Significance was set at *p* Values < 0.05 and is highlighted in boldface. Abbreviations: BMI = Body Mass Index; Ca = Calcium; CHO = Carbohydrates; P = Phosphorous; PG = Placebo Group; ZG = Zinc Group; Zn = Zinc.

Table 14. Biochemical parameters of the study by groups.

Features	Reference values	PG (*n* = 25)		ZG (*n* = 26)		*p* Value PG Follow-up	*p* Value ZG Follow-up	*p* Value inter-groups
		Baseline (Mean ± SD)	Follow-up (Mean ± SD)	Baseline (Mean ± SD)	Follow-up (Mean ± SD)			
Glucose (mg/dL)	70.0 – 110.0	97.1 ± 19.5	96.3 ± 19.4	90.6 ± 16.0	91.0 ± 14.7	0.32	0.67	0.31
Transferrin (mg/dL)	200.0 – 360.0	289.5 ± 38.2	272.8 ± 43.4	273.0 ± 48.6	274.3 ± 50.2	0.89	0.65	0.93
Prealbumin (mg/dL)	20.0 – 40.0	27.2 ± 4.61	24.7 ± 5.01	24.6 ± 3.50	24.7 ± 3.71	**0.001**	0.93	0.97
Albumin (mg/dL)	3.50 – 5.20	4.50 ± 0.21	4.45 ± 0.27	4.37 ± 0.22	4.43 ± 0.26	0.62	0.22	0.77
Homocysteine (µmol/L)	< 13.0	12.3 ± 6.51	12.5 ± 4.86	11.1 ± 3.35	11.8 ± 6.93	0.23	0.75	0.75
Creatinine (mg/dL)	0.50 – 0.90	0.76 ± 0.17	0.75 ± 0.16	0.65 ± 0.91	0.67 ± 0.99	0.15	0.49	**0.045**
Total bilirubin (mg/dL)	0.10 – 1.20	0.50 ± 0.13	0.55 ± 0.21	0.47 ± 0.17	0.46 ± 0.15	0.13	0.85	0.12
LDH (U/L)	110.0 – 295.0	181.1 ± 28.8	181.1 ± 26.1	183.2 ± 69.8	183.3 ± 51.2	0.96	0.99	0.87
Urea (mg/dL)	10.0 – 50.0	36.9 ± 9.97	37.2 ± 9.42	33.3 ± 8.20	34.3 ± 9.51	0.73	0.47	0.32
Uric acid (mg/dL)	2.40 – 5.70	4.47 ± 0.99	4.70 ± 1.02	4.28 ± 1.00	4.48 ± 0.99	0.21	0.28	0.48
Triglycerides (mg/dL)	50.0 – 200.0	116.6 ± 70.3	112.3 ± 62.2	98.2 ± 82.7	98.9 ± 39.9	0.79	0.96	0.38

HDL (mg/dL)	40.0 – 60.0	62.9 ± 11.3	64.1 ± 12.3	70.4 ± 19.3	66.9 ± 17.4	**0.04**	**0.04**	0.55
LDL (mg/dL)	70.0 – 190.0	134.4 ± 36.1	137.6 ± 30.5	119.6 ± 31.3	121.6 ± 27.7	0.76	0.64	0.08
Total cholesterol (mg/dL)	110.0 – 200.0	224.2 ± 40.6	221.4 ± 31.4	212.8 ± 33.1	210.4 ± 31.3	0.86	0.59	0.25
Leptin (ng/mL)	3.6 – 11.1	12.7 ± 5.20	6.90 ± 3.30	13.4 ± 5.04	9.55 ± 3.27	**0.001**	**0.001**	**0.008**
Osteocalcin (ng/mL)	15.0 – 46.0	17.9 ± 9.44	18.7 ± 6.94	11.7 ± 8.89	19.5 ± 11.8	0.71	**0.005**	0.79
PTH (pg/mL)	20.0 – 70.0	51.2 ± 16.1	54.5 ± 34.9	65.2 ± 33.1	52.8 ± 18.3	0.41	0.97	0.83
P (mg/dL)	2.70 – 4.50	3.50 ± 0.41	3.53 ± 0.54	3.59 ± 0.51	3.68 ± 0.40	0.95	0.53	0.33
Ca (mg/dL)	8.60 – 10.2	9.30 ± 0.32	9.08 ± 0.39	9.05 ± 0.43	8.95 ± 0.44	0.13	0.39	0.29
Plasma Zn (mg/dL)	3.70 – 4.10	3.04 ± 1.24	2.49 ± 0.79	3.02 ± 1.15	4.76 ± 1.77	**0.03**	**0.001**	**0.001**
Erythrocyte Zn (mg/dL)	7.30 – 7.60	6.27 ± 2.17	8.87 ± 2.50	6.13 ± 1.94	9.62 ± 1.89	**0.001**	**0.001**	0.26
25(OH)D (ng/mL)	30.0 – 100.0	23.4 ± 8.98	24.5 ± 7.85	23.5 ± 7.57	24.4 ± 7.39	0.89	0.48	0.97
$25(OH)D_3$ (ng/mL)	> 20.0	18.5 ± 8.11	20.0 ± 8.04	17.1 ± 6.63	19.3 ± 6.71	0.64	**0.049**	0.76
$25(OH)D_2$ (ng/mL)	> 10.0	5.40 ± 2.07	4.55 ± 2.74	6.38 ± 3.94	4.75 ± 2.73	0.32	**0.037**	0.81

n = 51. Baseline and follow-up values are expressed as mean ± Standard Deviation (SD). Both for intra-group and inter-groups *p* Value, paired and unpaired *t*-student test was used. Significance was set at *p* Values < 0.05 and is highlighted in boldface. Abbreviations: Ca = Calcium; HDL = High-Density Lipoprotein; LDH = Lactate Dehydrogenase; LDL = Low-Density Lipoprotein; P = Phosphorous; PTH = Parathyroid hormone;

Table 15. Phosphorus-Calcium metabolism parameters of the study by age of menopause by groups.

Features	PG (*n* = 25)				ZG (*n* = 26)			
	< median age		> median age		< median age		> median age	
	Baseline (Mean ± SD)	Follow-up (Mean ± SD)	Baseline (Mean ± SD)	Follow-up (Mean ± SD)	Baseline (Mean ± SD)	Follow-up (Mean ± SD)	Baseline (Mean ± SD)	Follow-up (Mean ± SD)
Osteocalcin (ng/mL)	15.2 ± 7.42	18.1 ± 9.09	21.0 ± 10.9	19.3 ± 4.33	9.03 ± 4.54	17.2 ± 9.78[a]	14.7 ± 11.5	21.8 ± 13.5
PTH (pg/mL)	49.3 ± 12.5	47.7 ± 16.4	53.4 ± 20.0	61.4 ± 47.1	67.3 ± 41.2	52.2 ± 9.68[b]	62.6 ± 21.6	53.4 ± 24.5
Leptin (ng/mL)	12.5 ± 5.20	5.63 ± 3.61	13.1 ± 5.47	8.43 ± 2.20	12.7 ± 5.77	7.76 ± 2.17[a]	14.2 ± 4.30	11.3 ± 3.29[a,b]
Calcium (mg/dL)	9.37 ± 0.27	9.16 ± 0.51	9.23 ± 0.36	9.01 ± 0.22	9.09 ± 0.41	9.16 ± 0.48	9.01 ± 0.46	8.73 ± 0.28[b]
P (mg/dL)	3.47 ± 0.40	3.52 ± 0.65[a]	3.52 ± 0.44	3.54 ± 0.45	3.44 ± 0.64	3.53 ± 0.48	3.74 ± 0.28	3.82 ± 0.25
Plasma Zn (mg/dL)	2.85 ± 1.17	2.25 ± 0.59	3.25 ± 1.34	2.74 ± 0.93	2.96 ± 1.14	4.88 ± 1.70[a,b]	3.09 ± 1.21	4.65 ± 1.91[a,b]
Erythrocyte Zn (mg/dL)	5.05 ± 2.31	7.87 ± 2.64[a]	7.49 ± 1.13	9.86 ± 2.00[a]	5.57 ± 1.61	8.78 ± 0.92[a]	6.69 ± 2.13	10.4 ± 2.25[a]
25(OH)D (ng/mL)	25.4 ± 9.88	25.1 ± 6.74	20.9 ± 9.53	24.0 ± 9.10	21.9 ± 8.45	23.7 ± 6.86	24.9 ± 6.72	25.1 ± 8.10
25(OH)D_3 (ng/mL)	20.2 ± 8.2	20.3 ± 6.02	16.6 ± 8.01	19.8 ± 9.88	14.6 ± 6.56	17.8 ± 5.46[a]	19.3 ± 6.10	20.7 ± 7.66

25(OH)D_2 (ng/mL)	5.87 ± 2.08	4.44 ± 3.60	4.30 ± 1.94	4.62 ± 2.24	7.07 ± 4.57	5.34 ± 3.06	5.31 ± 2.57	3.85 ± 1.94

n = 51. Baseline and follow-up values are expressed as mean ± Standard Deviation (SD). Both for intra-group and inter-groups p Value, paired and unpaired t-student test was used. [a]Comparison baseline vs follow-up. [b]Comparison PG vs GM. Statistical signification = $p < 0.05$. Abbreviations: Ca = Calcium; P = Phosphorous; PG = Placebo Group; PTH = Parathyroid hormone; ZG = Zinc Group; Zn = Zinc. 25(OH)D = 25-Hydroxyvitamin D; 25(OH)D_2 = 25-Hydroxyvitamin D_2; 25(OH)D_3 = 25-Hydroxyvitamin D_3.

Initially, 58.3% and 54.2% of the total postmenopausal women showed a deficient status of Zn in plasma and erythrocyte, respectively. As seen in ***Figure 12***, when categorized by groups, both at baseline and follow-up following the intervention, the percentage of women with deficient plasma Zn levels decreased a 50% in ZG. On the other hand, while 2/3 of the women were initially deficient in terms of erythrocyte Zn levels, this variable dropped to almost zero after the intervention.

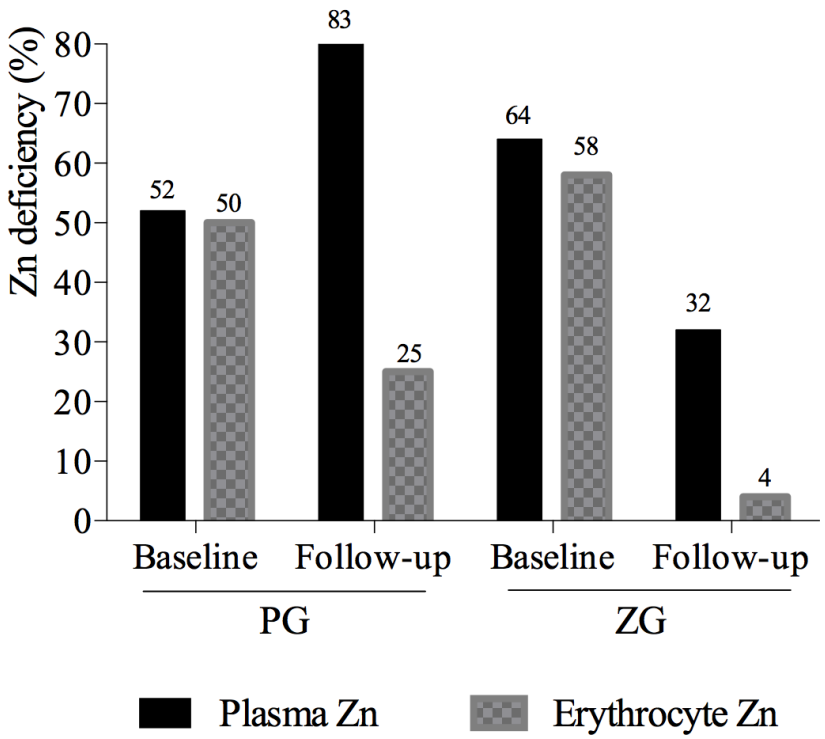

Figure 12. Zn deficiency by groups (% of women). Abbreviations: PG = Placebo group. ZG = Zinc Group. Zn = Zinc.

Figure 13 shows the bivariate correlations between intake and plasma Zn levels with leptin levels in ZG. The results show an inverse relationship between intake and plasma Zn levels with leptin levels – the association being more significant in women with older age at menopause ($r = –0.565$; $p = 0.044$ and $r = –0.675$; $p = 0.011$, respectively) –.

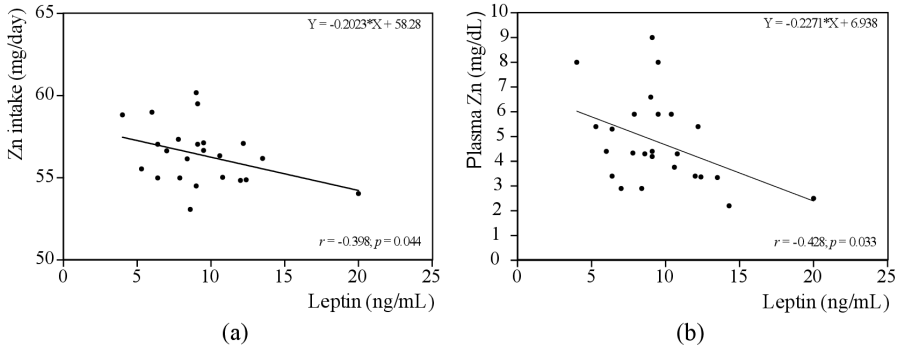

Figure 13. Correlations between intake and plasma Zn levels with leptin levels in ZG **(a)** Pearson's bivariate correlation of Zn intake with leptin in ZG after intervention; **(b)** Pearson's bivariate correlation of plasma Zn with leptin in ZG after intervention. Abbreviations: Zn = Zinc.

Figure 14 shows the simple mediation analysis to determine whether plasma Zn mediates the 25(OH)D and the 25(OH)D_3 levels upon Zn supplemented postmenopausal women. In the first regression equation (a), the Zn plasma levels showed to be positively associated to the Zn supplemented postmenopausal women ($\beta = 0.69$; $p < 0.001$). In the second equation (b), the Zn plasma levels were seen to be directly associated to 25(OH)D ($\beta = 0.47$; $p = 0.042$) **(*Figure 14a*)** and 25(OH)D_3 ($\beta = 0.52$; $p = 0.023$) **(*Figure 14b*)**. Lastly, the confidence intervals

referred to the indirect effect suggest that Zn in plasma mediated in the relationship between the supplementation (ZG vs PG) and 25(OH)D levels (IE β = 0.32; SE = 0.14; 95% CI = 0.01 to 0.57) **(*Figure 14a*)**, and 25(OH)D_3 (IE β = 0.36; SE = 0.13; 95% CI = 0.06 to 0.59) **(*Figure 14b*)**.

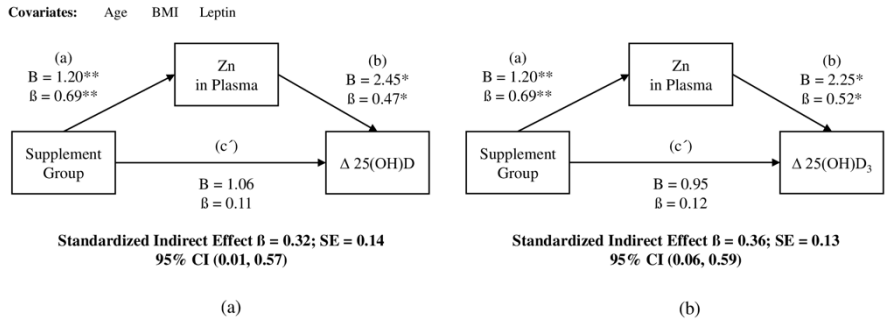

Figure 14. Simple mediation analysis to determine whether plasma Zn mediates the 25(OH)D and the 25(OH)D_3 levels upon Zn supplemented postmenopausal women **(a)** Simple mediation analysis to determine whether plasma Zn mediates the 25(OH)D levels upon Zn supplemented postmenopausal women. **(b)** Simple mediation analysis to determine whether plasma Zn mediates the 25(OH)D_3 levels on Zn supplemented postmenopausal women. *Statistically significant at $p < 0.05$. **Statistically significant at $p < 0.001$. Abbreviations: B = Unstandardized regression coefficients; ß = Standardized regression coefficients. CI = Confidence interval; SE = Standard error; Zn = Zinc; 25(OH)D = 25-Hydroxyvitamin D; 25(OH)D_3 = 25-Hydroxyvitamin D_3.

4. Discussion

The scientific evidence reflects that the postmenopausal population is at risk of suffering numerous micronutrient deficiencies, most notably referred to Zn. Furthermore, given the importance of monitoring Zn status at this stage, its association to key parameters such as vitamin D and leptin has been little investigated to date. In our study, a high percentage of the population was found to be deficient in vitamin D and vitamin D_3, improving its status after Zn intervention in ZG. In younger menopausal women, vitamin D_3 status responded better to Zn supplementation than in older menopausal women. On the other hand, our results also showed that as both intake and plasma Zn levels increased, the leptin levels decreased.

Zn is a mineral that predominates in protein foods, specifically those of animal origin (meat, fish, and dairy products). Western countries (including Spain) follow a diet pattern based primarily on the consumption of protein foods [35]. During menopause, women may experience weight gain and a redistribution of FM, restricting food intake. Added to the hormonal alteration, this could adversely affect Zn intake and status [3, 30]. In our study, considerably low Zn intake values were recorded **(*Table 12*)**. Studies such as those carried out by Ferueso et al. and Kumssa et al. [36,37], considered that deficiencies in the intake of micronutrients such as Zn are seen in Africa and Asia, where dairy product consumption is not as widespread, and the diet is based more on

foods of plant origin – which are poorer in Zn than those of animal origin –. Kim et al. [38], in a Korean menopausal population, recorded an intake of 10 mg Zn/day, which is very close to the RDA, highlighting an eastern diet pattern that is very different from the western one. Other authors such as Lim et al. [39], in the Australian women in their study, also recorded a Zn intake close to the RDA. In our study, we found the vitamin D intake values to be well below the RDA **(*Table 12*)**.

In reference to the biochemical parameters, our study population reflected levels within the reference ranges for the healthy female population. However, some parameters of lipid metabolism were found to be elevated, possibly as a result of the high Body Mass Index (BMI) shown by some women in our study [40]. The present study recorded a high prevalence of plasma and erythrocyte Zn deficiency. Studies such as that published by Darling et al. [41] in a female population of childbearing age, documented lower rates of Zn deficiency than in menopausal women, so the hormonal changes that occur during the menopausal stage could be a key factor in establishing deficient Zn status. Other authors such as Mahdavi-Roshan et al. [8] found their menopausal population to be deficient in several minerals, including particularly Zn, thus suggesting the need for adequate Zn supplementation, admitting that this deficient situation is difficult to reverse through the diet alone. Beiseigel et al. [42] observed that at baseline of Zn supplementation in a postmenopausal population, the

expected increase in Zn status was not observed, due to a lack of effective absorption – indicating that as supplementation progressed, Zn tended to be absorbed more steadily over time –. In our study, the Zn intervention significantly improved plasma and erythrocyte Zn levels in the postmenopausal population. Authors such as Neggers et al. [43], considered that an increase in Zn intake produces a decrease in erythrocyte Zn deficiency, but no such association was observed for plasma Zn.

Researchers such as Ortega et al. [44], assessed vitamin D intake in the Spanish menopausal population, choosing a representative sample from several Spanish provinces and, in coincidence with the results of our study (3.2 μg/day), reported vitamin D intake to be very low during the menopausal period – a pattern that is also repeated in adolescent females – [45]. Regarding vitamin D status, our study initially found a high prevalence of deficiency, with 80% of the population failing to reach sufficiency. This is surprising when comparing our results corresponding to an Andalusian postmenopausal population with those of a menopausal population in the United Kingdom [41], where a vitamin D deficiency similar to ours was observed in women living at a higher latitude and therefore with fewer hours of light than in southern Spain. Other studies such as that carried out by Dadoniene et al. [46], have reflected a lower percentage of deficiency (60%) in an obese postmenopausal population, possibly due to higher food intake than among women of normal weight.

On the other hand, González et al. [47], studied the prevalence of vitamin D deficiency throughout the Americas, reporting that while there is widespread deficiency, the latter decreases in proximity to the equator, and increases with age.

In our study, our mediation analysis revealed that Zn supplementation optimized both the plasma levels of vitamin D and vitamin D_3. In this regard, Kebapcilar et al. [48] found that vitamin D would be directly related to serum Zn status, and that both of these are involved in bone mineralization, but without clarifying the role of Zn in vitamin D_3 status. On the other hand, Potocnik et al. [49] recorded a synergistic action on plasma Zn status by supplementing with vitamin D and Zn combined, due to vitamin D enhances the intestinal absorption of Zn. In turn, Lutz et al. [50] found that an improved Zn status increased vitamin D_3 activity because Zn modulates the structure and binding of the Deoxyribonucleic Acid (DNA) binding domain of the active form of vitamin D receptor to specific vitamin D response element DNA. However, they did not record an increase in vitamin D_3 concentrations with increasing Zn levels as seen in our study. Other authors suggest that genes implicated in Zn homeostasis are largely increased by vitamin D_3 treatment [21]. Based on the above mentioned, vitamin D seems to have a synergistic action with Zn, due to vitamin D signals through the Vitamin D Receptor (VDR), a specific zinc-finger nuclear receptor,

modulating the immune system [51]. So that Zn supplementation could have a potential effect upon vitamin D_3 behavior.

Due to the hormonal imbalance, menopause can also lead to alterations in lipid metabolism, including an increase in FM, with the consequent increase in leptin levels [52]. Excessive adipose tissue has been associated with a reduction of the secretion of Zn-α2-glycoprotein, an adipokine with anti-inflammatory and lipid-mobilizing activity [53]. In this line, a high status of Zn has been demonstrated to have a preventive role on maintaining an adequate lipid profile, being a Zn supplementation an effective strategy to enhance lipid metabolism and to improve lipid parameters homeostasis [54]. In the present study, we found the Zn levels, both in intake and in plasma, to be inversely correlated with the leptin levels. Argani et al. [55] evaluated 100 mg/day of Zn supplementation during 8 weeks and recorded a decrease in leptin levels in patients with kidney disease. On the other hand, in studies such as that of Marreiro et al. [56] carried out at short term (4 weeks) and involving supplementation with 30 mg/day Zn, no reduction in leptin levels was noted in obese women. Another study in children [57], involving 8-week supplementation with 20 mg/day of Zn, recorded an inverse association with leptin. It is suggested that long-term Zn supplementation is the most effective strategy for reducing leptin levels, and that such supplementation works better in women than in men, since they have more fatty tissue and therefore higher leptin levels [58].

Therefore, assuring an adequate status of Zn could improve lipid metabolism, decreasing leptin levels and increasing vitamin D_3 status which is inversely related with body fatness [32].

In addition to described findings, the present study has some strengths and limitations. As strength, the study is a randomized, Pb-controlled study in which nutritional intake of energy, macronutrients and related Zn and vitamin D minerals were controlled at baseline and follow-up. In this regard, we found that nutritional intake and high compliance to supplementation remained stable during the nutritional intervention. The present study used LC–MS/MS which is the gold standard analytical method, offering greater sensitivity, flexibility, and specificity. Despite, this study has some limitations that should be considered such as the small sample size. Although initially 82 women were randomly assigned to be supplemented, a total of 51 postmenopausal women completed the study (***Figure 11***). Although the primary outcome of the trial was to assess the influence of a Zn diet strategy on vitamin D_3 status in postmenopausal women, the sample size in each group would allow us to preliminarily obtain significant results, although the results should be carefully considered. Clinical trials of the same nature and a similar sample size have shown a positive effect of different interventions on some parameters status in postmenopausal women [59,60]. Likewise, the sample size limitation did not allow us to make a more complex statistical approach since we did not have enough

power to perform multivariate analyses and to be able to adjust our model based on possible confounding variables.

5. Conclusions

In sum, Zn intervention in a group of postmenopausal women from the province of Granada (Spain), reduced deficiency of low plasma Zn levels by 50% and almost entirely resolved erythrocyte Zn deficiency. Vitamin D levels were deficient in approximately 80% of the population, and vitamin D_3 was seen to improve as a result of Zn supplementation. Of note is the fact that higher levels of both intake and plasma Zn were associated to lower leptin levels, being significantly decreased in women with lower age at menopause. Experimental studies involving longer term supplementation periods and larger sample sizes are needed to more precisely establish the role of Zn in the behavior of vitamin D_3 and leptin status.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (protocol code 149/CEIH/2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Title: Erythrocyte Zn concentration and antioxidant response after supplementation with Zn in a postmenopausal population. A double-blind randomized trial

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Abstract

Introduction: Menopausal hormonal changes increase the risk of deficiencies of minerals such as Zinc (Zn), which could further worsen the decreased antioxidant defense of postmenopausal women. This study was aimed to assess the effect of 8 weeks of Zn supplementation upon the antioxidant status and clinical nutritional parameters of a postmenopausal population. **Materials & Methods:** A total of 51 postmenopausal women were divided into 2 groups: Placebo (Pb) Group (PG) and Zn Group (ZG). Mineral status was determined by flame atomic absorption spectrophotometry. Total Antioxidant Capacity (TAC) and Superoxide Dismutase (SOD) were analyzed by kinetic colorimetric methods. Glutathione Peroxidase (GPx) was assessed by an enzymatic immunological method. **Results:** Poor Zn status was initially observed in erythrocyte samples. TAC showed a significant correlation ($r = 0.730$; $p < 0.05$) with erythrocyte Zn after the intervention (ZG: $r = 0.96$; $p < 0.001$). Moreover, erythrocyte Zn concentration in ZG was positively correlated to GPx after the intervention ($r = 0.61$; $p < 0.01$). **Conclusions:** The postmenopausal women initially presented Zn deficiency, and the status of this mineral improved after the intervention. Zn supplementation may be an effective approach for correcting the observed deficiencies, enhancing antioxidant defense in this risk population.

Keywords: postmenopausal women; antioxidant status; zinc; supplementation; total antioxidant capacity; superoxide dismutase, glutathione peroxidase

1. Introduction

Menopause is a natural phase of the female aging process, triggered by the cessation of ovarian hormone secretion. It refers to the disappearance of menstrual cycles and the appearance of physiological changes, with an increase in the risk of chronic degenerative disorders [1]. Due to the hormonal alterations, particularly estrogen depletion, this stage in life is characterized by an increased risk of deficiencies in the nutritional status of certain nutrients such as Zinc (Zn) [2].

Over the last decades, researchers have postulated that menopause itself contributes to molecular oxidation caused by free radicals, particularly Reactive Oxygen Species (ROS) [3]. Antioxidant status has been reported to be lowered during the menopausal period [4]. Estrogens are sexual hormones that function as antioxidants. In this sense, during menopause, the lowered estrogen levels could cause a reduction of antioxidant status [5]. On the other hand, it has been reported that a lower antioxidant status is involved in the deficient status of Zn [6]. Moreover, Superoxide Dismutase (SOD), which depends on Zn, has been mentioned to be present at low levels in the menopausal process. In this regard, expression of SOD can be modulated by estrogen and progesterone, which are decreased in menopause [7].

The mineral Zn is widely deficient in the general population, and this constitutes a serious global public health problem [8]. It acts as a

cofactor of more than 300 enzymes in the human body, regulates thousands of genes, controls numerous cell-signaling pathways and is also a key mineral for the immune, nervous, and reproductive systems [9]. Zn participates in antioxidant defense and counteracts the Oxidative Stress (OS) induced by ROS [10]. In this line, Zn is an inhibitor of NADPH oxidase, a co-factor of SOD, and an inducer of metallothionein [11].

Cellular Zn regulation is complicated and could imply several mechanisms to consider for controlling cellular Zn turnover [12]. The World Health Organization (WHO) has suggested to further expand on already known clinical markers of Zn status as there is no universally accepted single measure to assess Zn status and findings from studies assessing Zn status biomarkers are often contradictory and inconsistent [13]. Although cellular biomarkers such as erythrocyte Zn concentrations may not be a sensitive and a reliable marker of Zn status over a long period of time [9], erythrocyte Zn analysis remains informative and easy to use [14], presenting a good sensitivity in the assessment of Zn status in premenopausal women [15]. Moreover, Zn in erythrocytes is not influenced by several factors (e.g., post-meal status, stress, physical efforts and hormones), revealing more precisely Zn deficiency and being suggested to be a good marker of Zn status [16,17], although an increase in erythrocyte Zn concentrations might not sometimes be a realistic indicator of enhanced Zn status through Zn supplementation, especially

due to the lack of established reference values, making Zn in erythrocytes levels to be interpreted with caution [9].

Previous studies have reported the beneficial effects of a Zn intervention on antioxidant enzymes in adult populations [18]. Estrogen depletion in postmenopausal women increases the risk of Zn deficiency, which could be prevented through Zn supplementation [19]. Ageing is associated with an accumulation of free radical damage [20] and with a decrease in antioxidant minerals as Zn – which could lead to physiological and clinical modifications – in healthy elderly populations [21]. In fact, it has been demonstrated that a Zn intervention could have beneficial effects upon Total Antioxidant Capacity (TAC) in aged populations, but this effect has not been demonstrated in a population at risk of nutritional deficiency such as postmenopausal women [22]. Furthermore, due to the Zn mineral deficiency in postmenopausal women and the key role of this mineral in several physiological functions, supplementation with Zn in the menopausal setting is highly recommended [23].

Thus, given the lack of evidence on the effect of key mineral interventions with Zn upon antioxidant status in healthy postmenopausal women, the present study was carried out to assess the effect of 8 weeks of a Zn supplementation intervention upon antioxidant defense in this healthy yet risk population, and its association to nutritional, clinical and antioxidant parameters. We hypothesized that Zn in erythrocytes could

be correlated to the analyzed antioxidant defense parameters in our postmenopausal population.

2. Methods

2.1. Study Design and Intervention

This is an 8-week, double-blinded, Placebo (Pb)-controlled, randomized intervention trial. Participants were randomly assigned to 1 of 2 treatment groups: Pb Group (PG: 25 women); Zn Group (ZG: 26 women) – 50 mg/day of Zn – (600% of Recommended Dietary Allowances (RDAs)). Zn supplements were supplied by SM Natural Solutions, Sabadell, Spain (Number 0B62713821) following the period of 8 weeks, dosage and mode of application recommended by the manufacturer and based in previous studies with similar dosage and period of supplementation [24,25]. Pb capsules contained lactose and were made of the same size and color as Zn supplements for identical appearance and taste. The intervention was carried out in winter from January 15th to March 15th. The study was registered at the US National Institutes of Health (ClinicalTrials.gov) NCT03672513.

2.2. Study Participants

A total of 51 healthy postmenopausal women volunteers from the province of Granada, Spain aged between 44 and 76 were recruited once had been informed about the protocol. Women were excluded if they were unwilling to accept the randomization procedure. Women were included according to the following criteria: (I) to present postmenopausal status (with at least 12 months of amenorrhea), (II) not

to present any pathology related to disorders in nutrients absorption and metabolism that could affect their nutritional status, (iii) not to be subjected to Hormone Replacement Therapy (HRT), (iv) not to take vitamin and mineral supplements, (v) not to present systemic inflammatory status (C-Reactive Protein (CRP) was included as a reference biomarker to assess inflammation status of the participants at baseline). The present study was conducted according to the principles of the Declaration of Helsinki and the approval by the Ethics Committee of the University of Granada (149/CEIH/2016), in accordance with the International Conference on Harmonization/Good Clinical Practice Standards. Written informed consent was obtained from all patients taking into account the approval of the Ethics Committee and the Research Committee of the Centre. Randomization was performed in a 1:1 ratio using a table of random numbers, prepared by a researcher who did not participate in the data collection. Allocation concealment was ensured, as the referred researcher did not release the randomization code until the participants were recruited into the trial after all baseline measurements were completed. Women were randomly assigned (simple randomization) to study groups (parallel design). In order to ensure comparable distribution across the treatment arms. Women were stratified to balance baseline covariates including age (≥ 58 or < 58 years). Both study participants and investigators were blinded to the group allocation. Eligible participants of this study are represented in (***See Figure 11***).

2.3. Compliance Evaluation

Adherence/compliance of nutritional intervention was determined as the percentage of all of the supplement capsules ingested throughout the study period. Moreover, biochemical and clinical-nutritional parameters were taken at baseline and follow-up to evaluate the safety of the product and to verify the adverse effects. In addition, subjects were asked to keep daily records about side effects or other problems related to the supplements.

2.4. Data Collection

All qualitative data were obtained through the use of manual questionnaires administered by the interviewer that reflected information on personal data, sociodemographic aspects, an adequate diagnosis of the postmenopausal situation, smoking habits and physical activity.

2.5. Body Composition Analysis

Anthropometric recorded data were height (m) (SECA® Model 274, Hamburg, Germany) and weight (kg) (by bioelectrical impedance (Tanita MC-980 Body Composition Analyzer MA Multifrequency Segmental, Barcelona, Spain). BMI was then calculated as (weight in kg/ height in m^2). The analyzer complies with the applicable European standards (93/42EEC, 90/384EEC) for use in investigation. Participants were informed in advance of the required conditions prior to the measurement:

(I) no alcohol less than 24 hours before the measurement, (II) no vigorous exercise less than 12 hours prior to the measurement, (III) no food or drink less than 3 hours prior to the measurement, and (IV) no urination immediately before the measurement [26].

2.6. Intake Rating

A manual 24 Hours Recall (24HR) administered by a professional interviewer was used to assess nutrient intake, taking into account 1 holiday and 2 non-holidays days at baseline and follow-up. Recall accuracy was recorded with a set of photographs of prepared foods and dishes that are frequently consumed in Spain [27]. The Dietowin software program (7.1. version, Barcelona, Spain) was used in order to adjust food intakes to absolute and percentage values of the Recommended Dietary Allowances (RDAs) of nutrient intended for individual subjects. Dietary intake was compared with the RDAs for the female Spanish population within the age range included in our study [28].

2.7. Biochemical Parameters Analysis

A blood extraction was performed in the morning in fasting conditions at baseline and follow-up and was centrifuged at 4 °C for 15 minutes at 3,000 rpm obtaining plasma and erythrocyte compartments. The erythrocytes were washed 4 times with 3 mL of 0.9% sodium chloride solution, centrifuging for 15 minutes at 3,000 rpm after each wash. Then,

the supernatant saline solution was removed from the last wash and the erythrocytes were obtained with a Pasteur Pipette. The samples were frozen at –80 ºC for further analysis. All samples were measured in one run, in the same assay batch and blinded quality control samples were included in the assay batches to assess laboratory error in the measurements. Clinical parameters were glucose (mg/dL), urea (mg/dL), uric acid (mg/dL), Triglycerides (TG) (mg/dL), Total Cholesterol (TC) (mg/dL), total proteins (g/dL), transferrin (mg/dL), albumin (mg/dL) and CRP (mg/dL) by routine analytical hospital assays. Zn content (mg/dL) was analyzed by Flame Atomic Absorption Spectrometry (FAAS) (Perkin Elmer A. Analyst'300 Norwalk, Connecticut, United States of America) previous wet-mineralised way in the Scientific Instrumental Center (SIC) from the University of Granada. Accuracy of the method was evaluated by analysis of a Certified Reference Material (Seronorm^{TM} Trace Elements ref. MI0181 SERO AS, Billingstad, Norway). Levels of Zn were analyzed at different optimal wavelengths for each element (slit 0.7 nm), using a flow rate (Air/C_2H_2) of 10/1.9 L·min^{-1}, and a 5-point calibration curves (r^2 = 0.9997) [26]. 7.3 mg/dL was the cut-off point for determining a low erythrocyte Zn content. Antioxidant status parameters measured were TAC, GPx, and SOD, analyzed by commercial kinetic colorimetric methods. TAC determination in plasma samples was carried out evaluating the reduction power of Cu^{2+} from the action of antioxidants present in samples (TAC kit, Jaica, Shizuoka, Japan). Variability was tested repeatedly conducting 5 samples and considering

lower variability than 5% to be included [29]. GPx activity was determined by enzymatic immunological method using the Bioxytech GPx-340™ kit (OxisResearch™), an indirect colorimetric assay of the activity of GPx [30]. SOD activity was analyzed by colorimetric method based on cytochrome c reduction using the Randox Ransod kit (RANDOX Laboratories Ltd., Dublin, Ireland, United Kingdom) [31]. The reference values for each antioxidant, clinical, and mineral parameter were provided by the manufacturer, the hospital and the SIC, respectively.

2.8. Statistical Analysis

Data were obtained using SPSS 22.0 Software for MAC (SPSS Inc. Chicago, Illinois, United States of America). GraphPad Prism 9 software (GraphPad Software, San Diego, California, United States of America) was used for plotting the graphs. We performed sample size calculation for our primary aim of the double-blinded, Pb-controlled, randomized intervention based on the influence of oral Zn supplementation upon antioxidant status in a postmenopausal population by using G*Power software (version 3.1.9.6, Kiel, Germany). To the best of our knowledge, there were no available information on changes in TAC in postmenopausal women treated with Zn and based on their erythrocyte levels. Therefore, the number of participants to be included in the study was calculated based on the main statistical method used (unpaired T-test). An a priori power analysis indicated that at least a total of 82

participants were required. This calculation was based on a moderate effect size (effect size d = 0.65), an alpha level of 0.05, and a beta value of 0.80 for unpaired T-test calculating the difference between two independent means (two groups) [32]. Categorical variables were summarized as Frequencies (N) and Percentages (%), and continuous variables using Mean ± Standard Deviation (Mean ± SD). The hypothesis of normal distribution was accepted using the Kolmogorov-Smirnov test as a previous step to the execution of a parametric model or not. To compare the changes intergroup before and after intervention, unpaired T-test was used. To compare the differences in change over time among the two groups, paired T-test was employed. Correlation analyses and partial correlation coefficients were performed with Pearson test. A *p*-Value less than 0.05 was considered statistically significant.

3. Results

The women included in our intervention showed 100% adherence to supplementation and reported no side effects during the 2-month intervention. ***Table 16*** describes the baseline characteristics of the different postmenopausal groups. Based on the BMI, 37.9% of the postmenopausal women presented type I overweight before the intervention. Regarding the initial adequacy of mineral intake, 40.1% presented an insufficient intake of Zn (below 75% of the RDAs). The rest of the nutrients were within the RDAs, though energy intake was rather below the reference values. In the case of Zn status, approximately 60% of the women in ZG presented erythrocyte Zn deficiency, which was corrected to 4% after Zn supplementation. No intergroup differences were found for the studied parameters at baseline (all $p > 0.05$).

Figure 15 shows the correlation analysis between Zn in erythrocytes and antioxidant parameters after 2 months of supplementation. Of the different antioxidant status parameters considered, TAC showed a significant correlation to erythrocyte Zn in the total sample ($r = 0.81$; $p < 0.001$) after the intervention (***Figure 15A***), and also stratified by ZG after the intervention ($r = 0.96$; $p < 0.001$) (***Figure 15B***). Moreover, erythrocyte Zn in ZG was positively correlated to GPx after the intervention ($r = 0.61$; $p < 0.01$) (***Figure 15C***).

Table 16. Baseline characteristics of the study population.

	Reference Values	PG (n = 25) Mean ± SD (%RDA)	ZG (n = 26) Mean ± SD (%RDA)	p Value
Sociodemographic				
Age (years)	-	59.5 ± 9.00	57.0 ± 8.00	0.327
BMI (kg/m^2)	22.0-27.0	28.0 ± 4.30	26.2 ± 4.50	0.231
Blood pressure				
Normal blood pressure n (%)	–	11 (42)	16 (70)	–
High blood pressure n (%)	–	14 (58)	10 (30)	–
Physical exercise	–			–
Sedentary n (%)	–	9 (36)	6 (23)	–
Non-sedentary n (%)	–	16 (64)	20 (77)	–
Smoking habit	–			–
Non-smoker n (%)	–	18 (75)	21 (81)	–
Smoker n (%)	–	7 (25)	5 (19)	–
Educational level	–			–
Basic educational level n (%)	–	11 (42)	8 (31)	–
Secondary or high level n (%)	–	14 (58)	18 (69)	–
Dietary Intake				
Energy (Kcal/day)	2000.0	1339.0 ± 277.1 (67.0)	1487.9 ± 385.5 (74.5)	0.121
CHO (g/day)	275.0	145.5 ± 39.8 (53.2)	154.0 ± 39.8 (55.5)	0.452
Proteins (g/day)	50.0	59.7 ± 13.9 (145.7)	63.9 ± 14.4 (140.3)	0.307
Fats (g/day)	70.0	56.4 ± 16.9 (77.3)	67.7 ± 26.2 (93.0)	0.076
Zn (mg/day)	12.0	6.67 ± 5.06 (46.0)	5.80 ± 1.40 (40.2)	0.411
Biochemical parameters				
Glycemia (mg/dL)	70.0-110.0	96.0 ± 19.8	90.6 ± 16.0	0.295
Creatinine (mg/dL)	0.50-0.90	0.76 ± 0.18	0.66 ± 0.10	0.012
Urea (mg/dL)	10.0-50.0	36.2 ± 10.2	33.3 ± 8.20	0.262
Uric acid (mg/dL)	2.40-5.70	4.50 ± 1.00	4.36 ± 1.01	0.436
Triglycerides (mg/dL)	50.0-200.0	115.8 ± 68.9	98.2 ± 82.7	0.419

Cholesterol (mg/dL)	110.0-200.0	224.1 ± 39.7	212.8 ± 33.1	0.279
Transferrin (mg/dL)	200.0-360.0	289.9 ± 29.5	274.2 ± 49.2	0.381
Albumin (mg/dL)	3.50-5.20	4.48 ± 0.21	4.37 ± 0.21	0.069
Total proteins (g/dL)	6.60-8.70	7.10 ± 0.42	7.00 ± 0.62	0.705
Total bilirubin (mg/dL)	0.10-1.20	0.50 ± 0.13	0.47 ± 0.11	0.533
Prealbumin (mg/dL)	20.0-40.0	26.6 ± 5.03	24.8 ± 6.45	0.133
C-reactive protein (mg/L)	0.02-5.00	0.29 ± 0.25	0.29 ± 0.39	0.994
Erythrocyte Zn (mg/dL)	7.30-7.60	6.39 ± 2.12	6.13 ± 1.94	0.657

$n = 51$. Quantitative data are expressed as the mean ± Standard Deviation (SD) (percentage of RDA) unless specified otherwise. Qualitative data are expressed as n (%) of subjects. For obtaining inter-groups p Value, unpaired t-student test was used. Significance was set at p Values < 0.05 and is highlighted in boldface. Abbreviations: BMI = Body Mass Index; CHO = Carbohydrates; PG = Placebo group; RDA = Recommended Dietary Allowance; ZG = Zinc group; Zn = Zinc. Unpaired T-test was used for inter-groups analysis of quantitative variables. Statistical significance was considered for $p < 0.05$.

Figure 16 shows the mean values of the 3 antioxidant parameters studied according to median age and the intervened groups following supplementation. TAC was significantly lower ($p < 0.05$) in those postmenopausal women below the median age (58 years) in ZG. No significant changes were observed after the intervention in both groups with regard to the rest of the antioxidant parameters.

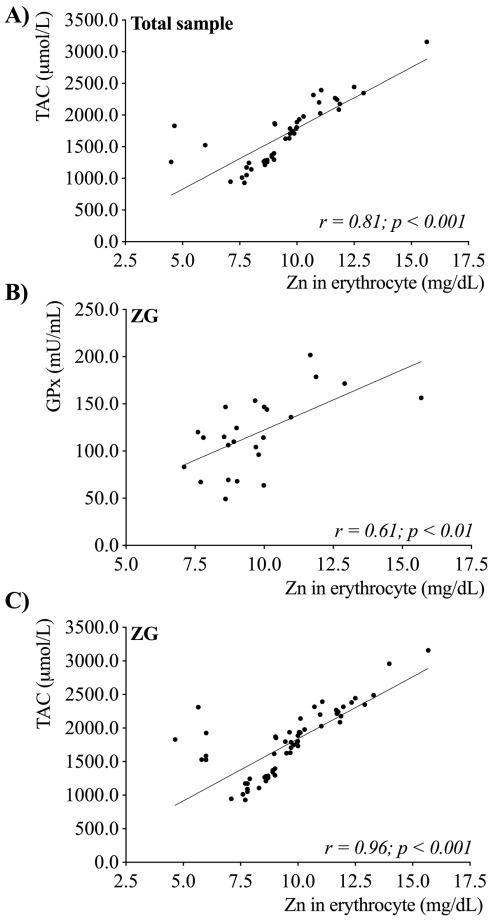

Figure 15. Scatter plots of the relationship between erythrocyte zinc status after two months of supplementation and the different antioxidant parameters. (A) Zn in Erythrocyte-TAC in total population; (B) Zn in Erythrocyte-TAC in ZG; (C) Zn in Erythrocyte-GPx in ZG. Correlation analyses and partial correlation coefficients were

performed with Pearson test. A *p* Value less than 0.05 was considered statistically significant. Abbreviations: GPx = Glutathione peroxidase; TAC = Total Antioxidant Capacity; ZG = Zinc group; Zn = Zinc.

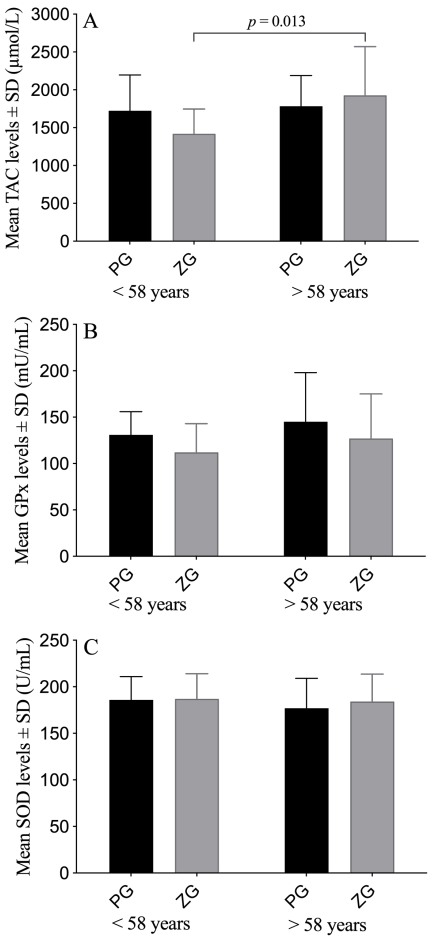

Figure 16. Mean values of antioxidant parameters after intervention by groups and median age. (A) mean TAC levels by intervened groups and age; (B) mean GPx levels by intervened groups and age; (C) mean SOD levels by intervened groups and age. All data are expressed as the mean (Standard Deviation (SD)). Paired T-test was used for comparing intra-groups and unpaired T-test for comparting inter-groups. The model was adjusted for erythrocyte Zn as covariate. Statistical significance considered for $p < 0.05$. Abbreviations: GPx = Glutathione peroxidase; PG = Placebo Group; SOD = Superoxide dismutase; TAC = Total Antioxidant Capacity; ZG = Zinc Group.

4. Discussion

There is a growing interest in maintaining optimal trace element status in menopause. In this regard, Zn is associated to a number of indirect antioxidant functions, suggesting that it may play a preventive role in certain diseases associated with menopause [33]. The aim of this study was to investigate the effects of short-term Zn supplementation upon the antioxidant status of healthy postmenopausal women. The results showed that a large percentage of postmenopausal women had alterations in Zn status that were partially corrected after the Zn intervention, observing significant improvement of antioxidant status in those Zn-supplemented postmenopausal women in the higher age range. Likewise, the observed relationship between the erythrocyte Zn concentrations after supplementation and antioxidant status (mainly TAC) may contribute to preserve antioxidant defenses during the postmenopausal process.

In line with other investigations (albeit to a lesser extent), the present study showed postmenopausal women to have insufficient Zn intake (***Table 16***), as previously reported accompanied by low biochemical levels of this mineral [34]. According to our results, at baseline the mean erythrocyte Zn levels were below the reference values and improved significantly after the Zn intervention in ZG. Regarding Zn supplementation, significantly increased erythrocyte Zn concentrations were recorded in ZG over the course of the intervention, together with the achievement of optimum Zn intake. Supplementation had a major

effect in restoring Zn homeostasis and consequently reducing the percentage of women who presented low Zn status. In this sense, a study involving a cohort of 387 middle-aged volunteers (55% women) aged 55–85 years showed Zn concentrations – which increased significantly in the case of serum Zn following Zn supplementation – to be no different between supplemented and non-supplemented individuals in the case of erythrocyte Zn [35]. This observation could be explained in part by the implication of Zn in many metabolic pathways. Thus, the decrease in the concentration of the mineral in erythrocytes could be due to the larger demand for the mineral [9]. Therefore, the significant increase observed in erythrocyte Zn after supplementation in ZG in our study could suggest that the demand for this mineral is mainly focused on promoting the transport of blood Zn to different tissues and enzymatic systems before restoring the erythrocyte reserves. In this regard, taking into account that erythrocytes present a lifespan of 3 months, Zn content in erythrocytes could be a useful biomarker of Zn status according to our 2 months intervention period [36]. In this line, another possible explanation could be the potential effect which metallothionein could perform upon Zn homeostasis, as it is the main regulator of the intracellular transport and mobilization, storage and transferring of Zn [37]. Metallothioneins are more expressed when Zn is in higher amounts and bind Zn intracellularly, serving as markers of Zn status [38]. Together with their well-known relationship with Zn homeostasis, metallothioneins are considered too as antioxidant Zn-related factors due to their influence on decreasing

oxidative damage [39]. Regretfully, metallothionein levels, that could have helped to clarify the assessment of Zn status and their potential involvement in increasing antioxidant defenses, were not assessed in this study.

Positive associations were found between erythrocyte Zn and TAC after the intervention in ZG ($r = 0.96$; $p < 0.001$) (***Figure 15A*** and ***15B***), confirming the potent role of Zn as an antioxidant. Previous evidence [2] points to increased TAC levels after Zn interventions in menopausal populations compared to Pb, demonstrating the potential influence of Zn upon antioxidant status. Moreover, erythrocyte Zn was directly correlated to GPx levels in ZG (***Figure 15C***). Mariani et al., [40] performed a Zn supplementation in healthy old population to assess its effect upon GPx levels. In their study, no age and gender-related differences in the activity of GPx was observed, but, contrary to our findings, they did not observe a direct relationship between the Zn intervention and GPx activity. Unfortunately, no relationship was found between erythrocyte Zn and SOD activity. Opposite to our findings, one study involving a Zn supplementation in postmenopausal women, evidenced increased SOD levels after the Zn intervention, considering SOD as a proper biomarker of Zn status in postmenopausal women [6]. In this line, the abovementioned study only suggests short period interventions as the one performed in our study, because of the possible adverse effects of long-term Zn interventions.

OS is a risk factor for clinical outcomes in menopausal women that could be ameliorated through enhanced antioxidant status parameters [41]. In our study, TAC was significantly lower in those postmenopausal women below the median age in ZG after the intervention. Metabolic changes occurring with advancing age may increase OS because of a decrease in antioxidant capacity [42]. In this sense, the increase in OS during menopause may be due to lower levels of hormones and their antioxidant role [43]. It has recently been demonstrated that the decrease in antioxidant defense capacity depends on the time of the menopausal phase, possibly through the mentioned hormonal alteration [44]. Alterations in metabolism, redox imbalance, and dysregulation of the inflammatory response in elderly women can be risk factors in the development, progression, and clinical manifestations of different disease conditions [45]. In this regard, the observed differences in antioxidant status related to age could be due to the time of the menopausal phase. More trials are needed to further elucidate our findings.

The present study has strengths and limitations. As limitations, mention must be made of the use of questionnaires for assessing intake, without registering images and real weights of food (gold standard) [46], which could be affected by recall bias, since the results are conditioned by the memory and educational level of the subject. In this regard, the authors considered energy intake to be underestimated. One of the major

limitations of the 24 Hours quantitative Recall (24HR) is based on the fact that the subject tends to report lower intakes amounts. This could be one reason why no association between mineral intake and clinical parameters was found. In turn, the present study has a small sample size, recruiting less women than expected from the sample size estimation mainly due to the loss of participants during the follow-up. Another limitation is the lack of established reference values for these cellular Zn concentrations which makes interpreting the results difficult. Moreover, it could have been interesting to include in our study OS markers or metallothionein assessment with the aim of evaluating the influence of increased TAC in ZG upon OS markers as ROS. On the other hand, it would be relevant to reproduce this study in the context of long-term supplementation. As strengths, this is a randomized, Pb-controlled study in which intakes of energy, macronutrients, and related Zn minerals were controlled at baseline and over two months of follow-up. Ours is one of the few studies to have been conducted in this context, assessing the effect of a Zn intervention upon antioxidant parameters in a population such as postmenopausal women, at risk of suffering deficiencies of key micronutrients.

5. Conclusions

The results of our study show that an 8-week Zn intervention reduces the high percentage of postmenopausal women with low erythrocyte Zn levels observed in the analyzed population. Zn in erythrocytes was related to TAC levels whether or not the women received Zn supplementation and was reinforced among those who were supplemented. Moreover, Zn in erythrocytes was directly related to GPx levels after the Zn intervention. These observations should be investigated more in depth, due to the lack of evidence of the influence of Zn supplementation upon antioxidant parameters. Further studies involving long-term antioxidant mineral interventions, including the evaluation of OS parameters, are needed in order to preserve the health of postmenopausal women and improve their quality of life.

Author Contributions: Conceptualization, H.V.-L. and E.P.; Methodology, L.H.-Q., H.V.-L., and E.P.; Software, H.V.-L., L.H.-Q., and J.M.-L.; Formal analysis, L.H.-Q., H.V.-L., E.P., and J.M.-L.; Investigation, B.L.G. and Y.G.-M.; Resources, B.L.G., Y.G.-M., E.P., and J.M.-L.; Writing–original draft preparation, L.H.-Q., B.Q.-O and H.V.-L.; Writing–review and editing, L.H.-Q., H.V.-L., Y.G.-M., B.L.G., E.P., and J.M.-L.; Supervision, E.P. and J.M.-L.; Project administration, B.Q.-O and E.P.; Funding acquisition, E.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (protocol code 149/CEIH/2016).

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12.3. CAPÍTULO III

El capítulo 3 de la presente Tesis Doctoral pretende evaluar el efecto de la suplementación con Mg sobre una de las variables principales del estudio, la cual es el estatus plasmático y eritrocitario de Mg, así como una posible reducción del porcentaje de deficiencia en este mineral. Adicionalmente, se evalúa el efecto que tiene dicha intervención sobre las variables secundarias del estudio como el estatus nutricional de la vitamina D y la defensa antioxidante y otros parámetros rutinarios asociados, así como la relación existente entre estos parámetros con el estatus bioquímico del Mg.

Se ha incluido 1 artículo experimental cuyos objetivos se describen a continuación:

Artículo 7: evaluar la influencia de una intervención con Mg sobre el estatus de la vitamina D en una población postmenopáusica de la provincia de Granada (España).

12.3.1. Manuscript 7

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Title: Response of Vitamin D after Magnesium Intervention in a Post-menopausal Population from the Province of Granada, Spain

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Abstract

Introduction: Menopause is a stage of hormonal imbalance in women which, in addition to other physio pathological consequences, poses a risk of deficiency of key micronutrients such as Magnesium (Mg) and vitamin D. We aimed to evaluate the influence of a Mg intervention upon vitamin D status in a postmenopausal population from the province of Granada (Spain). **Materials & Methods:** A total of 52 healthy postmenopausal women between 44–76 years of age were included and 2 randomized groups – Placebo (Pb) group and Mg group (500 mg/day) – were treated for 8 weeks. Nutrient intake was assessed using questionnaires based on 24 Hours Recall (24HR). Vitamin D was analyzed by liquid chromatography–tandem mass spectrometry. **Results:** Baseline vitamin D proved deficient in over 80% of the subjects. The administration of Mg resulted in significantly increased vitamin D levels in the intervention group versus the controls ($p < 0.05$). **Conclusions:** Our intervention improved vitamin D status in the studied postmenopausal women.

Keywords: vitamin D; magnesium; liquid chromatography–tandem mass spectrometry; post-menopause

1. Introduction

Menopause is characterized by physiological changes with important variations in hormone levels. If left unchecked, this situation can lead to disease [1], including an increased risk of different types of cancer, cardiovascular disorders, osteoporosis, and type 2 diabetes, among other conditions [2,3]. During this stage of life, women may experience weight gain and a redistribution of Fat Mass (FM). Added to the hormonal alteration, this could adversely affect the status of different key micronutrients such as Magnesium (Mg) and vitamin D in this population [4,5].

The mineral Mg is necessary for most reactions in the human body and is a cofactor of more than 300 enzymes [6]. Mg is essential for the functioning of Parathyroid Hormone (PTH) and vitamin D. Hypomagnesemia in postmenopausal women needs to be monitored together with the status of those minerals closely related to phosphorus-calcium metabolism, to optimize homeostatic equilibrium and bone health. Mg supplementation may offer benefits in this regard [7,8]. The inclusion of Mg supplementation in postmenopausal women in the event of deficiency has been suggested by several authors, as it seems to improve postmenopausal symptoms, avoiding long-term systemic consequences [9–11]. In recent years, the interest in vitamin D has increased, due to the high prevalence of vitamin D deficiency worldwide [12]. Vitamin D plays a key role in phosphorus-calcium metabolism, improving the intestinal absorption of Calcium (Ca), and regulating bone mineralization and the renal excretion of Ca [13,14]. To prevent poor vitamin D status, the monitoring of risk populations such as postmenopausal women is recom-

mended with a view to preserving bone health [15]. However, new studies have also addressed the role of vitamin D in non-skeletal diseases [16,17].

Nowadays, the routine analytical determination of vitamin D is recommended in healthy risk groups such as postmenopausal women. However, such determinations are characterized by variability of the results obtained – thus suggesting the need to standardize the laboratory test protocol employed – [18]. One of the methods currently used to measure vitamin D is Enzyme Immuno-Assay (EIA), which is the most widely used method in hospitals [19,20]. Use is currently also made of chromatography, which yields stable and reproducible results, and distinguishes between 25-Hydroxyvitamin D_3 ($25(OH)D_3$) and 25-Hydroxyvitamin D_2 ($25(OH)D_2$)[12]. In this respect, Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) is regarded as the gold standard, offering greater sensitivity, flexibility and specificity [18]. Unfortunately, LC–MS/MS cannot always be used, due to its high cost [20,21]. The technique of choice is therefore conditioned by the availability of resources [22,23]. In general, all techniques measure mainly 25-Hydroxyvitamin D (25(OH)D), because of its long half-life (one month) in plasma. Plasma vitamin D concentrations are conditioned not only by homeostatic regulation but also by lifestyle, environmental and sociocultural factors such as the use of sunscreens, the female gender, postmenopausal status, and FM [24–28]. During post menopause, vitamin D supplementation could be recommended in women with confirmed vitamin D deficiency, since it seems to be associated with an increase in bone mineral density and could improve future quality of life [29].

Therefore, the postmenopausal period could be associated with a genuine risk of deficiency of various minerals and vitamins, particularly Mg and vitamin D [30,31]. The present study was carried out to assess vitamin D status in a population of postmenopausal women in the province of Granada (Spain), with evaluation of the influence of a Mg intervention.

2. Materials and Methods

2.1. Study Design and Intervention

This is an 8-week, double-blinded, Placebo (Pb)-controlled, randomized intervention trial (***Figure 17***). Participants were randomly assigned to 1 of the 2 treatment groups: Pb group (PG: 25 women) and Mg Group – 500 mg/day – of Mg (MG: 27 women) . Randomization was performed in a 1:1 ratio using a table of random numbers, prepared by a researcher who did not participate in the data collection. Allocation concealment was ensured, as the referred researcher did not release the randomization code until the participants were recruited into the trial after all baseline measurements were completed. Mg supplements were supplied by Botánica Nutrients SL, Seville, Spain (Number B91070797), following the period of 8 weeks recommended. Pb capsules were made of the same size and color as Mg supplements for identical appearance and taste. The intervention was carried out in winter from January 15th to March 15th. The study was registered at the US National Institutes of Health (ClinicalTrials.gov) NCT03672513.

2.2. Study Participants

A total of 52 healthy postmenopausal women volunteers from the province of Granada, Spain aged between 44 and 76 years were recruited once they had been informed about the protocol. Inclusion criteria were (I) to present postmenopausal status (with at least 12 months of amenorrhea), (II) to present low status in Mg obtained in a previous biochemical assessment, (III) not to present any pathology that could affect their nutritional status, (IV) not to be subjected to Hormone Replacement Therapy (HRT), (V) not to take vitamin and mineral supplements. Women were

excluded if they were unwilling to accept the randomization procedure. Written informed consent was obtained from all patients considering the approval of the Ethics Committee and the Research Committee of the Centre. The present study was conducted according to the principles of the Declaration of Helsinki and the approval by the Ethics Committee of the University of Granada (149/CEIH/2016), in accordance with the International Conference on Harmonization/Good Clinical Practice Standards.

Eligible participants of this study were 121 participants. Of these, 39 menopausal women were excluded because 18 women did not meet the inclusion criteria and 21 women declined to participle in the study after the initial interview, and so, 82 menopausal women were enrolled in the study and randomly assigned to the 2 arms (***Figure 18***). Of the 41 postmenopausal women that were allocated to intervention in the PG, a total of 16 women withdrew the study due time and supplementation commitment. In reference to the 41 postmenopausal women allocated into MG, a total of 13 postmenopausal women withdrew due to time and supplementation commitment, illness severity or not giving any reason. Of the 28 women included in the data analysis, 1 woman was excluded from the analysis because of insufficient blood sample was collected. Thus, 25 women in PG and 27 women in MG were enrolled in the present study.

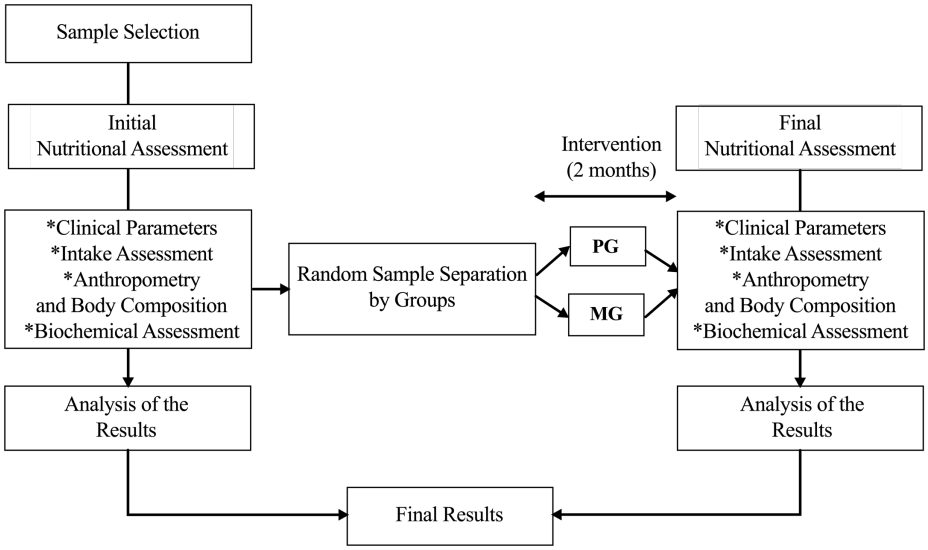

Figure 17. Study design. MG = Magnesium Group. PG = Placebo Group.

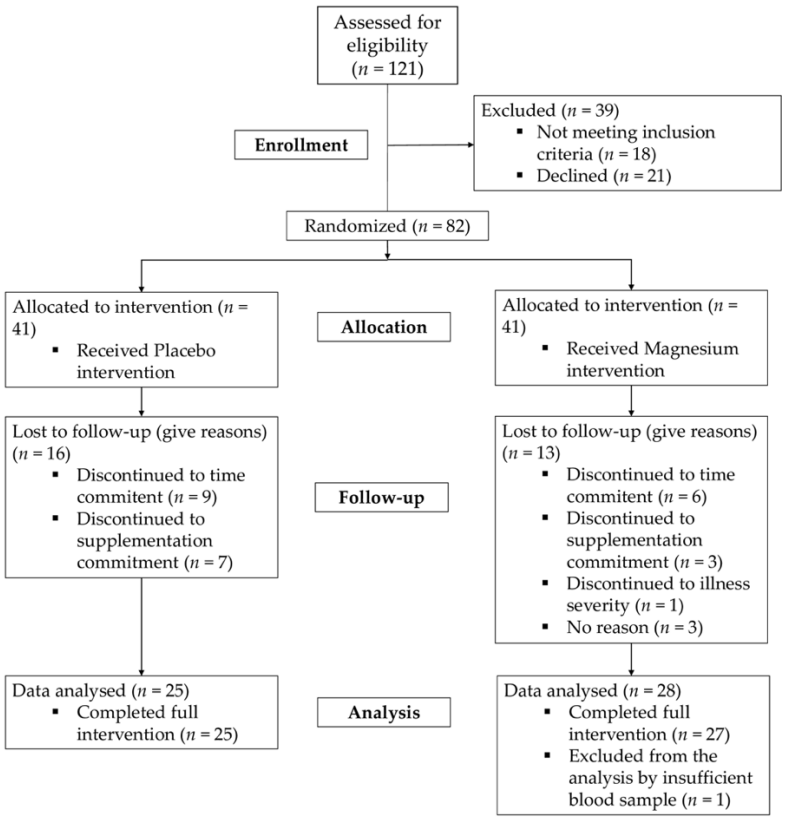

Figure 18. Flowchart of participants recruited, enrolled, and involved in the clinical study.

2.3. Randomization and Blinding

Women were randomly assigned (simple randomization) to study groups (parallel design). To ensure comparable distribution across the treatment arms, women were stratified to balance baseline covariates. Both study participants and investigators were blinded to the group allocation. Initial and follow-up visits for evaluating dietary intake, Body Composition (BC), and biochemical status parameters were performed at baseline and after 2 months of intervention.

2.4. Sample Size

We performed sample size calculation for our primary aim of a randomized controlled trial based on the influence of a Mg supplementation on vitamin D status. The number of participants to be included in the study was calculated on the basis of the change in vitamin D status after Mg intervention. To the best of our knowledge there were no available information regarding group difference changes on vitamin D in Mg intervened postmenopausal women. Therefore, we assumed a difference of 2.63 ng/mL as clinically meaningful based on previous observations in our group (unpublished data). A total of 68 participants were needed to detect a mean group difference of 2.63 ng/mL and a Standard Deviation (SD) of 3.85 ng/mL in vitamin D with a power of 80% and an alpha of 0.05, and assuming a maximum loss of 20% of participants (n = 82).

2.5. Compliance Evaluation

Adherence/compliance to nutritional intervention was determined as the percentage of all of the supplement capsules ingested throughout the study period. In addition, subjects were asked to keep daily records about

side effects or other problems related to the supplements. Moreover, biochemical and clinical-nutritional parameters were taken at baseline and follow-up to evaluate the safety of the product and to verify the adverse effects.

2.6. Data Collection

All recorded data were obtained through the use of manual questionnaires administered by the interviewer that reflected information on personal data, sociodemographic aspects, an adequate diagnosis of the postmenopausal situation, smoking habits, and physical activity [30].

2.7. Body Composition Analysis

Anthropometric recorded data were height (m) (SECA® Model 274, Hamburg, Germany), Waist Perimeter (WP) (cm) (SECA® Model 201, Hamburg, Germany), and BC by bioelectrical impedance (Tanita MC-980 Body Composition Analyzer MA Multifrequency Segmental, Barcelona, Spain). The analyzer complied with the applicable European standards (93/42EEC, 90/384EEC) for use in the medical industry. Participants were informed in advance of the required conditions prior to the measurement: no alcohol less than 24 hours before the measurement, no vigorous exercise less than 12 hours prior to the measurement, no food or drink less than 3 hours prior to the measurement, and no urination immediately before the measurement. All measurements were taken simultaneously during the morning in fasting conditions. Weight (kg) and Body Mass Index (BMI) (calculated as weight (kg)/height m^2) measurements were obtained.

2.8. Intake Rating

Dietary nutrient intake was assessed using a manual 24 Hours Recall (24HR) [30], taking into account 1 holiday and 2 non-holidays days, both at baseline and follow-up, which was administered by the interviewer. Recall accuracy was recorded with a set of photographs of prepared foods and dishes that are frequently consumed in Spain. The food intake assessment was converted to both energy and nutrients, determining the adequacy of the macronutrient and micronutrient intake according to the Recommended Dietary Allowances (RDAs) for the female Spanish population within the age range included in our study [32] using the Dietowin software (7.1. version, Barcelona, Spain).

2.9. Sample Treatment

A blood extraction in the morning in fasting conditions was performed at baseline and follow-up, being centrifuged at 4 °C for 15 min at 3,000 rpm to extract the plasma. Once the plasma was removed from the tube, it was frozen at –80 °C until the analytical determination of the different parameters. All samples were measured in one run, in the same assay batch and blinded quality control samples were included in the assay batches to assess laboratory error in the measurements.

PTH (pg/mL) and osteocalcin levels (ng/mL) were measured using EIA by colorimetric method (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany). Vitamin D levels (ng/mL) were measured by LC–MS/MS (Acquity UHPLC System I-Class Waters, Milford, United states of America) [33]. The biochemical values of vitamin D obtained were classified according to the reference

values of 25(OH)D in plasma, being sufficiency > 30 ng/mL, insufficiency 20–30 ng/mL and deficiency < 20 ng/mL for total vitamin D [33]. Total Antioxidant Capacity (TAC) (µmol/L) was measured via the reduction power of Cu^{2+} from the action of antioxidants present in plasma samples (TAC kit, Jaica, Shizuoka, Japan). An enzymatic immunological method (Bioxytech GPx-340™ kit, OxisResearch™, Portland, Oregon, United States of America) was used to determine Glutathione Peroxidase (GPx) (mU/mL) activity. Superoxide Dismutase (SOD) (U/mL) activity was analyzed by the colorimetric method based on cytochrome c reduction using the Ransod kit (RANDOX Laboratories Ltd., Dublin, Ireland, United Kingdom). The remaining biochemical parameters such as glucose (mg/dL), urea (mg/dL), uric acid (mg/dL), creatinine (mg/dL), Triglycerides (TG) (mg/dL), Total Cholesterol (TC) (mg/dL), High Density Lipoprotein (HDL) (mg/dL), Low Density Lipoprotein (LDL) (mg/dL), transferrin (mg/dL), albumin (mg/dL), Homocysteine (Hcy) (µmol/L), and bilirubin (mg/dL), were determined in the Analysis Unit at the Virgen de las Nieves Hospital, Granada (ECLIA, Elecsys 2010 and Modular Analytics E170, Roche Diagnostics, Mannheim, Germany).

2.10. Statistical Analysis

Data were obtained using SPSS 22.0 Software for MAC (SPSS Inc. Chicago, Illinois, USA). GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA) was used for plotting the graphs. Descriptive analysis has been used for data expression, indicating the results of the numerical variables such as arithmetical Mean ± Standard Deviation (X ± SD), and the results of the categorical variables were expressed in Frequencies (%). As a previous step to the execution of a parametric model

or not, the hypothesis of normal distribution was accepted using the Kolmogorov-Smirnov test. For the comparative analysis based on categorical variables, chi square test was used. For the comparative analysis based on baseline and follow-up, the paired t-test for parametric samples was used. For the comparative analysis based on groups, the unpaired t-test for parametric samples was used. Correlation analyses and partial correlation coefficients were performed with Pearson test. A *p* Value less than 0.05 was considered statistically significant.

3. Results

The mean levels of plasma and erythrocyte Mg were 1.9 ± 0.2 (1.7 – 2.6) and 4.0 ± 0.7 (4.2 – 6.7) respectively. Our results showed that 27% of the postmenopausal women were deficient in plasma Mg and 67% were deficient in erythrocyte Mg at the beginning of the study. Given the deficiency justified here, the study population was randomly supplemented with Mg, reaching 0% of Mg deficiency in both groups after intervention.

Table 17 shows the general characteristics of the study population by groups. In both study groups, BMI was above and energy intake was below the reference values. Regarding Ca, 16.7% and 10.7% of the postmenopausal women in PG and MG did not reach two-thirds of the RDAs at baseline. After the intervention, 20.0% of the women in PG and 11.1% of those in MG did not reach 2/3 of the RDA referred to Ca intake. With regard to Mg intake, 41.7% and 46.4% of the women in PG and MG, respectively, were below 2/3 of the RDAs at baseline. Nevertheless, after Mg supplementation, 30% of the postmenopausal women in PG and 100% of those in MG reached more than 2/3 of the RDAs for Mg. In the case of vitamin D intake, our results showed that 87.5% and 81.5% of the postmenopausal women in PG and MG, respectively, were below 2/3 of the vitamin D recommendations. After Mg supplementation, these figures were 75% and 92.6%.

Table 18 shows the biochemical parameters of the study by intervention groups. In both groups, TC was above its reference values, and prealbumin significantly decreased in PG ($p < 0.05$) after the intervention. When using the LC–MS/MS method, the 25(OH)D levels were seen to have increased significantly after the intervention comparing baseline

and follow-up ($p < 0.05$), though the levels were still below the recommended values. However, although an increase in 25(OH)D_3 and 25(OH)D_2 levels was also seen intra (MG) and inter-groups, the results were not statistically significant. Finally, no changes in both PG and MG for antioxidant parameters were observed after Mg supplementation (all $p \geq 0.05$)

Figure 19a shows the distribution of 25(OH)D in the study population at baseline and after Mg intervention. Lesser data dispersion of the 25(OH)D levels was obtained after Mg supplementation when compared with baseline. ***Figure 19b*** shows the percentage of postmenopausal women with different vitamin D statuses by group. We found 80.8% of the study population to initially have vitamin D deficiency as established by LC–MS/MS. After the Mg intervention, the percentage of women in MG lacking in vitamin D decreased by about 20%.

Table 17. General characteristics of the study population by group.

Features	Reference Values	PG (*n* = 25)		MG (*n* = 27)		*p* Value PG Follow-up	*p* Value MG Follow-up	*p* Value Inter-Groups
		Baseline (Mean ± SD)	Follow-up (Mean ± SD)	Baseline (Mean ± SD)	Follow-up (Mean ± SD)			
Sociodemographic								
Age (Years)	–	59.7 ± 9.15	59.7 ± 9.15	57.7 ± 7.58	57.7 ± 7.58	–	–	–
Weight (Kg)	–	69.1 ± 11.0	67.8 ± 11.2	69.3 ± 14.2	69.7 ± 13.6	0.23	0.43	0.62
Height (cm)	–	157.2 ± 6.01	157.2 ± 6.01	160.2 ± 6.11	160.2 ± 6.11	–	–	–
BMI (Kg/m^2)	22.0 – 27.0	28.0 ± 4.30	27.7 ± 4.41	26.9 ± 4.88	27.7 ± 4.41	0.21	0.49	0.65
Blood pressure *n* (%)								
Normal blood pressure	–	10 (40)	–	14 (51)	–	–	–	–
High blood pressure	–	15 (60)	–	13 (49)	–	–	–	–
Physical exercise *n* (%)								
Sedentary	–	9 (36)	–	6 (23)	–	–	–	–
Non-sedentary	–	16 (64)	–	21 (77)	–	–	–	–
Smoking habit *n* (%)								
Non-smoker	–	18 (75)	–	23 (82)	–	–	–	–
Smoker	–	6 (25)	–	5 (18)	–	–	–	–
Educational level *n* (%)								
Basic educational level	–	10 (40)	–	11 (40)	–	–	–	–
Secondary or high educational level	–	15 (60)	–	16 (60)	–	–	–	–
Dietary Intake								
Energy intake (Kcal)	2000.0	1339.5 ± 283.1	1232.9 ± 285.5	1307.1 ± 323.3	1323.4 ± 264.5	0.17	0.66	0.27
CHO intake (g/day)	275.0	146.6 ± 40.3	146.5 ± 33.4	149.5 ± 48.2	150.5 ± 48.5	0.84	0.89	0.75
Protein intake (g/day)	50.0	59.7 ± 14.2	57.1 ± 10.4	61.1 ± 17.6	63.4 ± 15.6	0.47	0.42	0.12

Fat intake (g/day)	70.0	56.1 ± 17.2	47.9 ± 18.5	53.2 ± 14.7	51.8 ± 12.7	0.15	0.66	0.41
Cholesterol intake (mg/day)	< 300.0	150.6 ± 61.6	151.7 ± 64.4	154.1 ± 64.6	158.3 ± 76.8	0.87	0.81	0.75
Fiber intake (g/day)	> 25.0	17.1 ± 10.6	16.2 ± 4.07	15.7 ± 7.72	16.5 ± 7.50	0.42	0.53	0.88
P intake (mg/day)	800.0	996.1 ± 257.7	993.9 ± 219.1	1002.6 ± 318.9	1038.8 ± 282.5	0.86	0.57	0.55
Ca intake (mg/day)	800.0 – 1000.0	728.2 ± 223.1	679.9 ± 168.6	873.5 ± 250.2	832.5 ± 210.6	0.69	0.56	**0.01**
Mg intake (mg/day)	320.0	237.4 ± 87.0	232.7 ± 55.8	219.3 ± 71.1	726.4 ± 59.9	0.99	**0.001**	**0.001**
Vitamin D intake (µg/day)	10.0	3.36 ± 3.00	4.34 ± 2.91	3.89 ± 3.62	3.62 ± 2.57	0.35	0.72	0.57

n = 52. Baseline and follow-up values are expressed as mean ± Standard Deviation (SD). Both for intra-group and inter-groups *p*-value, paired and unpaired t-student test was used. Categorial variables are expressed as continuous as sample size (*n*) and percentage of subjects (%), and chi-square test was used. Significance was set at a *p* Value < 0.05 and is highlighted in boldface. Abbreviations: BMI = Body Mass Index; Ca = Calcium; CHO = Carbohydrates. MG = Magnesium Group; Mg = Magnesium; P = Phosphorous. PG = Placebo Group.

Table 18. Biochemical parameters of the study population by group.

Features	Reference Values	PG (*n* = 25)		MG (*n* = 27)		*p* Value PG Follow-up	*p* Value MG Follow-up	*p* Value Inter-groups
		Baseline (Mean ± SD)	Follow-up (Mean ± SD)	Baseline (Mean ± SD)	Follow-up (Mean ± SD)			
Glucose (mg/dL)	70.0 – 110.0	96.0 ± 19.8	95.8 ± 19.9	90.1 ± 11.1	95.8 ± 18.9	0.63	0.25	**0.047**
Transferrin (mg/dL)	200.0 – 360.0	285.9 ± 39.7	272.8 ± 43.4	284.2 ± 47.7	272.8 ± 43.4	0.89	0.18	0.71
Prealbumin (mg/dL)	20.0 – 40.0	26.6 ± 5.03	24.7 ± 5.01	24.8± 6.45	24.7± 5.01	**0.001**	0.06	0.62
Albumin (mg/dL)	3.50 – 5.20	4.50 ± 0.20	4.45 ± 0.27	4.50 ± 0.21	4.45 ± 0.27	0.62	0.055	0.57
Homocysteine (µmol/L)	< 13.0	12.5 ± 6.45	12.7 ± 4.78	11.5 ± 4.25	12.7 ± 4.78	0.27	0.66	0.46
Creatinine (mg/dl)	0.50 – 0.90	0.75 ± 0.16	0.75 ± 0.16	0.68 ± 0.11	0.75 ± 0.16	0.31	0.81	0.08
Total bilirubin (mg/dL)	0.10 – 1.20	0.49 ± 0.13	0.55 ± 0.21	0.47 ± 0.11	0.55 ± 0.21	0.13	0.95	0.26
LDH (U/L)	110.0 – 295.0	182.8 ± 29.3	181.1 ± 26.1	192.5 ± 26.7	181.1 ± 26.1	0.96	0.41	0.81
Urea (mg/dL)	10.0 – 50.0	36.2 ± 10.2	36.7 ± 9.35	34.3 ± 8.98	36.7 ± 9.35	0.87	0.65	0.44
Uric acid (mg/dL)	2.40 – 5.70	4.51 ± 0.98	4.70 ± 1.02	4.43 ± 1.23	4.70 ± 1.02	0.21	0.26	0.21
Triglycerides (mg/dL)	50.0 – 200.0	115.8 ± 68.9	112.3 ± 62.2	111.1 ± 50.6	112.3 ± 62.2	0.79	0.41	0.80
HDL (mg/dL)	40.0 – 60.0	62.6 ± 11.2	64.1 ± 12.3	66.6 ± 14.4	64.1 ± 12.3	**0.04**	0.06	0.95
LDL (mg/dL)	70.0 – 190.0	134.5 ± 35.3	137.6 ± 30.5	130.4 ± 26.4	137.6 ± 30.5	0.76	0.06	0.19
Total cholesterol (mg/dL)	110.0 – 200.0	224.1 ± 39.7	221.4 ± 31.4	224.7 ± 30.1	221.4 ± 31.4	0.86	0.09	0.64
Osteocalcin (ng/mL)	15.0 – 46.0	17.4 ± 9.45	18.1 ± 7.21	16.8 ± 10.4	18.1 ± 7.21	0.69	0.22	0.91
PTH (pg/mL)	20.0 – 70.0	50.7 ± 15.8	53.3 ± 34.4	52.9 ± 17.2	53.3 ± 34.4	0.46	0.07	0.29
Ca (mg/dL)	8.60 – 10.2	9.31 ± 0.31	9.14 ± 0.44	9.27 ± 0.51	9.13 ± 0.49	0.31	0.07	0.98
P (mg/dL)	2.70 – 4.50	3.45 ± 0.45	3.57 ± 0.55	3.42 ± 0.53	3.60 ± 0.49	0.49	0.07	0.88

25(OH)D (ng/mL)	30.0 – 100.0	23.0 ± 8.99	24.2 ± 7.71	23.6 ± 5.70	27.8 ± 7.56	0.81	**0.049**	0.14
25(OH)D_3 (ng/mL)	> 20	18.0 ± 8.37	19.7 ± 8.00	17.7 ± 6.25	21.1 ± 7.40	0.52	0.13	0.57
25(OH)D_2 (ng/mL)	> 10	4.99 ± 2.11	4.55 ± 2.74	5.86 ± 3.05	6.80 ± 7.16	0.31	0.41	0.22
TAC (µmol/L)	1500.0	1453.7 ± 364.6	1559.5 ± 423.7	1508.9 ± 471.5	1535.1 ± 466.4	0.152	0.107	0.586
GPX (U/mL)	120.0	115.4 ± 60.9	119.4 ± 43.5	108.7 ± 35.4	94.6 ± 47.4	0.407	0.102	0.073
SOD (U/mL)	164.0 – 240.0	178.9 ± 29.7	181.1 ± 29.1	187.4 ± 38.6	179.2 ± 32.3	0.677	0.241	0.837

n = 52. Baseline and follow-up values are expressed as mean ± Standard Deviation (SD). For intra-group and inter-groups *p* Value, paired and unpaired t-student test were used respectively. Significance was set at a *p* Value < 0.05 and is highlighted in boldface. Abbreviations: Ca = Calcium; HDL = High density lipoprotein; LDH = Lactate dehydrogenase; LDL = Low density lipoprotein. MG = Magnesium Group; P = Phosphorous; PG = Placebo Group; PTH = Parathyroid hormone; 25(OH)D = 25-Hydroxyvitamin D; 25(OH)D_2 = 25-Hydroxyvitamin D_2; 25(OH)D_3 = 25-Hydroxyvitamin D_3.

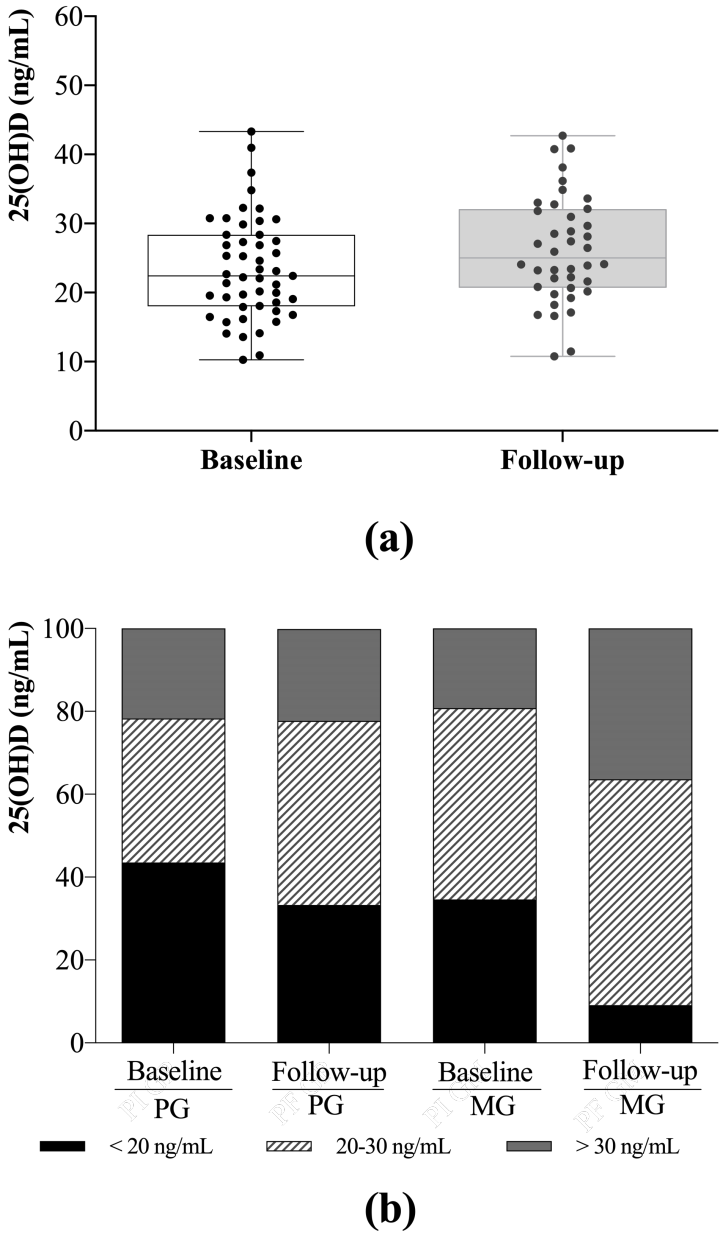

Figure 19. Levels of vitamin D in the evolutive study and by groups. (**a**) Data distribution of 25(OH)D levels at baseline and follow-up. (**b**) Vitamin D status in the study population by groups. Abbreviations: MG = Magnesium Group; PG = Placebo Group; 25(OH)D = 25-Hydroxyvitamin D.

4. Discussion

Previous scientific evidence indicates that postmenopausal women are at risk of suffering numerous micronutrient deficiencies. However, although vitamin D and Mg could be candidates for deficiency in this population, there is currently not enough evidence of the interaction between them. On the other hand, a large part of our study population was deficient in vitamin D as evidenced by LC–MS/MS and the vitamin D status was seen to improve in MG after the Mg intervention.

Authors such as Rosanoff et al. [34] have affirmed that western populations (including Spain) are characterized by a low intake of Mg, since the latter is a predominant mineral in vegetables, and current consumption trends are towards an increased intake of animal products. Our results evidenced a pattern of low Mg consumption below the RDAs, with the exception of the MG population following the Mg intervention. In our study, Ca intake was seen to be within the recommended range for postmenopausal women. This is in contradiction to the findings of another study conducted in Spanish postmenopausal women, in which Ca intake fell short of the RDA. However, the data coincided with our own observation of vitamin D intakes below 50% of the RDA [35]. Another study involving a sample of 144 African women, in which dairy product consumption was lower, found that over 90% of the menopausal women analyzed failed to reach the RDA for Ca [36]. On the other hand, it should be noted that vitamin D intake in menopause is very low, as evidenced by studies such as that published by Rizzoli et al. [37], in which vitamin D intake among most postmenopausal women was seen to be very low in 9 European countries. This is consistent with our own study, where most of the women failed to reach the RDA corresponding to vitamin D.

In the present study, Mg intake showed a significant correlation ($r = 0.451$; $p = 0.03$) to the 25(OH)D levels. Authors such as Deng et al. [38] have argued that Mg intake is inversely proportional to 25(OH)D deficiency, independently of whether Mg is administered alone or in combination with vitamin D. This association was suggested to be due to the close relationship between Mg and vitamin D metabolism. Moreover, in our population, Mg and Ca intake were directly correlated ($r = 0.689$; $p = 0.001$). Authors such as Olza et al. [39] have mentioned the fact that Mg and Ca intakes are based fundamentally on 2 food groups, namely cereals and dairy products, which are among the most widely consumed products in the Spanish population. Other authors such as Al-Musharaf et al. [40] have found vitamin D status to improve with the intake of Ca, as many food rich in Ca also have high a content of vitamin D. Nevertheless, we found no significant association between them, as well as no correlation to vitamin D intake, as this was too low – presenting high deficiency levels in almost all the women analyzed –. These results could be explained by the data from studies such as that of Harris et al. [41], indicating that such correlations with vitamin D intake cannot be made, since its contribution depends on other elements such as genetic factors, as well as on exposure to the sun.

Vitamin D levels are deficient in a large percentage of the population (***Figure 19***), and this pattern is observed in all parts of the world and at any latitude [42]. In our study, a large percentage of the population presented 25(OH)D levels below the references values when analyzed by LC–MS/MS. Authors such as Park et al. [43] analyzed 25(OH)D levels by LC–MS/MS in a population of postmenopausal women and recorded the same high prevalence of vitamin D deficiency (82%) as in our study. This confirmed that despite use of the gold standard for the analytical

determination of vitamin D, the levels of this vitamin were low. Schmitt et al. [44] studied 25(OH)D levels by EIA among 463 postmenopausal women and found only 32% of them to have sufficient levels. Vitamin D deficiency therefore is a generalized finding in the postmenopausal population, with high deficiency levels being reported by both chromatographic and immunological laboratory test methods.

Several authors [38,45–47] have reported Mg to exert synergic action with vitamin D, placing special emphasis on the effect of Mg upon the vitamin D Binding Protein (DBP), as well as on the enzymes that mediate in the hydroxylation of vitamin D in the liver and kidney. Thus, a high intake of either dietary or supplemented Mg could lessen the risk of vitamin D deficiency. According to our results, there was a significant increase in 25(OH)D levels after Mg supplementation ($p < 0.05$) when analyzed by LC–MS/MS. However, authors such as Melamed et al. [48] have pointed out that the administration of Mg supplements does not increase the 25(OH)D levels, despite the fact that Mg has a direct relationship with vitamin D metabolism. This could be explained by considering that if a population has very low vitamin D levels, the supplemented Mg might not be able to mediate the hydroxylation of enough vitamin D to improve vitamin D status. Hence, Mg supplementation is usually recommended together with vitamin D, advising Mg in greater proportion than vitamin D, in order to prevent all the Mg from being depleted by the hydroxylation of vitamin D [49].

On considering the results referred to vitamin D obtained with LC–MS/MS when comparing baseline with follow-up, different values were observed (***Figure 19***) according to the level of deficiency with respect to the reference values. The LC–MS/MS technique provided data

indicating higher vitamin D deficiency at baseline compared with the data obtained ($p < 0.05$) in MG after the intervention. Granado-Lorencio et al. [50] studied the 25(OH)D levels of 32,363 general population samples using the EIA method and suggested that the results obtained were unable to predict vitamin D deficiency, since the technique usually underestimates vitamin status, and even more so when 25(OH)D is present in low amounts. Other authors such as Klapkova et al. [51] consider that different methods other than LC–MS/MS likewise underestimate vitamin D status, thus affecting clinical decision making. Nikooyeb et al. [26] analyzed 275 general population serum samples using different methods and argued that although the chromatographic techniques are the gold standard for the laboratory test determination of vitamin D, the results are comparable, since there are no major differences among the techniques. However, other authors such as Garg et al. [52] stated that although the differences in results obtained by the various vitamin D analytical methods have been reduced in recent years, it is advisable to use chromatographic techniques until full harmonization of the analytical methods for vitamin D is achieved. To date, the immunochemical methods have not been able to match the precision and specificity of the chromatographic techniques [53]. Accordingly, LC–MS/MS would be a more appropriate method in this scenario, since the results exhibit less dispersion, and do not usually underestimate the values, in coincidence with Atef et al. [12], who found the LC–MS/MS technique to estimate within normality ranges in the studied adult subjects.

In addition to the described findings, the present study has some strengths and limitations. As strengths, the study is a randomized, Pb-controlled study in which nutritional intake of energy, macronutrients and related Mg and vitamin D minerals were controlled at baseline and

follow-up. In this regard, we found that nutritional intake and high compliance to supplementation remained stable during the nutritional intervention. The present study used LC–MS/MS which is the gold standard analytical method, offering greater sensitivity, flexibility, and specificity [18]. Despite it, this study has some limitations that should be considered as the small sample size. Although initially 82 women were randomly assigned to be supplemented, a total of 52 postmenopausal women completed the study (***Figure 18***). Although the primary outcome of the trial was to assess the influence of a Mg diet strategy on vitamin D status in postmenopausal women, the sample size in each group would allow us to preliminarily obtain significant results, although the results should be carefully considered. Clinical trials of the same nature and a similar sample size have shown a positive effect of different interventions on some parameter's status in postmenopausal women [54–56]. Likewise, the sample size limitation did not allow us to make a more complex statistical approach since we did not have enough power to perform multivariate analyses and to be able to adjust our model based on possible confounding variables such as previously described age or BMI.

5. Conclusions

Mg supplementation in the postmenopausal women of our study had a significant positive impact upon their vitamin D status. Most of the postmenopausal population presented inadequate plasma 25(OH)D levels. Future studies are needed to shed light on the vitamin D status of this risk population and to define protocols and strategies such as Mg intervention in postmenopausal women, with a view to improving their health and quality of life.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Granada (protocol code 149/CEIH/2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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13. LIMITACIONES

Los resultados de la presente tesis doctoral deben ser interpretados con precaución debido a las siguientes limitaciones:

- Un total de 3 de los 7 estudios que fueron incluidos presentan un diseño de tipo transversal, y, por tanto, no se puede establecer una relación causal a través de ellos.
- La población del estudio se limitó a mujeres posmenopáusicas de edades comprendidas entre (44-76 años), de raza caucásica, de una zona específica del sur de España, por lo que estos resultados pueden no ser generalizables a mujeres posmenopáusicas de diferentes regiones, razas o con edades no incluidas en nuestro rango de edad.
- Se reclutaron menos participantes de los deseados para estudiar los objetivos de estudio y, por tanto, el tamaño de la muestra pudo ser un factor limitante para la potencia estadística y la obtención resultados estadísticamente significativos.
- No se midieron otros biomarcadores de estatus de Zn y Mg, la forma activa de la vitamina D y otras hormonas, que podrían haber complementado el estudio.
- No disponíamos de información sobre el tiempo exacto de menopausia, ni se reportaron los síntomas a corto plazo de las mujeres del estudio.

14. CONCLUSIONES

14.1. Conclusión General

- La evaluación del estatus clínico-nutricional evidenció una mayor alteración de parámetros bioquímicos en aquellas mujeres que presentaron un peor perfil físico. La suplementación corrigió la deficiencia eritrocitaria de ambos minerales y de Mg a nivel plasmático en casi la totalidad de la población, afectando en menor medida a los niveles plasmáticos de Zn. La vitamina D fue el parámetro que más se vio afectado por la suplementación, mejorando su estatus en ambos grupos de intervención, efecto que no se observó para la defensa antioxidante.

14.2. Conclusiones Específicas

Al comienzo de la intervención

- Se detectó una ingesta hipocalórica acompañada de una elevada inadecuación de ingesta en Zn, Mg, y vitamina D. El IMC y la MG fueron las variables físicas más alteradas, relacionándose dichas alteraciones con algunas de las observadas en ciertos parámetros bioquímicos.

- Se reflejó un elevado porcentaje de deficiencia en Zn y Mg plasmático y eritrocitario, así como en la vitamina D, mientras que las mujeres posmenopáusicas presentaron una adecuada defensa antioxidante.

- En las mujeres posmenopáusicas del estudio se evidenció un peor perfil físico a medida que disminuían su estatus de vitamina D y su defensa antioxidante, la cual se vio adicionalmente afectada por parámetros del metabolismo proteico, observándose que la adecuación de ingesta estuvo influida por la edad.

Después de la intervención

- Los parámetros bioquímicos mejoraron en ambos grupos de intervención, sin evidenciarse cambios reseñables respecto al perfil dietético y físico.

- A diferencia del Zn plasmático, la deficiencia inicial de Mg plasmático y de Zn y Mg eritrocitarios se corrigió en la mayoría de las mujeres estudiadas. El estatus de vitamina D_3 y vitamina D total mejoraron con las intervenciones en Zn y Mg, respectivamente, efecto que no fue observado para la defensa antioxidante.

- La intervención con Zn mejoró otros parámetros clínico-nutricionales relacionados. Además, el Zn eritrocitario se correlacionó positivamente con parámetros de estatus antioxidante. La falta de asociación entre el Mg y las variables estudiadas confirmarían al Zn como el mineral que mayor relación guardó con una mejora del estado nutricional la mujer posmenopáusica.

- Finalmente, según los resultados obtenidos, serían necesarias intervenciones a largo plazo con un tamaño muestral más grande y heterogéneo incluyendo adicionalmente un grupo de cosuplementación y analizando más biomarcadores de estatus nutricional, con el fin de establecer programas de prevención y corrección de un posible estado nutricional inadecuado en situación de menopausia.

15. CONCLUSIONS

15.1. General Conclusion

- The evaluation of the clinical-nutritional status showed a greater alteration of biochemical parameters in those women with a poorer physical profile. The supplementation corrected erythrocyte deficiency of both minerals and plasma Mg levels in almost the entire population with plasma Zn levels being affected to a lesser extent. Vitamin D was the most influenced parameter by supplementation, improving its status in both intervention groups, an effect that was not observed for antioxidant defense.

15.2. Specific Conclusions

Before intervention

- A hypocaloric intake accompanied by a high inadequacy of Zn, Mg and vitamin D intake was detected. BMI and FM were the most altered physical variables, and these alterations were related to some of those observed in certain biochemical parameters.
- A high percentage of deficiency in plasma and erythrocyte Zn and Mg, as well as vitamin D, was found, whereas an adequate

antioxidant defense status was seen in the postmenopausal women.

- The postmenopausal women of the study showed a worse physical profile as their vitamin D status and antioxidant defense declined, which was additionally affected by parameters of protein metabolism, the intake adequacy being found to be influenced by age.

After intervention

- Several biochemical parameters improved in both intervention groups, with no notable changes in dietary and physical profile.
- In contrast to plasma Zn, the initial deficiency of plasma Mg and erythrocyte Zn and Mg was corrected in most of the women studied. Vitamin D_3 and total vitamin D status improved with Zn and Mg interventions, respectively, an effect that was not observed for antioxidant defense.
- Zn intervention improved other related clinical-nutritional parameters. In addition, erythrocyte Zn correlated positively with antioxidant status parameters. The lack of association between Mg and the variables studied would confirm Zn as the mineral which is most strongly related to an improved nutritional status in postmenopausal women.

- Finally, according to the results obtained, long-term interventions with a larger and more heterogeneous sample size including additionally a co-supplementation group and analyzing nutritional status related biomarkers would be necessary, in order to establish programs for prevention and correction of a possible inadequate nutritional status during menopause stage.

16. PERSPECTIVAS FUTURAS

A raíz de los resultados de la presente tesis doctoral, se proponen una serie de perspectivas futuras que podrían completar más el estudio y ayudar a entenderlo desde un punto de vista más integral:

- Sería interesante reproducir nuestro estudio con un tamaño muestral más grande con la suficiente potencia estadística para valorar si hubieran existido cambios significativos en otros parámetros tras la intervención.
- Son necesarios estudios que evalúen un grupo más de suplementación simultánea Zn y Mg, valorando un posible efecto sinérgico entre ambos minerales sobre diversos parámetros.
- Se requieren investigaciones que comparen los hallazgos de este estudio con mujeres premenopáusicas, bajo THS o menopausia artificial, para entender mejor el funcionamiento de la menopausia.
- Sería conveniente evaluar el efecto de la intervención con suplementación oral de los minerales del estudio más a largo plazo y compararlo con los efectos observados a corto plazo, ayudando a esclarecer un protocolo idóneo de suplementación.

17. ANEXOS

17.1. ANEXO I

Consentimiento Informado

Para satisfacción de los Derechos del Paciente, como instrumento favorecedor del correcto uso de los Procedimientos Diagnósticos y Terapéuticos, y en cumplimiento de la Ley General de Sanidad.

Yo, D/Dña....., como participante candidato/a (o D./Dña.....DNI…….....,
como su representante), en pleno uso de mis facultades, libre y voluntariamente, EXPONGO:

Que he sido debidamente INFORMADO/A por D./Dña....., en entrevista personal realizada el día...../...../....., de que es conveniente que se me efectúe la valoración del estado nutricional. Que he recibido explicaciones tanto verbales como escritas, sobre la naturaleza y propósitos del procedimiento, beneficios, riesgos, y medios con que cuenta la UGR para su realización, habiendo tenido ocasión de aclarar las dudas que me han surgido.

ANEXO I

MANIFIESTO Que he entendido y estoy satisfecho de todas las explicaciones y aclaraciones recibidas sobre el proceso médico citado Y OTORGO MI CONSENTIMIENTO para que me sea realizada la valoración del estado nutricional,

Entiendo que este consentimiento puede ser revocado por mí en cualquier momento antes de la realización del procedimiento.

Y, para que así conste, firmo el presente documento Granada, a….de…..de…….

Firma del paciente y Nº D.N.I.

Firma del investigador Informante (O su representante legal en caso de incapacidad) y Nº D.N.I.

En caso de negativa por parte del paciente a firmar el consentimiento.

Firma del testigo. D.N.I.

17.2. ANEXO II

Información para la Firma del Consentimiento Informado

Si usted participa de este estudio, será sometido a mediciones que permitirán conocer su situación, se evaluará la alimentación que recibe actualmente, valorando la adecuación de la misma.

También se estudiará, dentro de la analítica que se le realizará diariamente, indicadores bioquímicos de minerales para lo que se necesita obtener una cantidad extra de la que se le extraerá rutinariamente.

Estos procedimientos no suponen ningún riesgo para su estado clínico actual, no se realizará ninguna intervención medicamentosa ni nutricional derivada de este estudio.

Los resultados obtenidos nos permitirán conocer mejor la situación clínica-nutricional que presenta. Su participación contribuirá enormemente a ampliar los conocimientos científicos que se tienen hasta ahora y posibilitará mejorar la calidad del tratamiento brindado.

Si en algún momento quiere abandonar el estudio, podrá hacerlo libremente y sin perjuicio de tratamiento en el futuro.

17.3. ANEXO III

Currículum Vitae Abreviado

ESTUDIOS

***30/10/2019-28/10/2023:**

- ✓ Contratado FPU

***01/10/2018-30/06/2019:**

- ✓ Máster en Nutrición Humana. Facultad de Farmacia. Granada. Universidad de Granada.

***15/09/2014-30/06/2018:**

- ✓ Graduado en Nutrición Humana y Dietética (Premio extraordinario). Facultad de Farmacia. Granada. Universidad de Granada.

MENTORIZACIONES

- ✓ **Trabajo Fin de Máster:** Valoración de ingesta de nutrientes y hábitos alimentarios en población adulta con necesidades especiales, defendido en la convocatoria ordinaria de 2021 al ponente José Enrique Muñoz-Polanco Gómez. Tutora: Elena María Planells del Pozo. Mentor: Héctor Vázquez Lorente

PROYECTOS FINANCIADOS

- ✓ Colaborador/miembro del equipo de trabajo de un proyecto financiado. **Tipo:** Proyectos de I+D+i en el marco del Programa Operativo FEDER Andalucía Referencia: A-CTS-708-UGR20. **Título:** Respuesta a la intervención con N-acetilcisteína y otros antioxidantes, y estudio de biomarcadores pronóstico durante la estancia en UCI, en paciente crítico por COVID-19. **Organismo:** Consejería de Economía, Conocimiento, Empresas y Universidades. Junta de Andalucía. **Investigador responsable:** Elena María Planells del Pozo. **Subvención:** 25.000 €. **Periodo de participación:** desde el 01/07/2021 hasta el 30/06/2023.

PUBLICACIONES

***NOTA*:** Las publicaciones de la presente Tesis Doctoral están sombreadas en color negro mientras que aquellas realizadas durante las estancias en el extranjero están en cursiva.

* = Autor de correspondencia

† = Contribuyó de forma equitativa.

1) **Vázquez-Lorente H†, Herrera-Quintana L†*, Molina-López J*, López-González B, Planells E. Sociodemographic, Anthropometric, Body Composition, Nutritional, and Biochemical Factors Influenced by Age in a Postmenopausal Population: A Cross-Sectional Study. Metabolites. 2023 Jan 3;13(1):78. doi: 10.3390/metabo13010078. PMID: 36677003.**
2) Herrera-Quintana L†, Vázquez-Lorente H†*, Carranco Romo MJ, Flores Buitrón EP, Molina-López J*, Moya MT, Planells E. Imbalanced dietary patterns, anthropometric, and body composition profiles amongst adults with Down syndrome. Nutr Neurosci. 2022 Dec 29:1-10. doi: 10.1080/1028415X.2022.2161139. PMID: 36579765.
3) Gamarra-Morales Y*, Molina-López J*, Machado-Casas JF, Herrera-Quintana L, Vázquez-Lorente H, Castaño-Pérez J, Perez-Villares JM, Planells E. Influence of Nutritional Parameters on the Evolution, Severity and Prognosis of Critically Ill Patients with COVID-19. Nutrients. 2022 Dec 16;14(24):5363. doi: 10.3390/nu14245363. PMID: 36558522.
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9) Herrera-Quintana L, Vázquez-Lorente H, Molina-López J*, Gamarra-Morales Y, Planells E*. Selenium Levels and Antioxidant Activity in Critically Ill Patients with Systemic Inflammatory Response Syndrome. Metabolites. 2022 Mar 22;12(4):274. doi: 10.3390/metabo12040274. PMID: 35448461.

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17) **Vázquez-Lorente H, Molina-López J*, Herrera-Quintana L, Gamarra-Morales Y*, López-González B, Planells E. Association between Body Fatness and Vitamin D_3 Status in a Postmenopausal Population. Nutrients. 2020 Feb 29;12(3):667. doi: 10.3390/nu12030667. PMID: 32121398; PMCID: PMC7146150.**

18) Vázquez-Lorente H, Herrera-Quintana L, Quintero-Osso B, Molina-López J, Planells E*. Current trends in the analytical determination of vitamin D. Nutr Hosp. 2019 Dec 26;36(6):1418-1423. English. doi: 10.20960/nh.02713. PMID: 31657612.

CONGRESOS

- ✓ Vázquez-Lorente H, Herrera-Quintana L, Molina López J, Gamarra-Morales Y, Lobo- Támer G, Planells del Pozo E. Relación entre la edad y los niveles de vitamina D en una población de mujeres posmenopáusicas. “37 Congreso Nacional SENPE”. Granada (España), 2022.
- ✓ Vázquez-Lorente H, Herrera-Quintana L, Molina-López J, Gamarra-Morales Y, Planells E. Influencia de la edad sobre parámetros proteicos en una población postmenopáusica. "XXVI Jornadas Internacionales de Nutrición Práctica y XV Congreso Internacional de SEDCA 2022". Madrid (España), 2022.
- ✓ Vázquez-Lorente H, Gamarra-Morales Y, Molina-López J, Lobo-Támer G, Planells E. Influencia de la suplementación con zinc en los niveles de colesterol HDL en una población de mujeres posmenopáusicas. “"XXXVI Congreso Nacional SENPE". Madrid (España), 2021.

- ✓ Vázquez-Lorente H, Herrera-Quintana L, Gamarra-Morales Y, Molina-López J, Planells E. Evaluación del estatus de vitamina D en mujeres postmenopáusicas suplementadas con zinc. "XXV Jornadas Internacionales de Nutrición Práctica 2020". Virtual. Madrid (España), 2021.
- ✓ Vázquez-Lorente H, Gamarra-Morales Y, Herrera-Quintana L. Valoración del estatus de magnesio en una población posmenopáusica de la provincia de Granada. "IV congreso internacional de intervención e investigación en salud. Murcia (España), 2021.
- ✓ Vázquez-Lorente H, Herrera-Quintana L, Gamarra J, Molina-López J, Planells E. Valoración del estatus de zinc en una población posmenopáusica de la provincia de Granada. "XXIV Jornadas Internacionales de Nutrición Práctica 2020". Centro de Conferencias Fundación Pablo VI. Madrid (España), 2020.
- ✓ Vázquez-Lorente H, López-González B, Molina-López J, Herrera-Quintana L, Domínguez-García A, Lobo-Támer G, Planells E. Capacidad antioxidante de mujeres posmenopáusicas suplementadas con cinc. "XXXIV Congreso Nacional SENPE". Galicia (España), 2019.

- ✓ Vázquez-Lorente H, Herrera-Quintana L, Gamarra-Morales J, Domínguez-García A, Molina-López J. Mujer posmenopáusica y magnesio. ¿es necesaria la suplementación? "XXIII Jornadas Internacionales de Nutrición Práctica 2019 y XII Congreso Internacional de Nutrición, Alimentación y Dietética". Centro de Conferencias Fundación Pablo VI. Madrid (España) 2019.

CAPÍTULOS DE LIBRO

- ✓ Vázquez-Lorente H, Gamarra-Morales Y, Herrera-Quintana L. Mejora del estatus de vitamina D tras una intervención a corto plazo con magnesio en una población de mujeres posmenopáusicas. Publicado en el libro titulado Intervención para la mejora de la salud desde una perspectiva integradora. nuevos contextos y necesidades, editado por ASUNIVEP con número de ISBN: 978-84-09-27605-9. Capítulo 16, páginas 111-116.

BECAS

***2018-2019:**

- ✓ Beca de Colaboración para primer curso de Máster Oficial. (MEC-UGR)

***2017-2018 Granada:**

- ✓ Beca de Iniciación a la Investigación para estudiantes de Grado. Plan propio de investigación y transferencia. Universidad de Granada.

PREMIOS

- ✓ Premio UGR-Caja Rural a la excelencia en el rendimiento académico 2019. Sede de Caja Rural. Granada.
- ✓ 1º Premio Extraordinario Fin de Carrera en el Grado en Nutrición Humana y Dietética. Promoción 2014-2018. Facultad de Farmacia. Universidad de Granada.

ACTIVIDADES FORMATIVAS

- ✓ Asistencia a la actividad formativa "Investigación, innovación, propiedad intelectual y transferencia del conocimiento", impartida por José Antonio Morales Molina y Beatriz Clares Naveros de la Universidad de Granada dentro de las actividades formativas de la Escuela de Doctorado de Ciencias de la Salud y celebrada entre el 17 y el 20 de enero de 2023 con una duración de 20 horas presenciales, de las cuales ha asistido a 20, obteniendo la calificación de Sobresaliente.

- ✓ Asistencia a la actividad formativa “Diseños y análisis experimentales avanzados” impartida por Francesco del Petre (Centro de Investigación Mente Cerebro y Comportamiento) dentro de las actividades formativas de la Escuela de Doctorado de Ciencias de la Salud, celebrada del 14 al 18 de marzo de 2022 con una duración de 20 horas presenciales.
- ✓ Asistencia a la actividad formativa “Estrategias para optimizar la escritura, publicación y comunicación de artículos científicos” impartida por Jonatan Ruiz Ruiz y Francisco B. Ortega Porcel de la Universidad de Granada, dentro de las actividades formativas de la Escuela de Doctorado de Ciencias de la Salud, celebrada en las fechas del 11 al 18 de marzo de 2021 con una duración de 20 horas presenciales en formato virtual y 5 horas no presenciales.
- ✓ Asistencia a la actividad formativa “Revisión Sistemática”, impartida por Carmina Wanden-Berghe (Instituto de Investigación Sanitaria y Biomédica de Alicante (ISABIAL) y Javier Sanz-Valero (Instituto de Salud Carlos III, Escuela Nacional de Medicina del Trabajo, Madrid) dentro de las actividades formativas de la Escuela de Doctorado de Ciencias de la Salud, celebrada en las fechas de 28, 29 y 30 de enero de 2021 con una duración de 20 horas.

- ✓ Asistencia a la actividad formativa del Programa de Doctorado en Nutrición y Ciencias de los Alimentos denominada “Técnicas estadísticas básicas en el ámbito de la nutrición y de la salud” curso 2019-20, realizada en la Facultad de Farmacia de la UGR, del 14 al 16 y del 21 al 23 de enero de 2020.
- ✓ Asistencia a la actividad formativa del Programa de Doctorado en Nutrición y Ciencias de los Alimentos denominada “Diseños y análisis experimentales básicos” curso 2019-20, realizada en la Facultad de Farmacia de la UGR, del 13 al 15 de enero de 2020.
- ✓ Asistencia a la actividad formativa del Programa de Doctorado en Nutrición y Ciencias de los Alimentos denominada “Jornada de bienvenida e informativa a doctorandos/as’’ curso 2019-20, realizada en la Facultad de Farmacia de la UGR, el día 21 de enero de 2020.
- ✓ Participación en las IV jornadas de iniciación a la docencia universitaria para contratados/as predoctorales FPU y FPI. Organizado por la Unidad de Calidad, Innovación Docente y Prospectiva de la Universidad de Granada, desde el 21 de noviembre de 2019 al 22 de noviembre de 2019, con una duración de 20 horas.

ESTANCIAS

- ✓ **Financiada.** Ayudas complementarias de movilidad destinadas a beneficiarios del programa de Formación del Profesorado Universitario (FPU). Estancias Breves. **Lugar:** Universidad Mendel en Brno, Brno, Republica Checa. **Fechas:** 01/09/2022 – 30/11/2022. **Ranking Mundial Webometrics (2022):** 1479. **Ranking Continental Webometrics:** 563. **Ranking por país:** 11. **Impacto:** 2214. **Apertura:** 2156. **Excelencia:** 1623
- ✓ **Financiada.** Ayudas complementarias de movilidad destinadas a beneficiarios del programa de Formación del Profesorado Universitario (FPU). Estancias Breves. **Lugar:** Universidad de Belgrado, Belgrado, Serbia. **Fechas:** 22/09/2021 – 21/12/2021. **Ranking Mundial Webometrics (2021):** 451. **Ranking Continental Webometrics:** 187. **Ranking por país:** 1. **Impacto:** 958. **Apertura:** 582. **Excelencia:** 353

DOCENCIA

2022-2023:

- ✓ Experiencia en docencia por grado, asignatura, facultad e institución (3 créditos):
    - Grado en Farmacia:

- Teoría: Fisiología Celular y Humana 2, Facultad de Farmacia, UGR (1,5 créditos)
- Prácticas: Fisiología Celular y Humana 2, Facultad de Farmacia, UGR (0,75 créditos)

- Grado en Logopedia:
  - Prácticas: Fisiología de los Órganos de la Audición, Habla y Voz, Facultad de Psicología, UGR (0,75 créditos)

2021-2022:

- ✓ Experiencia en docencia por grado, asignatura, facultad e institución (3 créditos):
  - Grado en Farmacia:
    - Teoría: Fisiología Celular y Humana 2, Facultad de Farmacia, UGR (1,5 créditos)
    - Prácticas: Fisiología y Bioquímica Clínica, Facultad de Farmacia, UGR (1,5 créditos)

2020-2021:

- ✓ Experiencia en docencia por grado, asignatura, facultad e institución (6 créditos):
  - Grado en Farmacia:

- Teoría: Fisiología Celular y Humana, Facultad de Farmacia, UGR (1 créditos)
- Teoría: Fisiología Celular y Humana 2, Facultad de Farmacia, UGR (2 crédito)
- Prácticas: Fisiopatología, Facultad de Farmacia, UGR (0,14 créditos)
- Prácticas: Fisiología y Bioquímica Clínica, Facultad de Farmacia, UGR (0,20 créditos)

o Grado en Ciencias de la Actividad Física y del Deporte:

- Prácticas: Fisiología Humana, Facultad de Ciencias del Deporte, UGR (2,45 créditos)

o Grado en Ciencia y Tecnología de los Alimentos:

- Prácticas: Fisiología Celular y Humana, Facultad de Farmacia, UGR (0,21 créditos)

2019-2020:

✓ Experiencia en docencia por grado, asignatura, facultad e institución (6 créditos):

o Grado en Bioquímica:

- Prácticas: Fisiología Molecular de Animales, Facultad de Ciencias, UGR (1,36 créditos)

o Grado en Nutrición Humana y Dietética:

- Teoría: Fisiología Humana, Facultad de Farmacia, UGR (1,25 créditos)
- Prácticas: Fisiología Humana, Facultad de Farmacia, UGR (2,64 créditos)

- Grado en Ciencias de la Actividad Física y del Deporte:
  - Prácticas: Fisiología Humana, Facultad de Ciencias del Deporte, UGR (0,75 créditos)