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EDITORIAL
Importancia de las revisiones sistemáticas y metanálisis en la era de la información digital.

El aumento contínuo del número de publicaciones científicas y la proliferación de revistas en el área de biomedicina, favorecida por la modalidad del formato electrónico, han derivado en una abrumadora avalancha de información científica en todos los ámbitos, ocasionando que los especialistas en el área de la salud se enfrenten a un gran reto constante para seleccionar la información de mayor importancia y relevancia en su campo específico, y lograr "estar al día" invirtiendo el menor tiempo posible. Con el advenimiento de la *Web,* entre otras herramientas basadas en internet, se creó un espacio informativo sin fronteras geográficas ni físicas, con acceso a la información de forma inmediata y sin restricciones económicas, en algunos casos, pero con la desventaja que podría no ser la ideal para la toma de decisiones en el área de la salud. La introducción y aceptación universal de varias modalidades de publicación como "*preprint*", que permite la divulgación inmediata de un artículo reciente o incluso de datos preliminares antes de la revisión formal por pares, así como "*open access*", que permite acceso a la información sin requerimientos de registro o suscripción alguna, ha contribuido aún más a la gran cantidad de datos médicos que surgen diariamente.

Más recientemente, las redes sociales que constituyen plataformas digitales de comunicación e interacción entre los usuarios que las utilizan, se han convertido en sitios controversiales para la comunicación y divulgación de la información científica, lo que ha cambiado radicalmente la forma de difundir este tipo de conocimiento. Es indudable que la información que se expone en las redes sociales va dirigida más a la población general, sin embargo, cada día, más médicos y profesionales de otras áreas las utilizan para dar a conocer experiencias sobre su ejercicio clínico y su conocimiento sobre ciertos temas, como una manera de proyectarse y obtener beneficios económicos de la captación de pacientes. En el campo de la investigación, un perfil en una red social científica podría ayudar al investigador a visibilizar su productividad, a realizar alianzas con otros grupos e incrementar la posibilidad de ser citado en otras publicaciones. Si bien, esta información es de fácil acceso, adolece de innumerables deficiencias metodológicas que podrían ser fuente para decisiones erradas y peligrosas en el ámbito asistencial sanitario. Incluso, estudios publicados en revistas de gran impacto y prestigio internacional, a pesar de haber sido revisados cuidadosamente, pueden en ocasiones, tener deficiencias y limitaciones metodológicas, con resultados que podrían carecer de exactitud y rigurosidad científica.

Debido a los argumentos anteriormente expuestos, es imprescindible contar con estrategias que faciliten la búsqueda de la información más relevante y que sea basada en la mejor evidencia científica existente para la fecha. De igual manera, es fundamental que el personal de salud sea capaz de analizar, interpretar y manejar adecuadamente esta "sobredosis de información digital", para

aplicarla en su campo respectivo. Ante esta situación, las revisiones se han posicionado como una de las principales herramientas para la búsqueda, recopilación y síntesis de los datos de las publicaciones científicas, facilitando al personal de salud el mantenerse actualizado, especialmente en esta época de importantes y continuos avances en el diagnóstico y tratamiento de las enfermedades.

Las revisiones científicas pueden realizarse de forma narrativa, que son aquellas que no siguen un método sistemático y los resultados de investigaciones previas no se comparan para llegar a una síntesis cualitativa o cuantitativa, y debido a estas desventajas, actualmente se prefieren las revisiones sistemáticas (RS). Las RS están constituidas por investigaciones que reúnen la evidencia empírica a través de criterios de elegibilidad previamente establecidos, con el fin de responder a una pregunta específica de investigación, utilizando métodos explícitos y sistemáticos y minimizando los sesgos[1]. A partir de la búsqueda e identificación sistemática de estudios primarios originales y del análisis de sus resultados, se busca extraer conclusiones confiables, válidas y aplicables en un campo específico. Cuando se combinan los resultados de dos o más estudios mediante técnicas estadísticas, para proporcionar estimaciones más precisas que las aportadas por los estudios aislados, se denominan RS cuantitativas o metanálisis (MA) [1,2]. Está claro que no todas las RS deben incluir un MA, pero si deben establecer con suficiente detalle, el método sistemático que se utilizó para llevar a cabo la revisión, selección y análisis de la información, para que de esta manera, los lectores puedan entender con facilidad los pasos que fueron planificados. Sin embargo, estas publicaciones científicas tampoco están exentas de limitaciones y desventajas. En ocasiones, obtener conclusiones o estimaciones válidas puede ser difícil debido a que los resultados de los estudios analizados pueden aportar datos que no siempre son confiables, limitando en gran medida sus potencialidades.

Para corregir estas debilidades, se han llevado a cabo varios esfuerzos para afinar los métodos y lineamientos de las RS y elaborar guías con el fin de mejorar todo el proceso que conlleva su realización. Un grupo de expertos metodólogos e investigadores en 1999, desarrolló una guía que recibió el nombre de declaración QUORUM (*Quality of Reporting of Meta-analysis*) [3], con el propósito de establecer normas para mejorar la calidad de la presentación de los MA de ensayos clínicos aleatorizados. QUORUM no es más que una lista de verificación de 18 ítems, que explica los pasos que se deben cumplir para la elaboración de un MA. A pesar que QUORUM significó un adelanto importante para la unificación de los MA, muchos de ellos realizados posteriormente a la implementación de esta declaración, demostraron aún deficiencias importantes. Ante esta dificultad, surgió la necesidad de modificar la lista de verificación y desarrollar un nuevo instrumento, por lo que en el año 2009 apareció la declaración PRISMA (*Preferred Reporting Items for Systematic Reviews and Meta-Analyses*) [4], que se puede considerar como una forma actualizada y ampliada de QUORUM, ya que no solo se enfoca en la elaboración de MA de estudios aleatorizados, sino que también como su nombre lo indica, para otros tipos de RS. La lista de la declaración PRISMA, la conforma un total de 27 *items* y se acompaña de una extensa y detallada explicación de cada uno de los aspectos a considerar en la elaboración de una RS y MA. Dentro de los aspectos más importantes de esta declaración resalta la necesidad de disminución del riesgo de sesgo tanto en la selección de los estudios, como en los resultados, como un aspecto clave del proceso de elaboración de una RS[5]. La exclusión de estudios debido a que los resultados no son favorables o de algunos resultados de un estudio en particular, lo que se denomina como sesgo de publicación, podría conducir a la selección de una muestra sesgada, que por lo tanto no sea representativa de toda la información existente sobre el tema [5].

Se han presentado sucesivas modificaciones de esta declaración, con la finalidad de disminuir sus debilidades y mejorar cada vez más la calidad e integridad de este tipo investigación científica. Los MA en red o NMA (*Network Meta-analysis)* [6]*, son* una extensión de PRISMA con un total de 32 elementos*,* diseñada para mejorar la presentación de informes de RS que incorporan MA en red. Esta versión permite comparar el efecto de más de dos intervenciones a la vez, de gran utilidad cuando existen varias alternativas terapéuticas y se quiere conocer el efecto relativo de cada una de estas en el resultado final. Recientemente fue propuesta la declaración PRISMA 2020 [7], diseñada principalmente para la RS de estudios que evalúan los efectos de las intervenciones de salud, independientemente del diseño de los estudios incluidos, aunque también puede ser usada en el ámbito social o educativo.

Es indudable que el éxito de las RS dependerá no solo en la medida en que sean corregidos cada uno de los puntos débiles de estas guías o declaraciones, y se mantengan en constante adaptación y evolución conforme a la nueva evidencia publicada, sino también que los editores de las revistas científicas del área de la salud fomenten la realización de RS y MA, dándoles prioridad para su publicación sobre otros tipos de revisiones como las narrativas.

En conclusión, las RS y los MA constituyen herramientas muy útiles para seleccionar y sintetizar información y cuando son llevadas a cabo con una adecuada planificación, pueden aportar evidencia de alto nivel sobre la eficacia y seguridad de tratamientos u otras intervenciones en salud, y favorecer la toma de decisiones para el delineamiento de las políticas de administración en esta área. En el ámbito del ejercicio profesional las RS emergen como un método que facilita la tarea de "mantenerse al día" y de esta manera llevar a cabo la práctica clínica basada en la mejor evidencia disponible.

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Importance of systematic reviews and meta-analysis in the digital information age.

The digital information age has resulted in an overwhelming avalanche of scientific information in all areas, causing specialists in the health area to face a significant challenge to select and synthesize the most important and relevant information in their fields. Systematic reviews have positioned themselves as one of the main tools for searching, collecting, and synthesizing data from scientific publications, making it easier for health personnel to stay up-to-date and make health decisions based on the best scientific evidence available to date. Several guides have been developed seeking to establish guidelines and unify the way to carry out systematic reviews and meta-analyses to improve the quality of their presentation. However, this type of scientific publication is not exempt from limitations and disadvantages. The PRISMA statement constitutes an excellent guide for accomplishing scientific reviews and meta-analyses since it details each aspect to be considered in preparing this type of scientific publication. Since its appearance, this statement continues to experience modifications to update it and adapt it according to the new scientific evidence available.

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Induced differentiation of adipose-derived stem cells enhance secretion of neurotrophic factors.

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Keywords: differentiated ADSCs; Schwann cells; neurotrophic factors; P2X7; nerve damage.

Abstract. Adipose-derived stem cells (ADSCs) could be ideal seed cells for repairing nerve injury as they have the potential for multidirectional differentiation. However, it is still unclear whether the undifferentiated or the differentiated ADSCs have priorities in promoting axonal regeneration and myelin formation. In this study, the primary ADSCs from rats were cultured and differentiated. The morphology, differentiation potential, and secretion of neurotrophic factors of ADSCs were compared before and after induction. Undifferentiated ADSCs (uADSCs) were aggregated into bundles containing reticular, star, and polygonal structures. They contained a large number of lipid droplets and were positive for Oil red O staining. After differentiation, differentiation ADSCs (dADSCs) become long and spindle-shaped with decreasing protrusions around the cells, spiraling growth, and were negative for Oil red O staining. When comparing the groups the flow cytometer analysis showed: similar CD29 and CD45 surface markers in both groups; and CD44 and CD90 markers were very low in the undifferentiated groups. The levels of neurotrophin 3 (NT-3) and neuregulin 1 (NRG-1), and their receptors tropomyosin receptor kinase C (TrkC) and receptor protein-tyrosine kinase erbB-4 (ErbB-4) in dADSCs were higher than those in uADSCs. While the expressions of myelin protein zero (P0), myelin-associated glycoprotein (MAG), and purine receptor P2X7 (P2X7) were not significantly different before and after differentiation. It may be speculated that the dADSCs have enhanced abilities in nerve repairment which is associated with increased expression of neurotrophic factors.

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La diferenciación inducida de las células madre derivadas del tejido adiposo aumenta la secreción de factores neurotróficos.

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Palabras clave: ADSC diferenciadas, células de Schwann, factores neurotróficos, P2X7, daño nervioso.

Resumen. Las células madre derivadas del tejido adiposo (ADSCs) podrían ser una semilla ideal de células para la reparación de lesiones nerviosas, ya que tienen el potencial de diferenciación multidireccional. Sin embargo, aún no está claro si las ADSCs indiferenciadas o diferenciadas tienen prioridades en la promoción de la regeneración axonal y la formación de mielina. En este estudio, ADSCs primarias de las ratas fueron cultivadas y diferenciadas. Se compararon la morfología, el potencial de diferenciación y la secreción de los factores neurotróficos de las ADSCs antes y después de la inducción. Las ADSCs indiferenciadas (uADSCs) se encontraban agregadas en haces que contenían estructuras reticulares, estrelladas y poligonales. Contenían un gran número de gotitas de lípidos y fueron positivas para la tinción de Aceite Rojo O. Después de la diferenciación, las ADSCs (dADSCs) se vuelven largas y en forma de huso con un número decreciente de protuberancias alrededor de las células, crecimiento en espiral, y fueron negativas para la tinción de Aceite Rojo O. Cuando se compararon los dos grupos, análisis del citómetro de flojo muestra que los dos grupos de marcadores superficiales CD29 y CD45 eran similares; y los marcadores CD44 y CD90 eran muy bajos en el grupo indiferenciado. Los niveles de neurotrofina 3 (NT-3) y neuregulina 1 (NRG-1) y sus receptores, el receptor de tropomiosina quinasa C (TrkC) y el receptor de proteína tirosina quinasa erbB-4 (ErbB-4) en dADSC fueron más altos que los de uADSC. Mientras que las expresiones de proteína cero de mielina (P0), glicoproteína asociada a mielina (MAG) y receptor de purina P2X7 (P2X7) no fueron significativamente diferentes antes y después de la diferenciación. Se puede especular que las dADSC tienen capacidades mejoradas en la reparación nerviosa que se asocia con una mayor expresión de factores neurotróficos.

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INTRODUCTION

Regeneration and functional recovery after peripheral nerve damage are foci of research in neuroscience and a problem in clinical surgery [1]. Schwann cells (SCs) are the primary myelin-forming cells in the peripheral nervous system and play a prominent role in neuron survival and function. Schwann cells promote nerve regeneration by secreting neurotrophic factors and adhesion molecules [2]. However, the clinical use of Schwann cells is limited by the difficulty in obtaining adequate quantities. To overcome this, the ability of various types of stem cells to differentiate into Schwann cells is under investigation. Stem cells as seed cells combined with vector scaffolds can be used to

construct tissue-engineered nerves with biological activity and functionality; indeed, this research focuses on peripheral nerve repair. Adipose-derived stem cells (ADSCs) can be induced to differentiate into nerve cells [3], astrocytes, osteoblasts, and myofibroblasts in the appropriate type of medium [4-9]. Adipose tissue has the largest storage capacity in the body and is easily harvested and cultured. ADSCs are genetically stable, have low tumorigenicity, low immunogenicity, and show rapid expansion in vitro [10]. Therefore, ADSCs can be used to repair peripheral nerve damage.

ADSCs can be induced to differentiate into Schwann-like cells in vitro, which involves a changing from a flat to an elongated spindle shape and expressing s-100, GFAP, and P75. In coculture with spinal dorsal root ganglion (DRG) neurons, induced ADSCs promoted the axonal growth of DRG neurons and myelin sheath formation [11,12], indicating that the induced ADSCs had the phenotype and functionality of Schwann cells.

Repair by ADSCs of injured nerves has been confirmed in vivo. In mice with sciatic nerve injury, intravenous injection of ADSCs significantly increased the growth of sciatic nerve axons and ameliorated the inflammatory response [13]. ADSCs were transferred into artificial nerve conduits made of collagen [7], silica gel [8,9], PCL [14], and fibroin/collagen [15] and transplanted into sciatic nerve defects of rats. The regenerated axon of the sciatic nerve in the transplantation group was longer, and the walking gait, muscle weight, and nerve conduction velocity were significantly improved compared to that in the control group [7,15-17].

The mechanism by which ADSCs repair peripheral nerve injury is unclear. After differentiation, the mRNA and protein levels of the purine receptor P2X7 increased significantly in ADSCs, stimulating Ca2+ inflow and inhibiting the P2X7 receptor to prevent ATP-induced cell death [18].

The therapeutic effect and the underlying mechanisms of uADSCs and dADSCs on nerve injury are unclear. It is crucial to determine the phenotypic changes of ADSCs before and after differentiation is induced. Repair of peripheral injured nerves involves the secretion of neurotrophic factors, axon growth, and myelin sheath formation. Schwann cells secrete multiple growth factors that promote axonal regeneration, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) [19]. Stem cells repair damaged tissue by releasing several trophic factors in situ, which alters the local microenvironment [3]. To compare the efficacy of uADSCs and dADSCs in treating peripheral nerve injury, we investigated the effect of induction of differentiation on the differentiation potential, morphology, proliferation, and levels of Schwann cell-related proteins of ADSCs.

MATERIALS AND METHODS

Extraction, isolation, and culture of ADSCs

Male Sprague-Dawley (SD) specific-pathogen-free rats, of approximately 300 g of weight were euthanized by cervical dislocation and soaked in 75% ethanol for 10 min. Adipose tissue under the skin of the abdomen was dissected and placed in a Petri dish containing phosphate-buffered saline (PBS, pH=7.2) (Solarbio, Beijing, China). The cells were washed with PBS three times to remove blood and vessels. The adipose tissue was cut into one mm^3 pieces, digested with 0.075% type I collagenase (Invitrogen, Carlsbad, California, USA), and incubated at 37°C for 90min. After centrifugation, the upper undigested adipose tissue and the supernatant were discarded, and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM)/F12 (containing 1% penicillin/streptomycin and 10% fetal bovine serum). The cells were centrifuged, filtered through a 200-mesh sieve, and transferred to a $75cm^2$ culture flask. After 24h, half of the medium was replaced, and the cells were passaged at a ratio of 1:2. Then

they were cultured to the third generation to achieve the purity of the isolated cells, and the cell morphology (CKX41, Olympus, Tokyo, Japan) was examined. When the cells were grown at their best, they were digested and centrifuged with 0.25% trypsin, and collected in DMSO: FBS: DMEM/F12=1:2:7, and mixed with cryopreserved solution, which was added into the cryopreservation tube after resuspension, and placed at 4°C for 1 hour, -20°C for 4 hours, and -80°C overnight. Finally, they were transferred to the liquid nitrogen tank for cryopreservation for later use. The experiment was conducted in three batches of cells.

Cell viability assay

Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. ADSCs were cultured in a 96-well plate, and 20μL (0.6mM) of MTT solution was added to each well. The cells were incubated at 37°C for 4h, 150μL of dimethyl sulfoxide was added to each well, and the plates were shaken for 10 min to ensure that crystals were dissolved. The optical density was measured at 570nm. The experiment was conducted in three batches of cells.

Induction of differentiation of ADSCs

We hypothesized that adipose-derived stem cells are more conducive to axonal regeneration and myelination after differentiation. In order to verify this, this study mainly investigated the differences between undifferentiated and differentiated ADSCs in cell morphology and in promoting neurotrophic factor secretion and myelin-related protein expression. ADSCs were digested with 0.25% trypsin/ EDTA (Invitrogen, USA) at the third passage, centrifuged, resuspended in DMEM/F12, and transferred to a six-well plate (2×10^5/mL). After 24h, 2mL of 1mM β-mercaptoethanol (Sigma-Aldrich, USA) was added, followed by fresh medium containing 35ng/mL all-trans-retinoic acid (Sigma-Aldrich, USA). The cells were washed in PBS after cultivation for 72h, and 2mL of ADSC differentiation medium (DMEM/F12 containing 5 ng/mL platelet-derived growth factor, 10 ng/mL basic fibroblast growth factor, 14μM forskolin, and 200ng/mL heregulin) was added. The experiment was conducted in three batches of cells, the cells were maintained for 1 week under the same conditions and fresh medium was added at 48–72h intervals. The morphological characteristics of uADSCs and dADSCs were observed under a microscope.

Oil red O staining

uADSCs and dADSCs were cultured in six-well plates (2×10^5/mL), the medium was discarded, and the cells were washed in PBS three times and fixed in 10% formaldehyde for 40min. Next, the cells were washed three times in PBS, stained with Oil Red O (Worldbio, China), and incubated at room temperature for 40min. The cells were rinsed with 75% alcohol to remove excess dye. The cells were sealed with glycerin gelatin, and cell morphology was observed under a microscope. Three batches of cell morphological maps were collected and analyzed.

Flow cytometry

uADSCs and dADSCs were digested with 0.25% trypsin/EDTA, centrifuged, and the supernatant was discarded. The cells were washed three times in 2% bovine serum albumin (BSA; abcbio, China). The pellet was resuspended in 5% BSA and subjected to cell counting. The cell density was adjusted to 1×10^7/mL, and the cells were incubated with 10μL of antibodies against CD29, CD44, CD90, and CD45 (Bio-Rad) for 30min on ice in the dark. The cells were washed in 5% BSA, centrifuged (1500 rpm) for 5min, and resuspended in PBS for flow cytometry. Three batches of cells were collected and analyzed.

Western blotting

Cells were rinsed in 0.01M PBS and lysed in radioimmunoprecipitation assay lysis buffer. The lysates were centrifuged at

4°C at 12,000 rpm for 5min. The supernatant was collected and stored at -20°C. The protein concentration was quantified using a BCA Protein Assay Kit (Beyotime Biotechnology, China). The proteins were resolved by sodium dodecyl sulfate-polyvinylidene fluoride gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Primary antibodies against NRG-1 (1:1000, Affinity), NT-3 (1:500, Servicebio), P0 (1:500, Affinity), MAG(1:1000,Bioss), TrkC (1:500, GeneTex), ErbB-4 (1:500, GeneTex), and P2X7 (1:1000, ab109054, Abcam), were added and the membranes were incubated at 4°C for 24h. Next, the secondary antibody was added, followed by incubation for 2h.β-actin was used as the loading control for normalization. The grey values of the target protein were analyzed by Image J. The results were calculated by the ratio of accumulated gray values of target protein to the bands of β-actin, which represented the relative expression level of target protein. Bands of each target protein where appeared three times, were sorted out and analyzed.

Statistical analysis

Prism 5.0 was used for statistical analysis (GraphPad Systems, Inc., La Jolla, CA, USA), performed by t test. The data are means ± standard deviation. $P<0.05$ was considered indicative of statistical significance.

RESULTS

Morphological characteristics of ADSCs

ADSCs were isolated from subcutaneous abdominal fat of male SD rats and cultured in DMEM/F12. After 48h, the cells began to grow rapidly (Fig. 1A), adhered to the wall, and formed a short fusiform, star-shaped structure and irregular polygonal structure. After 5–7 days, the ADSCs were spindle-shaped and growing vigorously (Fig. 1B).

Growth of ADSCs

The ADSC growth curve was S-shaped. The ADSCs entered the logarithmic growth phase after 72h, and growth peaked at day 5 and decreased thereafter (Fig. 1C). In addition, ADSCs at passage 3 had the highest growth rate and those at passage 13 the low-

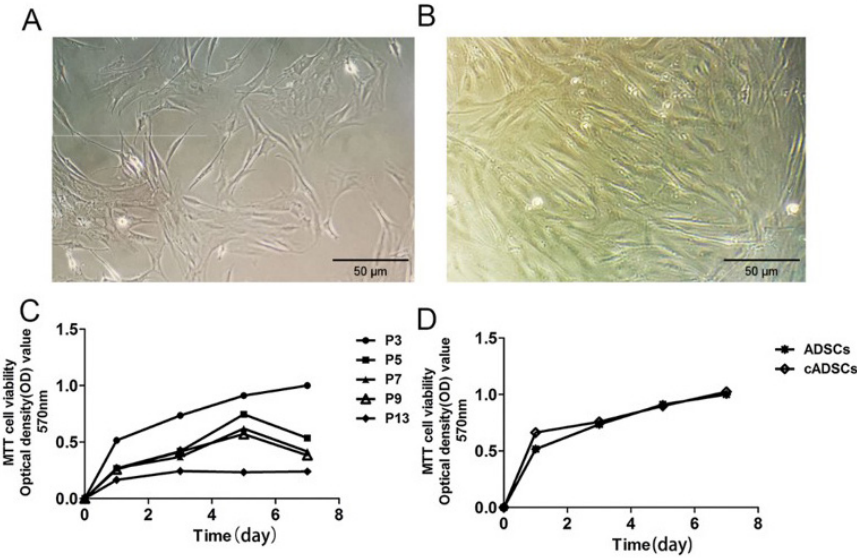

Fig. 1. Morphological characteristics and proliferation changes of ADSCs. A. After 48h, ADSCs were short and fusiform, with a star-shaped structure and irregular polygonal structure (×10). B. After 5–7 days, the primary ADSC cells exhibited a typical fibroblast-like morphology (×10). C. Growth curves of ADSCs at passages 3, 5, 7, 9 and 13. D. Growth curves before and after cryopreservation (cADSCs, ADSCs after cryopreservation).

est; therefore, the growth rate decreased with increasing passage number. At the initial stage of culture, the growth rate of cryogenically preserved ADSCs was similar to that of freshly prepared ADSCs (Fig. 1D).

Morphological characteristics of uADSCs induced to differentiate into dADSCs

The uADSCs aggregated into bundles containing reticular, star structures, and polygonal structures (Fig. 2A). After differentiation the cells were long and spindle-shaped, the number of protrusions around the cells decreased, spiraling growth, and showing a Schwann-like morphology (Fig. 2B).

Oil red O staining

The uADSCs contained a small number of lipid droplets (Fig. 3A). The dADSCs are Schwann cell-like cells and did not exhibit lipid droplets (Fig. 3B).

Expression of cell surface factors

Flow cytometry was performed to examine CD29, CD44, CD90 (stem-cell markers), and CD45 expression levels. In ADSCs, 96.0% expressed CD29, 60.1% expressed CD44, and 74.2% expressed CD90, indicating ADSCs have mesenchymal stem cell-related surface markers and have the potential of multi-differentiation of stem cells (Fig.4). As a marker of hematopoietic cells, the positive rate of CD45 was less than 50% (only 32.5%), suggesting they were uADSCs but could not differentiate into hematopoietic cells. After induction and differentiation into SCs, in dADSCs, 99.1% expressed CD29, 91.4% expressed CD44, 96.8% expressed CD90, and only 31.2% expressed CD45 (Fig.4). Compared with uADSCs, the expression levels of each marker in dADSCs were significantly increased, suggesting that the differentiation potential of ADSCs induce to differentiate into SCs was enhanced (Fig. 5).

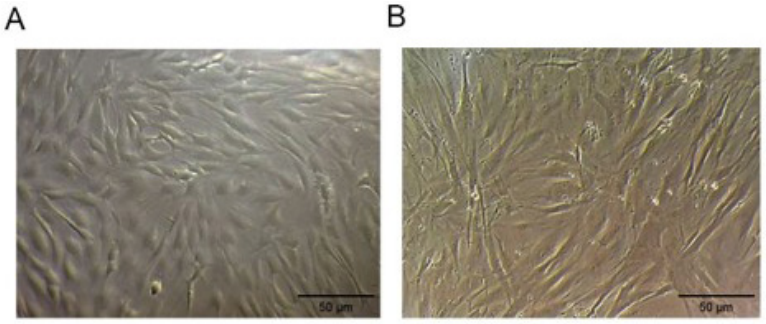

Fig. 2. Morphological changes of ADSCs after induced differentiation. A. Morphology of uADSCs. B. Morphology of dADSCs. After induction, the cells were long and spindle-shaped, the number of protrusions around the cells decreased, and spiraling growth (×10).

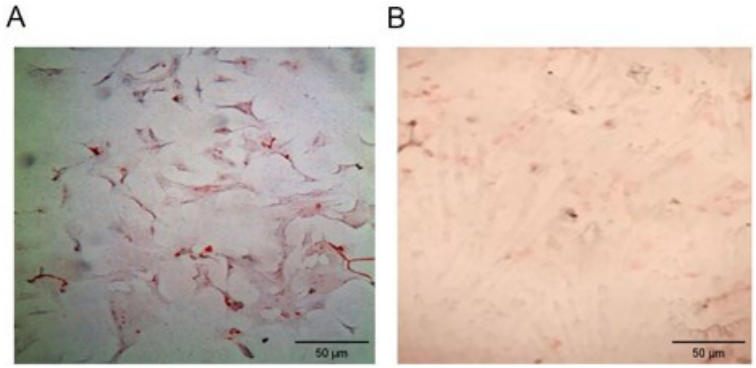

Fig. 3. Morphological changes after Oil red O staining. A.Oil red O staining of uADSCs. B.Oil red O staining of dADSCs (×10).

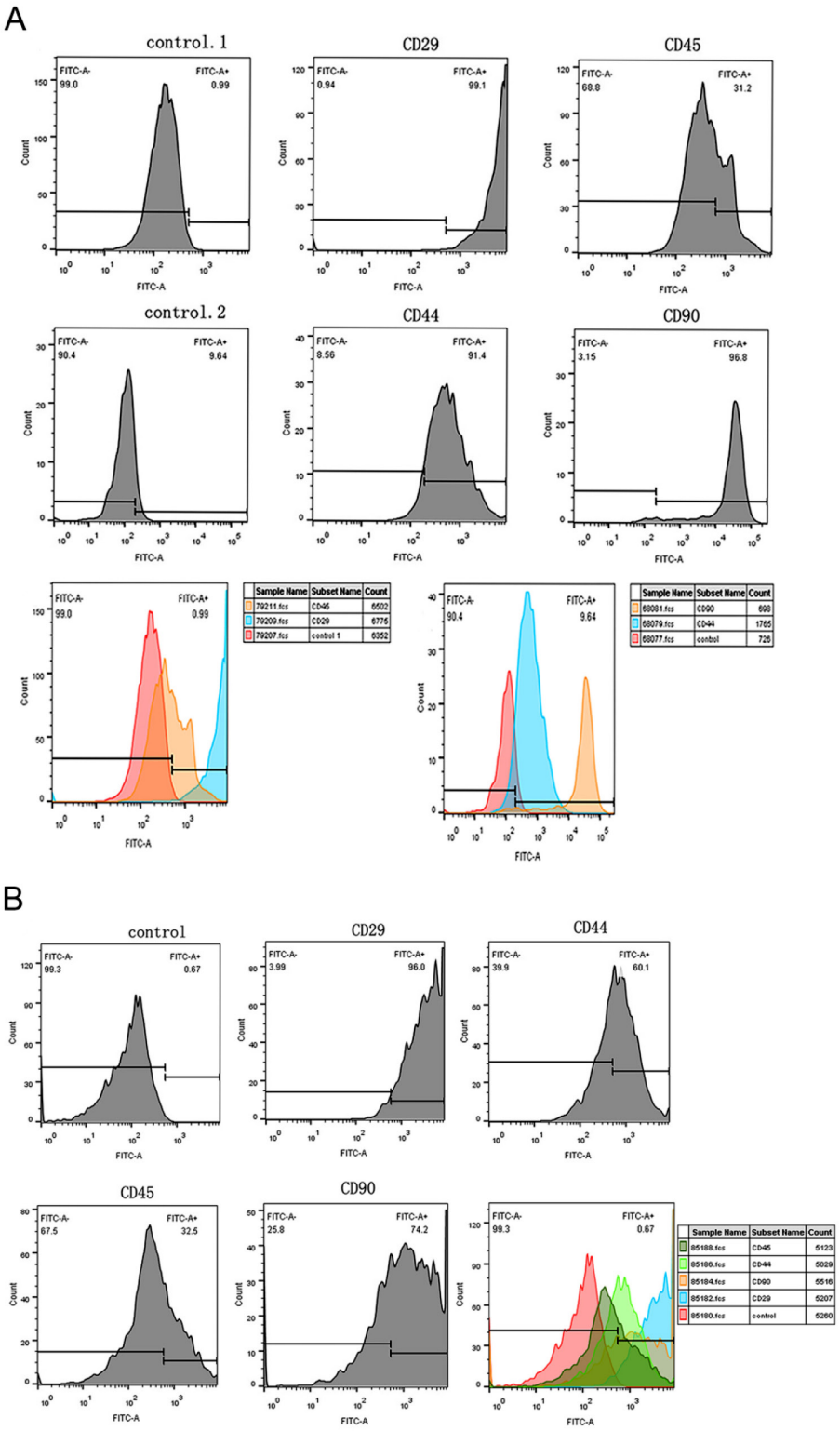

Fig. 4. CD29, CD44, CD45, and CD90 expression in uADSCs and dADSCs by flow cytometry. A. Expression of CD29, CD44, CD90, and CD45 in uADSCs. B. Expression of CD29, CD44, CD90, and CD45 in dADSCs. Blank control, cells treated with PBS but not anti-CD antibody.

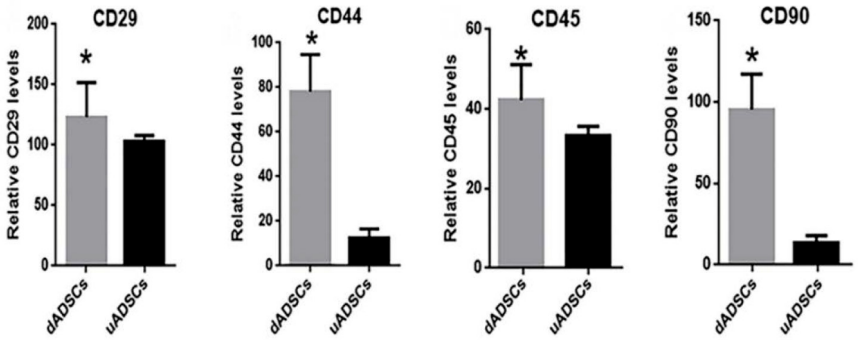

Fig. 5. Expression levels of CD29, CD44, CD45, and CD90 in uADSCs were lower than those in dADSCs (*p <0.05, n = 3).

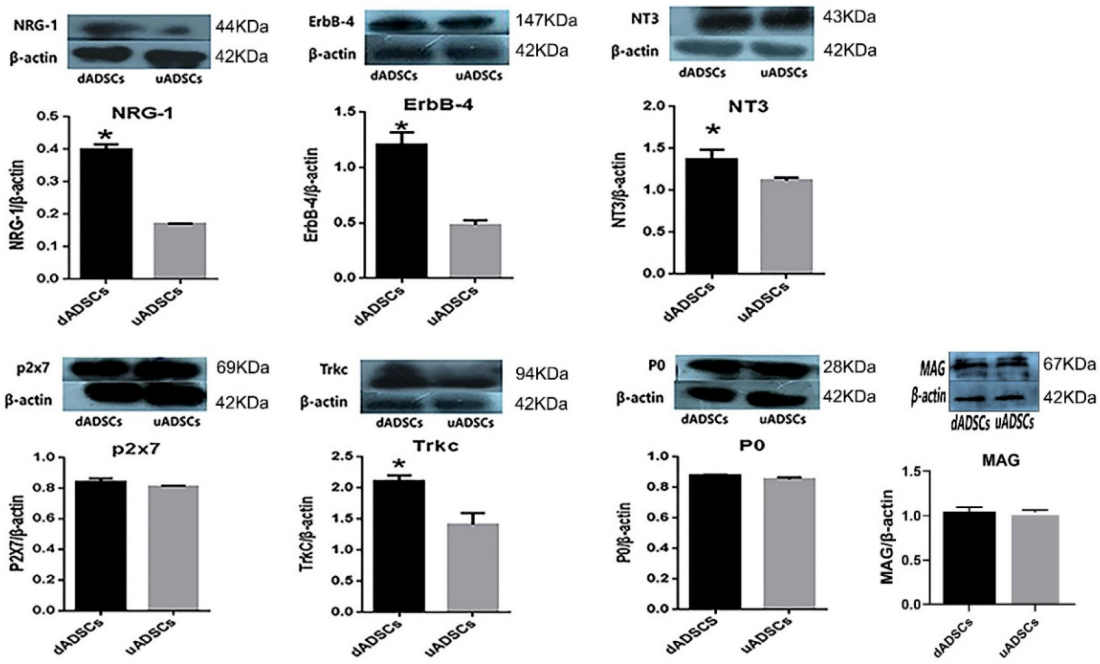

Fig. 6. Changes in NRG-1, NT3, TrkC, ErbB-4, P0, MAG and P2X7 protein levels. β-actin was used as the loading control (*p<0.05, n = 3).

Expression of uADSC- and dADSC-related proteins

Western blotting showed that NRG-1, ErbB-4, NT3, TrkC, P0, MAG and P2X7 were expressed on the surface of uADSCs and dADSCs (Fig. 6). The levels of NRG-1, ErbB4, NT-3, and TrkC in dADSCs were significantly higher than those in uADSCs ($p<0.05$). However, there were no significant differences in the levels of the myelin protein P0, MAG, and the purine receptor P2X7 before and after induction of differentiation.

DISCUSSION

Mesenchymal stem cells (also known as all-powerful mesenchymal stromal cells), as one of the stem cells, have attracted much attention in the field of stem cell therapy and regenerative medicine. ADSCs play an

important role in nerve injury and functional recovery and are considered ideal seed cells for nerve transplantation. However, it is currently unclear whether ADSCs differentiate into dADSCs after being stimulated by the in vivo injury environment or their direct effects on promoting nerve regeneration [20]. In this study, type I collagenase was used to isolate the subcutaneous adipose tissue of rat abdomen to evaluate the differentiation potential, morphology, and protein levels before and after differentiation. When the cells passed to the third generation, the cell growth activity was the best, so the third generation of ADSCs was selected as the research object. Cryopreservation is used for long-term storage of biological materials, such as oocytes, stem cells, vascular tissues, and embryos [21-23]. In this study, revived ADSCs undergoing cryopreservation did not show significant loss of viability or proliferation by MTT assay.

Some scholars compared the roles of dADSCs and uADSCs in nerve transplantation and believed that undifferentiated adipose stem cells (ADSCs) were easy to obtain and had the advantage of a short culture cycle in promoting neurotrophic factors secretion and repairing myelin sheath injury [24,25]. At the same time, studies have pointed out that differentiated adipose stem cells are more conducive to playing the role of stem cells in nerve injury repair [26]. In this study, adipose stem cells were differentiated and cultured to explore the advantages and disadvantages of undifferentiated and differentiated ADSCs in promoting axonal regeneration and myelination. The morphologies of uADSCs and dADSCs were significantly different. uADSCs cells presented star and polygonal structures. After 1-2 w of induction and differentiation by adding specific Schwann-inducing fluid, dADSCs cells presented a spindle shaped Schwann-like cell morphology, and the number of protuberances around the cells decreased. ADSCs expressed mesenchymal stem cell (MSC) markers and have similar properties to MSCs [6].

MSCs derived from some mammals can be transformed into Schwann cells in the presence of inducers or mixtures of growth factors. ADSCs express several stem cell surface molecules such as CD105, CD29, CD44, and CD45 [7]. In the recently published studies, just like our method, only positive markers were detected in the differentiation and identification of primary ADSCs cells, less involving negative markers such as CD34 [27, 28]. High expression of CD29, CD44, and CD90 and low expression of CD45 were found in uADSCs and dADSCs. Jiang *et al.* [29] reported that ADSCs isolated from SD mice show high CD29, CD44, and CD90 expression levels, but low or absent expression of CD45. This agrees with our finding that both uADSCs and dADSCs express MSC-associated surface markers and can undergo differentiation into multiple cell lineages. The uADSCs, but not the dADSCs, were positive for Oil red O staining, indicating that the former had a larger number of lipid droplets than the latter.

Repairing and regenerating damaged nerves involves a complicated pathophysiological process, mainly dependent on regulating various cytokines. When injured, the body can rely on its own nerve regeneration or granulation tissue hyperplasia and scar formation to achieve healing. ADSCs participate in various stages of tissue repair by virtue of their multiple physiological functions [20]. Multidirectional studies have confirmed that ADSCs can secrete various cytokines, which play a vital role in diverse physiological activities of ADSCs.

Schwann cells (SCs) are the most promising seed cells for peripheral nerve tissue engineering [30], which can promote peripheral axon regeneration after peripheral nerve injury (PNI). Recent research has found that salidroside may improve the regeneration effect on the sciatic nerve following a combined application of epimysium conduit and RSC96 Schwann cells in rats [31]. Epothilone B (EpoB) is an FDA-approved antineoplastic agent, which shows the capacity to induce

alpha-tubulin polymerization and improve microtubules' stability. The latest research found the potential therapeutic value of EpoB in enhancing regeneration and functional recovery in cases of PNI [32]. In addition, Schwann cells also play a significant role in promoting the regeneration of PNI. According to the latest research, SCs are integral in the regeneration and restoration of function following PNI. SCs are able to dedifferentiate and proliferate, remove myelin and axonal debris, and are supportive of axonal regeneration [33]. Moreover, 5% gastrodin/PU NGC efficiently promotes nerve regeneration, indicating their potential for use in peripheral nerve regeneration applications [34].

Schwann cells (SCs) secrete neurotrophin 3 (NT-3). NT-3 can promote the development and differentiation of neurons, and its binding with tropomyosin receptor kinase C (TrkC) receptor can maintain the survival of neurons [35], inhibit cell apoptosis, and promote the differentiation of SCs into neurons [36,37]. Several studies have shown that in vitro transfection of adenovirus carrying NT-3 (AdvNT-3) gene can promote the differentiation of MSCs into neuron-like cells. The role of NT-3 is mediated by its preferred binding receptor TrkC. In this study, the expression levels of NT-3 and TrkC were significantly increased after the induction of differentiation of ADSCs, consistent with the above. Western blot results showed that dADSCs could promote the expression of NT-3 and its receptor TrkC, thus maintaining the regeneration of injured nerves and reducing nerve apoptosis.

The neuregulin-1 (NRG-1)/receptor/tyrosine protein kinase ErbB (ErbB) system is an endothelium-controlled paracrine system. It has been found that NRG-1 can promote the recovery of nerve function after brachial plexus injury after contralateral C7 nerve root metastasis in rats, and NRG-1 has combined anti-inflammatory and anti-fibrosis effects in different organs, including skin, lung, and heart. There is increasing evidence that the NRG-1/ErbB system is active in various organs throughout the body. NRG-1 not only promotes neuronal activity but also acts on the receptor ErbB-4 in nerve endings. At nerve endings, NRG-1 enters the cell body through axoplasmic countercurrent and promotes the growth of neurons. In this study, NRG-1 and erbB-4 expression levels were significantly increased after induction of ADSC differentiation, consistent with a previous report [38]. Thus, the ability of dADSCs to secrete neurotrophic factors and promote the growth of axons likely explains their ability to repair peripheral nerve injury.

Repair of injured nerves is accompanied by myelin sheath formation, which involves the coordinated synthesis of a group of proteins related to myelin, including the transmembrane glycoprotein P0 and myelin-associated glycoprotein MAG. MAG is a major component of myelin-derived nerve growth inhibitor. MAG shows different functions at different stages of the nervous system development, promoting axon growth during development and inhibiting axon growth during maturation. In this study, the level of the myelin-sheath protein P0 and MAG in uADSCs and dADSCs was not significantly different before and after induction of differentiation. Synthesis by Schwann cells of P0 is dependent on contact with axons [39]. The synthesis of P0 in Schwann cells is regulated by neural developmental growth [40]. Therefore, we can speculate that our results may be related to this cause.

P2X7 is a non-selective cationic channel receptor expressed in neurons and smooth muscle; the ligand of this receptor is ATP [41]. P2X7 acts as a bridge between the nervous and immune systems when nerve damage occurs. After differentiation, the mRNA and protein levels of the purine receptor P2X7 increased significantly in ADSCs, and inhibiting the P2X7 receptor could prevent ATP-induced cell death [18]. However, in this study, the expression of P2X7 in uADSCs and dADSCs was not significantly different. This may suggest that P2X7 plays a fundamental role in maintaining the proliferation and dif-

ferentiation of uADSCs and dADSCs under physiological conditions.

In conclusion, there are no differences in myelin production in the two groups studied, despite the increased neurotrophic factors and their receptors in the dADSCs group being more potent inducers of axonal growth potential than uADSCs. dADSCs and SCs are similar in morphology and function in vitro and in vivo and are readily implanted and proliferate rapidly [42]. Nevertheless, the undifferentiated state of ADSCs enables multiple-lineage differentiation and the establishment of a favorable environment for nerve regeneration. uADSCs are easier to obtain, have shorter incubation periods, and are less costly than dADSCs, suggesting their potential for nerve regeneration. Further studies are needed to assess the potential of uADSCs and dADSCs.

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Conflict of interests

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

XZ: conceptualization, methodology, software, investigation, formal analysis, writing-original draft; YNL: conceptualization, methodology, formal analysis, writing-original draft; investigation; ZL: resources, writing-original draft; YH: conceptualization, visualization; FL: resources, supervision; SYZ: software, validation; JG: resources, data curation, visualization; LL: conceptualization, funding acquisition, supervision, writing - review & editing.

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Relationships between genetic vascular risk polymorphism and aging. A case-control study in Venezuela.

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Keywords: aging; cardiovascular homeostasis; lipid metabolism; polymorphism; blood coagulation.

Abstract: Aging is an irreversible process that produces the progressive decline of physiological functions favoring the development of cardiovascular complications associated with genetic Risk Alleles (RA). A case-control study using a sample of 90 Venezuelan individuals was performed to determine the correlation between the incidence of accelerated aging for 14 polymorphisms in genes associated with blood coagulation, lipid, and cardiovascular homeostasis. Odds Ratio (OR) results showed a 41% increase in the risk of presenting accelerated aging in subjects with the rs1800790 RA in the FGB gene. The CC genotype for the rs1800775 in the CETP gene was associated with a 62%, and the TT genotype for the rs1801133 in the MTHFR gene increased risk by two times. However, none of these results were statistically significant. Only a significant association was determined between the presence of the homozygous deletion genotype for the rs4340 RA in the ACE gene with an increased risk up to ten times (OR: 10.6; CI: 1.23 - 90.67; $p<0.05$). Multivariable analyses showed that gender, obesity, hypercholesterolemia, hypertriglyceridemia, smoking, age, body mass index, systolic hypertension, the rs662 RA in the APOB, rs693 RA in the PON1 and rs1801133 RA in the MTHFR genes were the main environmental and genetic factors associated with accelerated aging.

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Relación entre polimorfismos de riesgo genético vascular y el envejecimiento. Un estudio caso-control en Venezuela.

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Palabras clave: envejecimiento; homeóstasis cardiovascular; metabolismo lipídico; polimorfismo; coagulación sanguínea.

Resumen: El envejecimiento es un proceso irreversible que produce el declive progresivo de las funciones fisiológicas favoreciendo el desarrollo de complicaciones cardiovasculares asociadas con alelos de riesgo (AR) genéticos. Se realizó un estudio caso-control empleando una muestra de 90 individuos venezolanos para determinar la correlación entre la incidencia de envejecimiento acelerado para 14 polimorfismos en genes asociados a coagulación sanguínea, lípidos y homeóstasis cardiovascular. Resultados de razón de probabilidades (RP) mostraron en un 41% de sujetos con el AR rs1800790 en el gen FGB un incremento en el riesgo de presentar envejecimiento acelerado. El genotipo CC para el rs1800775 en el gen CETP fue asociado con un incremento de riesgo de 62% y el genotipo TT para el rs1801133 en el gen MTHFR con un incremento en el riesgo de 2 veces. Sin embargo, ninguno de estos resultados fue estadísticamente significativo. Sólo se determinó una relación estadísticamente significativa entre la presencia del genotipo de deleción homocigota para el AR de rs4340 en el gen ACE con un riesgo incrementado de hasta 10 veces (RP: 10,6; CI: 1,23 – 90,67; $p<0,05$). Los análisis multivariable mostraron que el género, obesidad, hipercolesterolemia, hipertrigliceridemia, hábito tabáquico, edad, índice de masa corporal, hipertesión sistólica, el AR rs662 en el gen APOB, el AR rs693 en PON1 y el AR rs1801133 en el gen MTHFR eran los principales factores ambientales y genéticos asociados a envejecimiento acelerado.

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INTRODUCTION

Aging is a complex time-depending process that causes the progressive decline of the organism's physiological function, affecting the cells' adaptability and their ability to maintain homeostasis and leading to a general decline of all body systems. Therefore, aging increases the susceptibility of the organism to suffer various diseases, usually related to cellular senescence [1,2]. Aging is a multifactorial process in which the interaction of several genetic and environmental variables can determine the growing old rate among different individuals or between organs and tissues of one specific individual [3]. Specific genotypes appear to be associated with accelerating the depletion of the organism's metabolism leading to the premature presence of degenerative diseases and the stimulation and acceleration of the natural aging course [4].

Alterations in the coding genes of proteins associated with endothelial function, blood coagulation, and lipid metabolism have proven to be highly related to the appearance of cardiovascular diseases (CVDs), known pathologies related to aging. More-

over, several alleles for some polymorphic variants in different candidate genes related to vascular risk have been postulated as genetic markers of premature aging [5,6], and many studies have been dedicated to establishing genetic variants which might be associated with healthy aging and longevity [7-12]. As a result, multiple variants in genes involved in different cellular processes and metabolic pathways have been postulated [8,11] with special attention on genes related to CVDs due to the close relationship between these diseases and aging [13]. However, due to the complexity of the aging process, there are some inconsistencies in the relationships proposed regarding the genetic factors involved in aging [10,11,14], probably because the environmental factors also play an essential role in aging progression as they can modulate the influence of the genetic risk factors [13,15].

Here, we evaluated the relationship between accelerated aging and different genotypes of 13 polymorphisms in the following genes: Apolipoprotein B (APOB; rs693), Apolipoprotein E (APOE; rs429358 and rs7412), Cholesteryl Ester Transfer Protein (CETP; rs1800775), Paraoxonase 1 (PON1; rs662), Fibrinogen Beta Chain (FGB; rs1800790 and rs1800791), Coagulation Factor II (F2; rs1799963), Coagulation Factor V (F5; rs6025), Coagulation Factor VII (F7; rs6046), Methylenetetrahydrofolate Reductase (MTHFR; rs1801133), Angiotensin Converting Enzyme (ACE; rs4340) Angiotensinogen (AGT; rs699), and Nitric Oxide Synthase 3 (NOS3; rs1799983) in a selected sample of 90 subjects from Caracas, Venezuela.

These gene variants have been correlated to diseases linked to aging, such as CVDs [5,6], and some of these variants have also been associated with longevity [2,4,6]. Knowledge of the genetic factors that may influence the susceptibility of an individual to develop CVDs would help identify, prevent, or slow down the disease. Additionally, early diagnosis of these diseases can be used to plan personalized treatments to avoid the progression of these and other diseases and favor healthy aging.

MATERIALS AND METHODS

Subjects

The sample comprised 90 randomly selected individuals unrelated to the "Centro Médico Antienvejecimiento" (CMA, Caracas, Venezuela), whose biological age was determined. This sample was classified into two groups: i) 30 control individuals, in which the biological age was equal or under the chronological age, and ii) 60 patients, which were subdivided into 30 patients with aging grade 1 (G1), in which the biological age was between 1 and 14 years over their chronological age, and 30 patients with aging grade 2 (G2), in which the biological age was between 15 and 28 years over their chronological age.

Determination of the biological age

The biological age was estimated using various biological and anthropometrical parameters such as body weight, body mass index (BMI), body fat percentage, stimuli response time, accommodation reflex, static balance, skin elasticity, and blood pressure. By comparing the estimated biological age with the chronological age, which corresponds to the time that has passed since the individual´s birth, the subjects were classified into different aging grade groups [16].

Blood Sampling

Peripheral blood was collected from all subjects after obtaining their signed consent. A standard *proforma* was filled up with their personal information, having particular emphasis on age, gender, smoking habit (current smokers or non-smokers), presence of hypertension (defined as a systolic blood pressure of at least 140 mm Hg and/or diastolic blood pressure of at least 90 mm Hg), diabetes mellitus (defined by a blood glucose level of at least 6.93 mmol/L) and obesity (BMI over 30).

Genes and Polymorphisms Studied

We evaluated the relationship between accelerated aging and different genotypes of 13 polymorphisms in the following genes: Apolipoprotein B (APOB; rs693), Apolipoprotein E (APOE; rs429358 and rs7412), Cholesteryl Ester Transfer Protein (CETP; rs1800775), Paraoxonase 1 (PON1; rs662), Fibrinogen Beta Chain (FGB; rs1800790 and rs1800791), Coagulation Factor II (F2; rs1799963), Coagulation Factor V (F5; rs6025), Coagulation Factor VII (F7; rs6046), Methylenetetrahydrofolate Reductase (MTHFR; rs1801133), Angiotensin Converting Enzyme (ACE; rs4340), Angiotensinogen (AGT; rs699), and Nitric Oxide Synthase 3 (NOS3; rs1799983).

Genotyping

Genomic DNA was extracted from total peripheral blood as described by Bowen y Keeney [17]. Details regarding identifying the polymorphisms for every specific gene are presented in Table 1.

Thirty cycles were performed following a denaturation step at 94°C for 5 min. Each cycle consisted of incubations at 94°C for 1 minute, annealing temperature for 1 minute, and 72°C for 1 minute. A final extension step was carried out at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 2.5% agarose gel containing SYBR Safe. Gel images were documented by using a digital camera equipped with ultraviolet filters.

The enzymatic digestions were carried out overnight at 37°C, and the digested samples were separated using an 8% polyacrylamide gel and visualized by silver staining [18].

Statistical Analysis

Values of continuous variables were expressed as means ± standard deviations (SD). The allelic frequency and the frequency of heterozygous and homozygous carriers of the studied polymorphisms were calculated in every subject group (control, G1, and G2). The number of cases and control subjects with a specific genotype was used to determine the risk, estimated as the Odds Ratio (OR) using the software PAST version 2.17c (2013) in both a recessive and dominant model. The OR represents the probability that the presence of accelerated aging occurs or not when we compared the patients with the control individuals, and it is defined as the ratio of occurrence of accelerated aging between the two groups [23]. Multivariable logistic curve regression analyses were used to monitor the risk of developing vascular disease as a result of accelerated aging under various conditions: genotype, age, gender, obesity, weight, body fat percentage, BMI, smoking, presence of hypertension, hypertriglyceridemia, hypercholesterolemia, and diabetes mellitus. The regression coefficients that were obtained represented the probability of suffering the disease because of the presence of the risk allele of the polymorphisms and the other variables studied. Statistical significance was set up at a $p \leq 0.05$.

RESULTS

General characteristics

The general and biological characteristics of the subjects conforming to the aging patient subgroups and control group are shown in Table 2. The patient subgroup 2 (G2) was mainly composed of young individuals, considering that the average chronological age (37.33 ± 11.8) was smaller than the other groups. The G2 group contained a higher percentage of individuals with smoking habits, diabetes, and obesity, as well as high blood pressure values. However, a higher percentage of individuals with hypercholesterolemia was observed in the control group (Control).

Genotyping

Except for the polymorphism in the APOB gene, all alleles and genotypes in the control group were within the Hardy-Weinberg equilibrium (data not shown).

Table 1
Detection techniques employed to determine genotype in the different polymorphism studied.

Gene	Polymorphism	Variant Type	Detection Technique	Primers Sequence	Ta	Possible Alleles	Reference
APOB	rs693	SNV	PCR-RFLP	3´-GATGAAACCAATGACAAAATCC-5´	58°C	G/A	19
				3´-AACAGTGAACCCTTGCTCTACC-5´			
APOE	rs429358	SNV	PCR-RFLP	3´-AGACGCGGGCACGGCTGTCCAAGGA-5´	62 °C	T/C	19
				3´-CCCRCG CGGGCCCCGGCCTGGTACAC-5´			
APOE	rs7412	SNV	PCR-RFLP	3´-AGACGCGGGCACGGCTGTCCAAGGA-5´	62°C	C/T	19
				3´-CCCRCGCGGGCCCCGGCCTGGTACAC-5´			
CETP	rs1800775	SNV	PCR-RFLP	3´- AGAATTGAAATGCCACAGACATTCC-5´	57°C	T/C	20
				3´-CCTTGATATGCATAAAATAACTCTGG-5´			
PON1	rs662	SNV	PCR-RFLP	3´- TTGAATGATATTGTTGCTGTGGGACCTGAG-5´	65°C	T/A/ C/G	19
				3´-CGACCACGCTAAACCCAAATACATCTCCCAGAA-5´			
FGB	rs1800790	SNV	PCR-RFLP	3´- GGTCTTTCTGATGTGTATT-5´	55°C	G/A	20
				3´-CTATTATTCTTTCTTGGTCTA-5´			
FGB	rs1800791	SNV	PCR-RFLP	3´-GTGTTCCTATTGATTCTTCTTGTAGG-5´	55°C	G/A	20
				3´-AATGAGGCCCATTTTCCTTGAAATT-5´			
AGT	rs699	SNV	PCR-RFLP	3´-GATGCGCACAAGGTCCTCTG-5´	61°C	T/C	19
				3´-CAGGGTGCTGTCCACACTGGCTCGC-5´			
F7	rs6046	SNV	PCR-RFLP	3´-CAGTCACGGMAGGTGGGAGAC-5´	56°C	G/A/ C/T	20
				3´-GGGGTAATTGACGTCTTCTT-5´			
MTHFR	rs1801133	SNV	PCR-RFLP	3´-GCCTCTCCTGACTGTCATCC-5´	61°C	C/T	21
				3´-CCCTTTTGGTGATGCTTGTT-5´			
NOS3	rs1799983	SNV	PCR-RFLP	3´-CATGAGGCTCAGCCCCAGAAC-5´	59°C	G/T	22
				3´-AGTCAATCCCTT TGGTGCTCAC-5´			
ACE	rs4340	Indel	PCR	3´-CTGGAGAGCCACTCCCATCCTTTCT-5´	58°C	Ins/ Del	19
				3´-GACGTGGCCATCACATTCGTCAGAT-5´			
				3´-TGGGACCACAGCGCCCGCCACTAC-5´ *	67°C		
				3´-TCGCCAGCCCTCCCATGCCCATAA-5´ *			
F2	rs1799963	SNV	ASPCR	3´-CACTGGGAGCATTGAGGCGC-5´	59°C	G/A	19
				3´-ATGAATAGCAATGGGAGCATTGAGGATT-5´			
				3´-ATGTGTTCCGCCTGAAGAAGTGGA-5´			
				3´-CCCACCTTCCCCTCTCTCCAGGCAAATGGG-5´ **			
				3´-GGGCCTCAGTCCCAACATGGCTAAGAGGTG-5´ **			
F5	rs6025	SNV	ASPCR	3´-CAAGGACAAAATACCTGTATTCAT-5´	58°C	C/A/T	19
				3´-CAAGGACAAAATACCTGTATTCTTT-5´			
				3´-GGCAGGAACAACACCATGAT-5´			
				3´-CCCACCTTCCCCTCTCTCCAGGCAAATGGG-5´ **			
				3´-GGGCCTCAGTCCCAACATGGCTAAGAGGTG-5´ **			

Abbreviations: PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; PCR: Polymerase Chain Reaction; SNV: Single Nucleotide Variant; In/Del: Insertion-Deletion; ASPCR: Allele Specific Polymerase Chain Reaction; Ta: Annealing Temperature. *Used for genotype verification; **Used as internal control.

Table 2
General and biological characteristics of the population.

Variables	Control (*n*=30)	G1 (*n*=30)	G2 (*n*=30)
Chronological age (X ± SD)	55.07 ± 8.08	50.43 ± 8.52	37.33± 11.8
Differential age (Biological age-Chronological age) (X ± SD)	6.58 ± 4.89	6.87 ± 3.94	21± 10.8
Mode (years)	59	48	27
Female (%)	83.3	90	70
Presence of smoking habits (%)	30	33.3	43.3
Presence of diabetes (%)	0	0	6.7
Presence of obesity (%)	0	26.7	36.7
Presence of hypercholesterolemia (%)[1]	43.3	40	23.3
Presence of hypertriglyceridemia (%)[1]	16.7	26.7	23.3
Body weight (Kg)	58.95 ± 8.25 (*n*=29)	69.18 ± 13.62 (*n*=30)	74.27 ± 18.81 (*n*=30)
Body fat percentage (%)	28.84 ± 13.28 (*n*=29)	37.93 ± 9.99 (*n*=29)	36.27 ±11.67 (*n*=30)
Body Mass Index (kg/m^2)	22.91 ± 2.98 (*n*=29)	26.34 ± 4.04 (*n*=27)	27.21 ± 5.42 (*n*=29)
Stimuli response time* (cm)	17.93·20.14·22.54 (*n*=28)	15.40·19.43·17.80 (*n*=30)	15.48·16.24·19.21 (*n*=29)
Accommodation reflex** (cm)	20.76 ± 8.15 (*n*=29)	21.07 ± 10.26 (*n*=29)	17.48 ± 4.65 (*n*=29)
Static balance *** (s)	7.86 · 5.90 · 7.14 (*n*=29)	4.45 · 4.45 · 6.10 (*n*=29)	6.07 · 4.03 · 5.93 (*n*=29)
Skin elasticity (s)	29.02 ± 41.65 (*n*=29)	12.28 ± 23.32 (*n*=30)	6.24 ± 12.37 (*n*=30)
Blood pressure (mmHg) Systolic/ Diastolic	124.86/79.21 (*n*=29)	127.21/77.86 (*n*=29/28)	132.93/81.50 (*n*=30)

[1]Values came from answering "yes" in the questionnaire regarding the presence of hypercholesterolemia and hypertriglyceridemia. *n*<30 indicates that results couldn't be obtained for all individuals for a specific variable while all the others consider the 30 individuals' population. *Values resulted from three measurements of the response speed of the upper extremities due to visual stimuli. **Values represent the distance between the eye and the text when the text can still be focus correctly. ***Time in which the patient started to oscillate or swing with the eyes close.

The frequency of the risk allele of the rs1800790 polymorphism (f: 0.15; CI: 0.13 – 0.17) in the FGB gene, rs1799963 polymorphism (f: 0.02; CI: 0.03 – 0.01) in the F2 gene, rs6025 polymorphism (f: 0.03; CI: 0.05 – 0.02) in the F5 gene, rs1801133 polymorphism (f: 0.30; CI: 0.27 – 0.33) in the MTHFR gene, and rs4340 polymorphism (f: 0.45; CI: 0.42 – 0.48) in ACE gene was more significant in the G2 group than in the control group. The frequency of the risk allele of the rs1799983 polymorphism (f: 0.30; CI: 0.245 – 0.354) in the NOS3 gene and rs662 polymorphism (f: 0.37; 0.34 - 0.40) in the PON1 gene was higher in the G1 group than in the control group. Finally, the risk allele frequency of the rs429358 and rs7412 polymorphisms in the APOE gene was greater in

the control group (f: 0.21; CI: 0.16 - 0.25) than in the other groups.

Fig. 1 shows schematically the OR value calculated for each polymorphic variant in the G1 and G2 subgroups. The associated risk calculated using the OR for the presence of the risk allele of the rs1800790 polymorphism in the FGB gene showed a 41% higher tendency to exhibit an accelerated aging (OR: 1.41; CI: 0.44-4.45) (Fig. 1), but these results were not statistically significant (p>0.05). Regarding the risk allele of the rs1799963 polymorphism in the F2 gene and of the rs6025 polymorphism in the F5 gene, we were unable to determine the OR value since the risk allele was only present in the G2 group. However, these two polymorphisms have been established as independent factors for vascular risk.

Moreover, the CC genotype for the rs1800775 polymorphism in the CETP gene was associated with a 62% increased risk of accelerated aging (OR: 1.62; CI: 0.40- 6.40; p>0.05). The calculated OR value for the rs662 polymorphism in the PON1 gene suggested that the occurrence of the risk allele is associated with a 15% increase in the risk of having accelerated aging (OR: 1.15; CI: 0.4-3.26; p>0.05) (Fig. 1). Also, the presence of the risk allele of the APOE gene was not associated with an increased risk of showing an accelerated aging (Fig. 1). All these results were not statistically significant (p>0.05).

Regarding the presence of the TT genotype for the rs1801133 polymorphism in the MTHFR gene, it was observed an increase by two times in the risk of exhibiting accelerated aging (OR: 2.07; CI: 0.18-24.15; p>0.05), however, this result was not statistically significant.

Similarly, the presence of the Del allele for the rs4340 polymorphism in the ACE gene was also associated with a two-time increase in the risk of exhibiting accelerated aging (OR: 2.07; CI: 0.18-24.15; p>0.05), showing no statistical significance. The risk was increased up to ten times (OR: 10.6; CI: 1.23-90.67; p<0.05) by the presence of the homozygote genotype for the risk allele displaying statistical significance.

The presence of the CC genotype for the rs699 polymorphism in the AGT gene was associated with a two time-increase in the risk of exhibiting accelerated aging (OR: 2.13; CI: 0.62-7.39; p>0.05) being this result not statistically significant.

Lastly, the presence of the T allele for the rs1799983 polymorphism in the NOS3 gene was associated with a 96% increase in

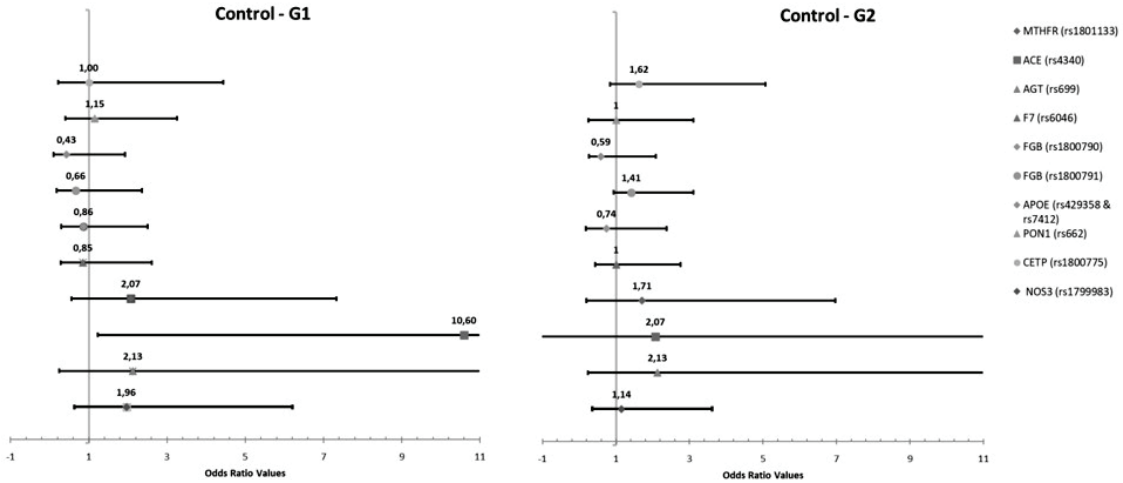

Fig. 1. OR values calculated comparing the control group with the G1 (Left) and G2 (Right) groups; with every value it shows the error bars which represent the confidence interval in each case.

the risk of exhibiting accelerated aging (OR: 2.13; CI: 0.62-7.39; p>0.05) being this result not statistically significant.

A multivariable statistical analysis was carried out to determine the association between the development of accelerated aging and each one of the genetic and environmental variables that define the sample. Table 3 shows the variables that were taken into consideration for the analysis. In the G1 subgroup, the environmental and genetic factors that proved to be associated with the development of an accelerated aging process were obesity, hypercholesterolemia and hypertriglyceridemia, age, BMI, APOE rs429358 and rs7412 polymorphisms, CETP rs1800775 polymorphism, FGB polymorphism rs1800790 and MTHFR polymorphism rs1801133. In the G2 subgroup, the environmental and genetic factors associated with accelerated aging were age, sex, body mass index, hypertriglyceridemia, smoking, systolic hypertension, F7 polymorphism rs6046, and MTHFR polymorphism rs1801133.

DISCUSSION

Recent studies in Latin American populations have shown the existence of differential ancestral contribution patterns between and within groups, which correlate with the indigenous population density before the conquest of America and with the current demographic growth patterns in these regions [24]. This agrees with genetic studies carried out in Venezuela, based on the analysis of blood group polymorphisms and DNA polymorphisms, which have revealed that, as in other Latin American countries, the conquest and colonization processes generated very heterogeneous populations. In general, the genetic component that prevails in these studies is the Mediterranean European, followed by the indigenous and, to a lesser extent, the African, and also with a marked inter- and intra-regional difference [25]. Specifically, for the population of Caracas, in a study carried out by Martínez *et al.* [26], who performed the analysis of five autosomal markers found in the high socioeconomic stratum, the European component (0.78) was found in a higher proportion than Sub-Saharan African, which was almost negligible (0.06); while for the low socioeconomic level, the Sub-Saharan, European, and Amerindian components were 0.21, 0.42 and 0.36, respectively. Therefore, to conduct genetic studies in a highly heterogeneous population like ours, it is imperative to understand the high degree of genetic variability of the different ethnic groups that inhabit the territory.

Attributable to the impact of the genetic constitution of any individual in the development of a particular phenotype, the sum of the genetic alterations or risk alleles of different genes can help us elucidate the effect of these in the evolution of the disease through molecular diagnosis. Likewise, the molecular diagnosis of risk alleles associated with vascular risk can help supplement the results of biochemical and clinical analyses and therefore provide an answer or an explanation to a disease or any given family history.

With the multivariable analysis we confirmed and demonstrated that age is directly related to accelerated aging, because as the years pass by diminishes the organism capacity to maintain homeostasis causing tissue failure and malfunction of the regulation systems, which in turn may produce an increase in the susceptibility to suffer various diseases. We also found that hypercholesterolemia and hypertriglyceridemia are risk factors associated with the development of accelerated aging, suggesting that the presence of high levels of cholesterol and triglycerides promote the rapid deterioration of the organism, mainly at the vascular level, given that high values of cholesterol and triglycerides are related to the formation of atheroma and the unfolding of atherosclerosis [27,28].

Other variables associated with an accelerated process of aging were obesity and

Table 3
Association between the different genetic and environmental factors and the development of vascular risk through an accelerated aging.

Variable	Association with G1 (n=60)	Association with G2 (n=60)
Sex	Not Associated	**Associated***
Diabetes *mellitus*	--	Not Associated
Obesity	**Associated***	Not Associated
Hypercholesterolemia	**Associated****	Not Associated
Hypertriglyceridemia	**Associated***	**Associated***
Smoking	Not Associated	**Associated***
Age	**Associated****	**Associated*****
Weight	**Associated****	Not Associated
Body fat percentage	Not Associated	Not Associated
BMI	Not Associated	**Associated*****
Systolic hypertension	Not Associated	**Associated*****
Diastolic hypertension	Not Associated	Not Associated
APOB (rs693) Allele 7545T	**Associated***	Not Associated
APOE (rs429358 y rs7412) Allele 388C y 526C	Not Associated	Not Associated
CETP (rs1800775) Allele -656C	Not Associated	Not Associated
PON1 (rs662) Allele 575G	**Associated***	Not Associated
FGB (rs1800790) Allele -455A	Not Associated	Not Associated
FGB (rs1800791) Allele -854[a]	Not Associated	Not Associated
F2 (rs1799963) Allele 20210A	--	Not Associated
F5 (rs6025) Allele 1691A	--	Not Associated
F7 (rs6046) Allele 10976G	Not Associated	Not Associated
MTHFR (rs1801133) Allele 655T	Not Associated	**Associated***
ACE (rs4340) Allele Del	Not Associated	Not Associated
AGT (rs699) Allele 803C	Not Associated	Not Associated
NOS3 (rs1799983) Allele 894T	Not Associated	Not Associated

* p< 0,05; ** p< 0,01; *** p< 0,001; -- excluded from the model.

BMI, being BMI a vascular risk indicator that is used universally to detect overweight and obesity. Obesity promotes the appearance of alterations in various mechanisms of hormonal regulation, being hyperinsulinemia and leptinemia, some of the most common hormonal alterations associated with obesity [29]. Moreover, obesity accelerates the aging of adipose cells increasing the formation of reactive oxygen species in fat cells, promoting inflammatory processes and insulin resistance. Aging and obesity not only favor the deregulation of the metabolism but also promote the development of hypertension, dyslipidemia, and cardiovascular complications [29,30].

Hypertension is known as a risk factor for the development of cardiovascular disease. In the present study, our results suggest that it is associated with accelerated aging, meaning that the deterioration of the vascular endothelium due to high blood pressure constitutes a significant risk factor for accelerating this process [31].

Regarding the allelic variant 20210A of the rs1799963 in the F2 gene, while an association could not be established in this investigation, this gene has been linked with premature aging, being reported with a decreased frequency in the middle to advanced-age individuals [32]. In turn, the allelic variant 1601A of the rs6025 polymorphism in the F5 gene has been associated as an independent factor that indicates blood hyper-coagulation.

The presence of this risk allele causes the production of a factor V protein that cannot be degraded by the activated protein C (APC), and consequently, an increase in the amount of factor V is obtained in the blood [33]. It is worth noting that similarly to rs1799963 in F2, the allelic frequency for the 1601A variant in F5 has been reported to decrease in individuals with advanced age (centenary individuals) [34]. Regarding the rs6046 polymorphism in the F7 gene, the presence of the mutant allele 1172A is beneficial for the prevention of cardiovascular diseases or the formation of vascular thrombus since this mutant allele produces a protein that has a deficient interaction with the tissue factor, causing a lower initiation of the secondary hemostasis [35].

Concerning the genes involved in lipid metabolism, it has been reported that the presence of the allele E4 in the APOE gene promotes the appearance of cardiovascular diseases, which is why many studies address the relationship between this gene and aging [7,8]. Although the OR values obtained here do not suggest that this risk allele is associated with accelerated aging, the multivariable analysis showed that the allele E4 is one of the risk factors that participate in the fast deterioration of the organism. This allele has been shown to decrease its frequency in advanced age centenary groups; in turn, the allele E2 which has been reported to have a protective effect, has been reported to increase in these groups, suggesting an association with the aging process [36]. The discrepancy might be because the multivariable analysis considers the interaction of various genetic variables in conjunction with environmental variables related to aging.

The risk allele -656C for the rs1800775 polymorphism in the CETP gene was found to be associated with accelerating aging. This polymorphism is linked to the transcription levels of the CETP gene, whose protein product is involved in the transference of cholesterol and other lipids from HDL to LDL and VLDL [37]. Thus, the risk allele -656C, which is the ancestral allele, has been associated with a higher transcription rate. The presence of this protein diminishes the cholesterol levels in the HDL [38], causing an increase in the risk of developing cardiovascular diseases and, therefore, increasing the risk of presenting an accelerated aging process.

Some studies have demonstrated that the antioxidant capacity of HDL and the enzymatic activity of PON1 decreases with age [38]. Likewise, the risk allele for the rs662 polymorphism in the PON1 gene has been associated with the reduction of the enzyme

arylesterase activity, which negatively affects the antioxidant capacity of HDL [39]. This could explain the relationship found in this study between the risk allele for the rs662 polymorphism and the accelerated process of aging. In this line of thought, the risk allele frequency has also been reported to decrease in nonagenarian and centennial individuals [14].

Concerning the genes involved in cardiovascular homeostasis, the allelic variant 655T of rs1801133 polymorphism in the MTHFR gene has been linked to a decrease in MTHFR enzymatic activity which in turn causes an increase in blood homocysteine [40], being reported a decrease of up to 70% in homozygotes individuals for the risk allele [41]. This homocysteine level change has been associated with a decline in physical functions. Proposed mechanisms regarding this outcome include direct endothelial damage caused by the generation of potent reactive oxygen species not only in endothelial tissues but also in proteins and DNA, along with an increase in the amount of telomere length loss[41]. The rs1801133 polymorphism has also been related to longevity with a decreased allelic frequency in centenary individuals for the risk allele 655T [42].

The allelic variant Del of rs4340 polymorphism in the ACE gene has been associated with an increase in plasmatic and cardiac ACE activity, causing an overexposition to high levels of Angiotensin II that on its own has been linked to a diverse repertoire of cardiovascular diseases such as hypertension and myocardial infarction[43]. It is worth noting that, while there has been a significant quantity of studies concerning this polymorphism, the mechanism by which it operates has not been elucidated completely; this due to the intronic nature of this polymorphic variant.

The allelic variant 803C of rs699 polymorphism in AGT gene has been reported to increase plasmatic AGT protein production up to 20% in homozygote individuals for the risk allele [44]; this increase has been adjudicated to a linkage disequilibrium between rs699 and rs5051 polymorphisms the last one located in the gene promoter imposing an augment in transcriptional activity of the AGT gene [45]. While this polymorphism has been linked with cardiovascular homeostasis alterations, few studies associate this variant with accelerated aging, some postulating it as a protection factor [46,47]. In contrast, others suggest there is no relationship at all. Similarly, to rs4340 polymorphism in ACE, the increase in AGT transcription rate is deeply linked to an increase in Angiotensin II levels, promoting the risk of developing cardiovascular diseases. However, in this case, the increased allelic frequency of the risk allele reported in advanced-age individuals suggest the existence of a protection element conferred by this variant; this protection has been postulated to exist due to the role as a skeletal muscle growth factor of angiotensin II [46–48].

Lastly, the allelic variant 894T of rs1799983 polymorphism in the NOS3 gene has been associated in previous studies with an increased probability of developing cardiovascular events and preeclampsia due to alterations in the NOS3 enzyme function [49]. In a previous case-control study we measured the nitric oxide concentration in serum through non-enzymatic colorimetric assays reporting that individuals carrying the 894T allelic variant showed a reduction of 46.47% in nitric oxide serum levels when compared with GG homozygote individuals. These results were statistically significant and showed that the presence of 894T can contribute to an increased risk of developing hypertension of up to four times in TT homozygote individuals (OR: 4.17; CI: 1.06-19.11; $p<0.05$) when compared to GG homozygote individuals [19].

In summary, according to the results of the OR analyses, the polymorphic variants considered in this study were not associated with the development of accelerated aging in a statistically significant way, except for the rs4340 polymorphism in the ACE gene

(recessive model). These results could be related to the sample size since it is essential to obtain statistically significant results. Nevertheless, the multivariable analysis showed a significant association between the variable's obesity, hypercholesterolemia and hypertriglyceridemia, age, body mass index, APOB rs693, and PON1 rs662 polymorphism with the development of accelerated aging in the G1 group. Also, the variables sex, hypertriglyceridemia, smoking, age, body mass index, systolic hypertension, and MTHFR rs1801133 polymorphism were linked with accelerated aging in the G2 group. Our findings showed that genetic and environmental factors are associated with an accelerated aging process.

The knowledge of the genetic profile is of great importance to complement the biochemical and clinical information of the individuals. The integral consideration of these parameters will allow the application of preventive antiaging medicine in an individualized way by making nutritional recommendations and modifications in lifestyle to reduce the incidence of diseases typically associated with age, such as cardiovascular diseases, and promote healthy aging.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Author's Contributions

CC direction and designed research. CA, AB, MAM, AV and AA performed research and analyzed data. CC, AP and AC wrote the paper. JCM analyzed the clinical data. All authors read and approved the final manuscript.

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Phenotypic and genotypic study of antibiotic-resistant *Escherichia coli* isolates from a wastewater treatment plant in Zulia state, Venezuela.

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Keywords: antibiotic re sistance; *E. coli;* wastewater; phylogroups.

Abstract. Antibiotic-resistance in bacteria is a global health problem, and wastewater treatment plants can play a role in their dissemination. In this work, we used PCR and plasmid transformation to characterize antibiotic-resistance and the phylogenetic groups of *Escherichia coli* isolated from a treatment plant in Zulia, a state in western Venezuela. Thirty-six bacteria isolates were analyzed, of which 27 resulted resistant by disc diffusion primarily to tetracycline and sulfisoxazole but also to trimethoprim, chloramphenicol, ampicillin, and ciprofloxacin. The *tetA*, *sul2*, *floR,* and *blaTEM* resistance genes were frequently present and, in most cases, transferable. *dfrA12*, *tetB*, *sul3*, *sul1,* and *aadA2* genes also were detected. The integrase gene *intI1* was common in multidrug-resistant isolates. These results suggest that *E. coli* from the treatment plant is a reservoir of antibiotic-resistance genes, which signify a potential health threat. Additionally, the phylogroup C was predominant, which is unusual and may represent an adaptation of this group to environmental conditions or perhaps the most frequent phylogroup entering from the influent.

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Estudio fenotípico y genotípico de aislados de *Escherichia coli* resistentes a antibióticos de una planta de tratamiento de aguas residuales del estado Zulia, Venezuela.

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Palabras clave: genes de resistencia; *E. coli;* aguas residuales; filogrupos.

Resumen. La resistencia bacteriana a antibióticos es un problema de salud global y las plantas de tratamiento pueden jugar un papel en su diseminación. En este trabajo caracterizamos, mediante PCR y transformación de plásmidos, la resistencia a antibióticos y los grupos filogenéticos de *Escherichia coli* aislada de una planta de tratamiento *en* el estado Zulia, Venezuela. Se analizaron 36 aislados bacterianos, de los cuales 27 resultaron resistentes por difusión en disco principalmente a tetraciclina y sulfisoxazol, pero también a trimetoprim, cloranfenicol y ampicilina. Los genes *tetA*, *sul2*, *floR* y *blaTEM* se encontraron comúnmente en los aislados resistentes y fueron en la mayoría de los casos transferibles; adicionalmente se detectaron los genes *dfrA12*, *tetB*, *sul3*, *sul1* y *aadA2*. El gen de integrasa *intI1* se detectó en la mayoría de los aislados multiresistentes. Estos resultados sugieren que *E. coli* en la planta de tratamiento es un reservorio de genes de resistencia a antibióticos, lo que significa una amenaza potencial para la salud. Adicionalmente predominó el filogrupo C, lo que es inusual y podría deberse a una adaptación de este a las condiciones ambientales o podría ser el mayoritario en el influente.

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INTRODUCTION

The indiscriminate use of antibiotics in human and animal medicine, as well as for prophylaxis and growth promotion in animal husbandry, threatens to reduce the effectiveness of these fundamental drugs. Antibiotic-resistant bacteria, although occurring naturally, are also released into the environment, where they may outcompete sensitive bacteria due to the presence of antibiotics and other chemical contaminants that are also released into the environment. The resistance genes in these bacteria can then be transferred to other pathogenic and non-pathogenic bacteria, thereby increasing the environmental reservoir of resistant bacteria and genetic resistance determinants.

Escherichia coli is a commensal bacterium that inhabits the intestines of humans and other animals and is often used to indicate environmental fecal contamination. Some *E. coli* are pathogens that can cause urinary tract, gastrointestinal or nosocomial infections. Antibiotic-resistance in environmental *E. coli* has also been proposed as an indicator to monitor the extent of antibiotic resistance in the environment [1].

The bacterial load of wastewater discharged into natural water bodies is significantly reduced by treatment plants. However, these plants may also promote the spread of antibiotic-resistant bacteria and resistance genes by providing favorable conditions for increasing the relative abundance of resistant bacteria and the horizontal transfer of

the genes conferring this resistance [2]. Although there are few treatment plants in Venezuela and much of the wastewater is discharged directly into the environment, there is a wastewater treatment plant in the state capital Maracaibo, located in the "El Tablazo" Petrochemical Complex of the Miranda municipality of Zulia state. The plant was designed so that the petrochemical industry could reuse some of its effluent water while the rest would be discharged into the giant Lake Maracaibo.There have been very few studies on antibiotic-resistance in bacteria isolated from raw or treated wastewater in Venezuela, but such studies represent essential surveillance measures to assess the extent of antibiotic-resistance in the environment and plan corrective strategies. Accordingly, we set out to perform a phenotypic and molecular study of antibiotic resistance in *E. coli* isolates from the wastewater treatment plant mentioned above.

MATERIALS AND METHODS

E. coli was isolated from water samples of the "El Tablazo" treatment plant (Miranda Municipality, Zulia State) collected from May to October 2012 for a microbiological quality evaluation. The system includes a pre-treatment to remove solids followed by absorption, biological oxidation, and a first chlorine injection. The water is then transported to the plant at "El Tablazo" and subjected to physical and biological treatment based on reactors where dissolved organic matter is removed, followed by a secondary settling. Then a first effluent is discharged into Lake Maracaibo. Another portion of the water to be used by the petrochemical complex is treated with a flocculant and chlorine.

Sampling sites were four different sections of the treatment plant: pre-treated influent (Site1); after physical processing (Site 2); after biological processing (effluent to the Maracaibo lake, Site 3); and the chlorine disinfection point (Site 4). There were six water samples from Site 1 and Site 4, four from Site 2, and five from Site 3.

The water samples were collected in sterile bottles and processed according to the procedures described in the Standard Methods for examination of Water and Wastewater to determine coliform by the fermentation technique [3]. Samples showing growth in EC broth were streaked onto EMB agar to select typical *E. coli* colonies, which were sub-cultured in nutrient agar tubes for transport. Re-isolation was performed on McConkey agar, and colonies were cultured in LB broth and then stored in 20% glycerol at -80°C. All assays were performed on the bacteria regrown from the frozen stocks.

Biochemical identification and antibiotic susceptibility testing

Bacterial isolates were first identified with the following biochemical tests: TSI, indole-motility, methyl red, Voges Proskauer, citrate, and urea.

Resistance was assessed with the Kirby Bauer disc diffusion method, using commercial discs with the following antibiotics: tetracycline 30 μg (TE), ampicillin 10 μg (AMP), ampicillin-sulbactam 10/10 μg (SAM), sulfisoxazole 250 μg (SF), chloramphenicol 30 μg (C), trimethoprim 5 μg (W), trimethoprim-sulfamethoxazole 1.25 μg /23.75 μg (SXT), ciprofloxacin 5 μg (CIP), aztreonam 30 μg (ATM) and imipenem 10 μg. *E. coli* ATCC 25922 was used as an antibiotic-susceptible control strain. The results were interpreted according to CLSI guidelines [4].

PCR amplification

PCR was used to confirm the bacteria as *E. coli*, determine phylogroups, and detect the presence of resistance genes and the integrase gene *intI1*. All PCR reactions were performed on boiled bacterial lysates, using Taq DNA polymerase with ThermoPol buffer (NEB), following the manufacturer's instructions, using previously reported specific primers, some of which were modified

as indicated below. PCR was performed to amplify genes conferring resistance to tetracycline (*tetA* and *tetB*) [5], sulfisoxazole (*sul1*, *sul2*, and *sul3*) [6-8], chloramphenicol (*floR* and *cat*) [6], ampicillin (*blaTEM*) [9] and trimethoprim (*dfrA12* and *dfrA7&17*) [10, 11]. The detection of the *intI1* integrase was with primers described by Moura[12]. In contrast while the phylogroup identification was performed using the quadruplex plus group C specific PCR described by Clermont *et al.* [13] Negative controls without template DNA were included in each PCR assay. A subset of the PCR products were confirmed by DNA sequencing (Macrogen, Korea) and used as positive controls for the detection of resistance genes and *intI1*, as well as the determination of phylogroups.

The reactions were performed with previously reported primers and conditions or with the following variations: Sul1-R. 5'-TGATCTAACCCTCGGTCTCT-3' temperature of annealing (Ta) 56°C, blaTEM-F 5'-GCATACACTATTCTCAGAATGA-3' blaTEM-R 5'-CTCACCGGCTCCAGATTTAT-3' Ta 56°C, dfr7&17-F 5'-CATTTGACTCTCTATGGGTGTTC TT-3' Ta 58°C.

To avoid analyzing duplicate resistant isolates, REP-PCR was performed on isolates showing the same phenotype and genotype, using the REP1 and REP2 primers as previously described [14]. *E. coli-specific* PCR was performed using primers to amplify *rrs* (75F and 619R) or *gad* [15, 16], with *E. coli* XL1-blue as a positive control.

Transformation and conjugation assays

Transferability of the detected antibiotic resistance genes was assessed by heat shock transformation using transformation competent *E. coli* DH5α as the recipient strain and plasmid DNA obtained by alkaline lysis from all isolates resistant to tetracycline, ampicillin, chloramphenicol, or trimethoprim in which PCR had detected a resistance gene. Transformants were selected on LB agar plates containing either carbenicillin (50 μg/mL), ampicillin (32 μg/mL), tetracycline (30 μg/mL), chloramphenicol (30 μg/mL), or trimethoprim (20 μg/mL) as appropriate. Phenotypic resistance was confirmed for each transformant, and the presence of plasmid DNA and the relevant resistance genes were verified.

In some cases, the capacity for conjugation was assessed in liquid medium using *E. coli* J62-2 as the recipient strain. Selection was performed on LB agar plates supplemented with tetracycline (30 μg/mL) and rifampicin (50 μg/mL). Transconjugants were confirmed by phenotypic resistance, amplification of the same resistance gene detected in the donor, and ERIC-PCR with primers described by Versalovic *et al.* [14].

RESULTS

Here, we characterized thirty-six isolates identified as *E. coli* with biochemical tests and identified as *E. coli* with biochemical tests and PCR amplification of rrs or gad. These isolates originated from the four sampling sites: 13 from Site 1, four from Site 2, nine from Site 3, and ten from Site 4.

Resistance phenotypes

As shown in Fig. 1, the highest frequency of resistance was to tetracycline and the lowest to ciprofloxacin and ampicillin-sulbactam, with intermediate prevalences of resistance to the other antibiotics tested, including ampicillin.

Twenty-four isolates (66.6%) were fully resistant to at least one antibiotic, corresponding to 7/13, 2/4, 7/9, and 8/10 isolates from sampling points 1 to 4, respectively (Table 1). Five of these 24 isolates (20.8%) also showed intermediate resistance to one or two additional antibiotics (two AMP-CIP, one CIP, and two SAM). Among the 12 remaining isolates, three had only intermediate resistance (two AMP and one TE), and nine (9/36, 25%) were fully sensitive to all antibiotics tested. There were also seven isolates (7/24, 29.1%) fully resistant to three or more antibiotics of different classes and

Table 1
Phenotypic and genotypic profiles of the bacterial isolates.

Sampling site	Resistance phenotype (FR/IR)	Resistance genotype	Phylogroup
1	TE	*tetB*	B1
1	TE-SF-W-TS	*tetA-sul2*	C
1	TE-SF-W-TS	*tetA-sul2*	C
1	TE-SF-W-TS/amp-cip	*tetA-sul2*	C
1	TE-SF- TS-C/amp-cip	*tetA-sul2-floR*	A
1	TE-SF-C/ cip	*tetA-sul2-floR*	A
1	TE-C	*tetA-floR*	A
1	amp	nd	B1
1	S	na	C
1	S	na	C
1	S	na	C
1	S	na	C
1	S	na	B2
2	TE	*tetA*	C
2	TE-SF-W-TS-AMP-C/sam	*tetA-sul1-blaTEM-floR-dfrA12-intI1*	C
2	S	*na*	A
2	S	*na*	A
3	TE	*tetA*	B1
3	TE-W	*tetA*	C
3	TE-SF-W- TS-AMP-C-CIP/sam	*tetA-sul3-blaTEM-intI1*	C
3	TE-SF-W-TS- AMP-SAM	*tetA-sul3-blaTEM-dfrA12-intI1*	C
3	TE-AMP	*tetB-blaTEM*	C
3	AMP-C	*blaTEM- floR*	B1
3	AMP-W	*blaTEM*	C
3	te	nd	C
3	S	na	A
4	TE	*tetA*	C
4	TE	*tetA*	A
4	TE-SF	*tetA*	B1
4	TE-SF	*tetA-sul2*	A
4	TE-SF	*tetA-sul2*	C
4	TE-SF	*tetA-sul2*	B1
4	TE-SF-W-TS-C	*tetA-sul3-intI1*	C
4	TE- W-C	*tetA- tetB- flor-intI1*	A
4	amp	nd	C
4	S	na	C

FR: Fully resistant, IR: intermediate resistance (lowercase), S: sensitive, nd: not determined, na: not apply. The abbreviations for the antibiotics are the same as in Fig. 1.

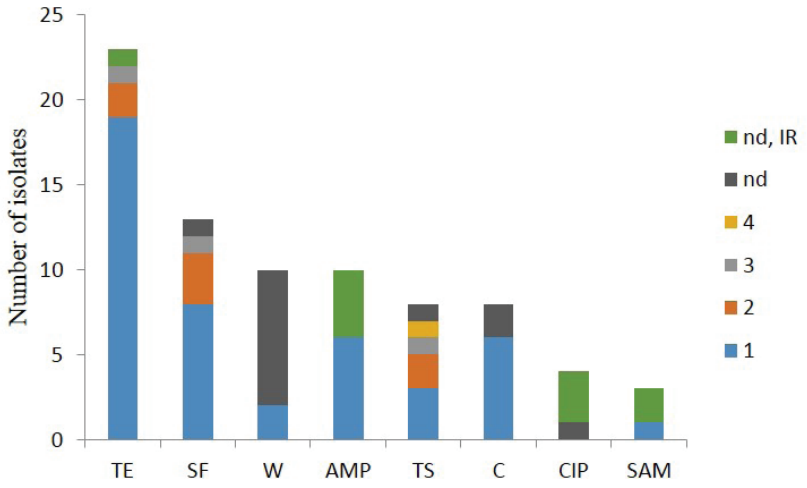

Fig. 1. Phenotypic and genotypic resistance per antibiotic. The numbers of phenotypically resistant isolates with corresponding genotypes (for full resistance) are indicated. Resistance genotypes are numbered from 1 to 4 according to the detection frequency from highest to lowest. 1: *tetA* (TE); *sul2* (SF and TS); *dfrA12* (W); *floR* (C); *or blaTEM* (AMP and SAM). 2: *tetB* (TE); or *sul3* (SF and TS). 3: *tetA-tetB* (TE); *sul1* (SF); *sul1-dfrA12*(TS). 4: *sul3-dfrA12* (TS). nd: Resistance genotype not determined, IR: intermediate resistance. TE, tetracycline; SF, Sulfisoxazole; W, trimethoprim; C, Chloramphenicol; TS, Trimethoprim-sulfamethoxazole; AMP, ampicillin; SAM, ampicillin-sulbactam; CIP, ciprofloxacin.

thus multi-drug resistant or MDR. Two of these originated from each of sampling Sites 1, 3, and 4, and one isolate was from Site 2 (see Table 1). All isolates were sensitive to aztreonam and imipenem.

Resistance genes

The genotypic resistance profiles and genes detected are described in Table 1. The most frequently detected genes were *tetA* (20/22) and *sul2* (8/12), while all isolates fully resistant to ampicillin contained *blaTEM* (Fig. 1). The *floR* gene was detected in most (6/8) of the chloramphenicol-resistant isolates, none of which contained the *cat* gene. The only amplified determinant associated with trimethoprim resistance was *dfrA12*, which was detected in just two of the ten trimethoprim-resistant isolates.

Of the seven MDR-resistant isolates, the *intII* gene was amplified from 5, representing 21% of the resistant isolates and 13.9% of total isolates (Table 1). In one of the isolates in which the *intII* gene was detected, amplification and sequencing with primers specific for conserved segments of class 1 integrons detected *dfrA12* and *aadA2*, which encodes an aminoglycoside adenyl transferase conferring streptomycin resistance.

Transfer of resistance determinants

Transformants were obtained from the isolated plasmid DNA of 20/24 resistant isolates. Most of the transformants (18/20) were recovered on media with tetracycline, while only 1/20 were recovered on media with chloramphenicol and 1/20 on media with trimethoprim. However, the major part of the genes conferring resistance to sulfisoxazole and chloramphenicol were co-transferred with the *tetA* gene (Table 2). Despite repeated attempts, no transformants were obtained from plasmid DNA isolated from the remaining four resistant isolates.

The *tetA* gene was transferred from almost all isolates in which it was detected (19/20). The *blaTEM* gene was transferred

Table 2
Antibiotic resistance profiles of transformants and transconjugants.

Genotype	Phenotype	Number of transformants (N=20)	Number of transconjugants (N=2)
tetA-sul2	TE-SF	8	-
tetA-sul3	TE-SF	1	-
tetA	TE	6	2
tetA-floR	TE-C	3	-
tetA- sul3- dfrA12	TE-SF-W	1	-
flor- blaTEM	C-AMP	1	-

from 1/6 of the isolates in which it was detected, the *sul* genes were transferred from 10/12 isolates (8 *sul2* and 2 *sul3*), *floR* from 4/6 and *dfrA12* from 1/2 isolates containing this gene. The *tetB* gene was not transferred under the conditions employed. The resistance genes most frequently detected in our isolates, *tetA* and *sul2*, hybridized with plasmid DNA isolated from most (18/20), or all isolates (8/8), respectively, in which the genes had been detected by PCR, confirming that they were carried on plasmids (not shown).

The transfer capability of the *tetA* gene was tested by conjugation experiments with five isolates in which the *tetA* gene was detected. Transconjugants were obtained from two isolates at the high frequencies of 7.5×10^{-3} y 1.95×10^{-2}, demonstrating that the tetA gene was carried on conjugative plasmids at least in these two isolates. One of these five isolates with the tetA gene generated neither transformants nor transconjugants; nevertheless, only scant plasmid DNA could be obtained from this isolate, perhaps because its plasmid was either very large or present at a very low copy numbers.

E. coli phylogroup analysis

Of the 36 bacterial isolates studied, 20 belonged to group phylogenetic C, nine to group A, six to group B1, and one to group B2 (Table 1).

DISCUSSION

In the present work, we analyzed phenotypic antibiotic-resistance, detected corresponding antibiotic-resistance genes, evaluated their transferability, and determined the phylogroups of 36 *E. coli* isolates obtained from a wastewater treatment plant. Similar to other studies of resistant *E. coli* isolates from wastewater and treatment plants [17-21], the most frequent resistance encountered was to tetracycline, sulfonamide, trimethoprim, and ampicillin. In contrast, resistance to the carbapenems and ciprofloxacin was infrequent or not detected. Our results differ from a previous phenotypic study of *E. coli* isolates from stabilization ponds in Maracaibo, Venezuela, that found a higher proportion of resistance for ampicillin (81.25%) followed by tetracycline and trimethoprim [21]. In comparison, a study of *E. coli* isolates from a treatment plant in Cumaná (Venezuela) found 73.3% and 23.3% of ampicillin and tetracycline resistance, respectively. These discrepancies may be due to differences in the treatment systems.

Although tetracycline and sulfonamide, to which we found medium to high frequencies of resistance (22% to 64%), have been, for the most part, replaced by newer agents in human medicine, they are still classified as highly important by the World Health Organization.[23] Ampicillin (28% resistance) is

still frequently used and considered critically important in human medicine, as are the antibiotics for which we found less frequent resistance (8% to 11%).

The antibiotic resistance we observed is common in *E. coli* isolated from healthy humans in low and middle-income countries [24], and the antibiotic-resistance in human isolates of *E. coli* has been correlated with the resistance in *E. coli* isolated from the local wastewater [25]. The resistance patterns may also be affected by selection or adaptation to the specific characteristics of the treatment plant [26].A high prevalence of the *tetA*, *sul2,* and *blaTEM* genes has been previously observed in *E. coli* isolated from other treatment plants.[17, 27] However, previous studies have predominantly found the *cat* gene in chloramphenicol-resistant isolates [17, 25], but most of our chloramphenicol resistant isolates carried the *floR* gene. In contrast, the *cat* gene was not detected.

We found the *intI1* gene in 13.9% of total isolates, similar to a study by Figueira et al. that found the gene in 22.3 % of *E. coli* isolates from treated wastewater [20]. Integrons contribute to the spread of multidrug resistance, and class I integrons are the most important in clinical isolates [28].

Horizontal transmission of plasmids carrying resistance genes is a crucial route for disseminating resistance. Similar to our results, a previous study of *E. coli* and other fecal coliforms isolated from treatment plants found that most plasmid transformants were resistant to tetracycline, followed by chloramphenicol and trimethoprim [29]. The resistance genes *tetA*, *blaTEM*, *tetB*, *sul*, *floR*, and *dfrA12* can be found on chromosomes, but as we observed, they are frequently carried on plasmids [30-35].

E. coli isolates can be classified into seven main phylogenetic groups: A, B1, B2, C, D, E, and F. Intestinal pathogenic *E. coli* strains have been associated with groups A, B1, and E, while extra-intestinal strains mainly belong to groups B2 and D[36]. However, Group C can also include human pathogenic strains, as shown in a study of human isolates from the USA and Europe, in which phylogroup C was associated with uropathogenic *E. coli*, although B2 and D predominated [37]. Jafari et al. found that group C was the most common (21.3%) among Shiga toxin-producing *E. coli* (STEC) patient isolates [38]. It has been suggested that some *E. coli* phylogroups, particularly groups A and B1 (or non-B2), are more prone to develop antibiotic resistance to traditional antibiotics and fluoroquinolones [39, 40].Most previous studies assigning *E. coli* phylogroups have used the triple PCR method [41] that identifies only four groups: A, B1, B2, and D. Several studies have found group A to predominate in wastewater, followed by D or B1[20]. Group A is also the phylogroup most frequently observed in human commensal isolates, followed by B2 group [42]. Researchers employing the quadruplex method plus the group C specific PCR, or in silico typing, have found that phylogroup C is less frequent than other phylogroups in samples from birds, humans, non-human mammals, domestic animals, wild animals, river and lake water[43-45]. Two studies on strains from wastewater found that group A or B2 predominated, while none or only 1% belonged to group C [17, 46].

Therefore, the presence of group C in most of our isolates differs from the findings in similar studies and could be due to geographical location or climate differences, as these factors may influence the distribution of phylogroups [45]. It is also possible that previous studies that used only the triple PCR method classified C group isolates as group A.

In conclusion, we observed *E. coli* isolates resistant to diverse antibiotics, including some clinically essential agents and found that many were associated with transferable genetic determinants and class 1 integrons. Also, in contrast to other studies, many of our isolates belonged to the phylogroup C. The characteristics of the isolates we studied may have been determined by the influent water, the nature of the treatment plant, and environmental conditions, but an evalu-

ation of the contribution of each of these aspects would require a much larger study with many more isolates. Similar studies should be repeated in the same treatment plant and undertaken in other treatment plants in Venezuela, and these studies should be extended to include untreated wastewater and focus on resistance to the newer, currently more commonly used antibiotics.

Conflict of interest

The authors declare that they have no conflict of interest.

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Effect of creatine kinase isoenzyme (CK-MB) on early prognosis after off-pump coronary artery bypass grafting.

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Keywords: coronary heart disease; beating coronary artery bypass grafting; myocardial enzymes; early prognosis.

Abstract. This study aimed to analyze the effect of elevated creatine kinase isozyme levels on early prognosis after off-pump coronary artery bypass (OPCAB) grafting. Based on the levels of creatine kinase isoenzyme (CK-MB), 116 patients were divided into two groups: one with a mild increase (n=85) and another group with a severe increase (n=31) in the enzyme. Clinical data, changes in CK-MB levels at 12, 24, and 48 hours after surgery, changes in left ventricular ejection fraction (LVEF) and left ventricular end-diastolic diameter (LVESD) before surgery, and seven days and three months after surgery were measured, and recorded. Also, the blood flow of the bridging vessel, vascular resistance, the diameter of the anterior descending branch, and the diameter of the distal target vessel were recorded during the operation (> 1.5 mm). A decrease in the level of LVEDD was recorded in both groups after the operation compared to the levels before. However, in the group with a mild increase in CK-MB, the LVEF after the operation increased compared to before the operation ($p<0.05$). The occurrence of angina pectoris 24 hours before surgery, high vascular resistance during surgery, and diameter of distal target vessel > 1.5 mm were related factors affecting the increase of CK-MB after surgery. The ratio of these factors was higher in the severe increase group than in the mild increase group ($p<0.05$). An increase in myocardial enzymes causes a slow recovery of myocardial function, so it can be used as a critical biological index to reflect the prognosis of patients.

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Efecto de la isoenzima de creatina quinasa (CK-MB) en el pronóstico temprano después de un injerto de revascularización coronaria sin circulación extracorpórea.

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Palabras clave: enfermedad cardiaca; injerto de revascularización coronaria con el corazón latiendo; creatine kinase isoenzyme (CK-MB); pronóstico temprano.

Resumen. Este estudio tuvo como objetivo analizar el efecto de los niveles elevados de la isoenzima de creatina quinasa (CK-MB) en el pronóstico temprano después de un injerto de revascularización coronaria sin circulación extracorpórea (OPCAB). Basados en el nivel de la isoenzima de creatina quinasa CK-MB, 116 pacientes fueron divididos en dos grupos: uno con un aumento leve de la enzima (n=85) y otro grupo con un aumento severo (n=31). Los datos clínicos, los cambios en los niveles de CK-MB a las 12, 24 y 48 horas posteriores a la cirugía, los cambios en la fracción de eyección del ventrículo izquierdo (LVEF) y el diámetro telediastólico del ventrículo izquierdo (LVESD) antes de la cirugía, 7 días y 3 meses después fueron medidos y registrados. Además, fueron registrados el flujo sanguíneo del puente venoso, la resistencia vascular, el diámetro de la rama descendente anterior y el diámetro del vaso diana distal durante la operación (> 1,5 mm). Se registró una disminución en el nivel de LVEDD en ambos grupos después de la operación en comparación con antes de la operación. Pero en el grupo con un ligero aumento, el nivel de LVEF después de la operación aumentó en comparación con el de antes (p<0,05). La aparición de angina de pecho 24 horas antes de la cirugía, la alta resistencia vascular durante la cirugía y el diámetro del vaso diana distal > 1,5 mm fueron los factores relacionados que afectaron al aumento de CK-MB después de la cirugía. La proporción de estos factores fue mayor en el grupo del aumento severo que en el grupo del aumento leve (p <0,05). Un incremento de las isoenzima de CK-MB provoca una recuperación lenta de la función miocárdica, por lo que puede utilizarse como un índice biológico crítico para reflejar el pronóstico de los pacientes.

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INTRODUCTION

Coronary heart disease is caused by blood flow obstruction caused by lumen stenosis based on coronary atherosclerosis, resulting in myocardial ischemia, hypoxia, or necrosis. Early, the patients have typical chest pain and dyspnea after fatigue or mood swings. If the obstruction cannot be relieved for too long, the patient may have cardiogenic shock at rest and even endanger the patient's life in severe cases [1,2]. In recent years, the incidence of coronary heart disease has increased and shows a younger patients' trend, and it has become a common disease seriously endangering people's health. Beating coronary artery bypass grafting is a frequent clinical cardiac surgery. It is of great value to improve the symptoms of myocardial ischemia, angina pectoris, and

cardiac pumping function; nevertheless, it can cause mechanical damage to the heart during the operation. Postoperative complications such as arrhythmia and cardiac insufficiency often occur, so early detection of myocardium damage after surgery is critical for successful clinical treatment [3,4]. Creatine kinase isoenzyme (CK-MB) exists mainly in the myocardium but also in small amounts in normal blood and tissues outside the heart. When the myocardium is damaged, it can be released into the blood immediately and may be used in the clinic as a biomarker to reflect the degree of myocardial injury [5]a CK-MB standard is needed to eliminate between-method bias. Because the in vitro expression of human creatine kinase generates three isoenzymes, CK-MM, CK-MB, and CK-BB, it is important to establish an effective method to purify the isoform CK-MB from the mixture. In this study, we aimed at using tandem affinity purification (TAP. Related data show that early detection of myocardial CK-MB after surgery can significantly improve the occurrence of complications in patients, which has an essential role in the clinical treatment of coronary heart disease [6]. Therefore, by exploring the changes in postoperative CK-MB levels in patients with off-pump coronary artery bypass (OPCAB) grafting, this study aims to analyze the impact of elevated levels on early prognosis after this surgical procedure.

PATIENTS AND METHODS

General information

We selected 116 patients who underwent OPCAB grafting in our hospital from January 2018 to January 2019. The inclusion criteria were as follows: 1) the age was ≤ 90 years old; 2) all patients in whom the left internal mammary artery was used as the bridging vessel during the operation and bypass with the anterior descending branch; 3) ventricular ejection fractions > 50%; 4) patients and their families knew and signed a consent form. The hospital ethics committee approved this study. There were 86 males and 30 females aged 40 to 86 years (mean (63.58 ±7.69) years).

Operation method

The patient was in the supine position, and endotracheal intubation and ventilator-assisted ventilation were performed after general anesthesia. After entering the chest through the median sternal incision, the left internal mammary artery was removed with an intramammary retractor as a spare. The skin of the leg was cut open to expose the great saphenous vein from the 2cm above the medial malleolus. After pericardiotomy and suspension, the local myocardium to be anastomosed was fixed with Octopus cardiac fixator, the internal diameter of the anterior descending branch was measured and recorded, and the distal end of the great saphenous vein was anastomosed on the ascending aorta with suture. After each arterial bridge completed the kiss, the blood flow was measured and recorded by transient time flow. After the anastomosis of all bridges, the total time from the first to the last bridging vessels was recorded, and heparin was neutralized by protamine (1:1). The chest was closed layer by layer, and a drainage tube was placed in the pericardium and mediastinum.

After surgery, the patients entered the extracardiac intensive care unit and were continuously pumped with nitroglycerin for 24 hours. The changes in CK-MB levels were monitored at 12 h, 24 h, and 48 h after the operation. The patients whose highest value of CK-MB was ≤ 5.31ng/mL were included in the mild enzyme elevated group, and those with values > 5.31ng/mL were included in the severely elevated group. Patients were followed for seven days postoperatively.

Observation index

1) General data of all patients on admission were collected. These included age, sex, body mass index, hypertension, and diabetes history, previous myocardial infarction, 24-hour angina pectoris, and patients that had

undergone percutaneous coronary intervention (PCI). The blood flow of the bridge, vascular resistance, the diameter of the anterior descending branch, and the diameter of the distal target vessel were recorded during operation > 1.5mm.

2) Color Doppler echocardiography (PHILIPS SONOS5500) was used to detect the changes in the left ventricular ejection fraction (LVEF) and left ventricular end-diastolic diameter (LVESD) before the operation and seven days and three months after surgery.

Statistical method

Quantitative data of this study were expressed by ($\bar{x} \pm s$). The data of the mild and severe elevated groups were compared with the t-test. All the counting data were expressed by n (%). The χ^2 test tested the data comparison between the two groups, and the logistic regression analysis analyzed the related factors affecting the increase of postoperative CK-MB. $p<0.05$ was considered statistically significant. The IBM SPSS21.0 software package analyzed the data of this study.

RESULTS

Preoperative general data between the two groups

The incidence of angina pectoris 24 hours before operation in the severe elevation group was high (32.26%) when compared with that in the mild elevation group (9.41%), and the difference was statistically significant ($p<0.05$). The two groups had no significant difference in other data ($p>0.05$). See Table 1.

Comparison of CK-MB levels between the two groups during the perioperative period

The levels of CK-MB in the two groups were higher than that before the operation ($p<0.01$), and the peak of CK-MB appeared 12 hours after surgery- The level of CK-MB in the severe elevated group was higher than that in the mild elevated group at each time point after the operation ($p<0.05$). See Table 2.

Table 1
Comparison of preoperative isoenzyme values between the two groups.

General data	Mild elevation ≤ 5.31ng/mL (n=85)	Severe elevation > 5.31ng/mL (n=31)	t/χ^2	p
Age*	63.25±7.18	64.78±7.36	1.009	0.315
Gender (male/female)	63/22	23/8	0.001	0.993
BMI(kg/m^2)*	23.67±3.12	24.55±3.29	1.325	0.188
Hypertension **	59(69.41)	20(64.52)	0.251	0.617
Diabetes **	35(41.76)	10(32.24)	0.761	0.383
Previous myocardial infarction **	30(35.29)	9(29.03)	0.399	0.528
Previous PCI **	7(8.24)	4(12.90)	0.576	0.448
Occurrence of angina pectoris 24 h before **operation	8(9.41)	10(32.26)	9.044	0.003
LVEDD (cm)*	5.67±0.37	5.58±0.59	0.978	0.330
LVEF (%)*	53.12±5.16	53.54±5.31	0.385	0.701

*($\bar{x} \pm s$), **[n (%)].

Table 2

Comparison of CK-MB levels between the two groups during the perioperative period.

Time	Mild elevation ≤ 5.31ng/mL (n=85)	Severe elevation > 5.31ng/mL (n=31)	*t*	*p*
Before operation (ng/mL)	0.93±0.54	0.90±0.53	0.266	0.791
12h after the operation (ng/mL)	2.26±0.85	10.77±12.77	6.153	<0.001
24h after the operation (ng/mL)	1.28±0.63	6.49±8.15	5.924	<0.001
48h after the operation (ng/mL)	0.86±0.49	2.57±1.52	9.199	<0.001

Values expressed as $\overline{x} \pm s$

Comparison of operation between the two groups

The proportion of patients whose diameter of the anterior descending artery ≤ 1.5mm and the diameter of the distal target vessel > 1.5mm in the severe elevated group were higher than those in the mild elevated group ($p<0.05$). The vascular resistance in the severe elevated group was significantly increased compared with that in the mild elevated group ($p<0.05$). See Table 3.

Logistic regression analysis of the related factors affecting the increase of postoperative CK-MB

Logistic regression analysis showed that the incidence of angina pectoris 24 hours before surgery, vascular resistance, and distal target vessel diameter > 1.5mm were related factors affecting the increase of postoperative CK-MB ($p<0.05$). See Table 4.

Comparison of cardiac function between the two groups before and after surgery

The level of LVEF three months after the operation in the mild elevated group was significantly higher than before surgery ($p<0.05$). In comparison, the level of LVEF at seven days and three months after operation in the severe elevated group was not significantly different from that before operation ($p>0.05$)- In contrast, the level of LVEDD seven days and three months after operation in the mild elevated group was significantly lower than that before operation ($p<0.05$). The level of LVEDD at three months after operation in the severely elevated group was decreased than before ($p<0.05$). See Table 5.

DISCUSSION

Currently, the clinical treatment of coronary heart disease is mainly by drug treatment, surgical treatment, and interventional stent treatment. Generally, different treatment schemes are chosen according to the severity of the patient's condition. Traditional coronary artery bypass grafting is performed under cardiopulmonary bypass, and the long-term typical rate of vascular anastomosis is high, as well as safety and effectiveness. However, cardiopulmonary bypass, cardiac arrest, and myocardial ischemia-reperfusion can cause systemic inflammatory reactions and multiple organ function damage, seriously endangering the life and health of patients [7]. In recent years, OPCAB surgery has entered a new period. During the operation, the anastomosis can be completed while maintaining the independent blood flow of the coronary artery and the beating heart, which reduces myocardial ischemia-reperfusion injury. Although the ascending aorta is not blocked during OPCAB grafting, the operation itself can cause myocardial ischemia-reperfusion injury during the anastomosis. Its effect on the prognosis of patients has become the focus of clinical schol-

Table 3
Comparison of surgery data between the two groups.

Operation condition		Mild elevation ≤ 5.31ng/mL (n=85)	Severe elevation > 5.31ng/mL (n=31)	t/χ^2	p
Internal mammary artery bridge flow (mL/min) *		30.12±13.58	29.58±17.11	0.135	0.788
Great saphenous vein bridge flow (mL/min) *		21.19±10.65	22.02±11.47	1.252	0.627
Vascular resistance (mmHg/mL/min) *		4.11±0.92	6.03±1.48	5.451	0.003
Diameter of the anterior descending branch	≤1.5mm **	6(7.06)	7(22.58)	5.500	0.019
	1.75~2.0 mm **	60(70.59)	18(58.06)	1.617	0.203
	≥2.25 mm **	19(22.35)	6(19.35)	0.121	0.728
Diameter of the distal target vessel	≤1.5mm**	65(76.47)	17(54.84)	5.130	0.024
	>1.5mm**	20(23.53)	14(45.16)	5.130	0.024

*($\overline{x} \pm s$), or **[n (%)].

Table 4
Logistic regression analysis of the related factors affecting the increase of postoperative CK-MB.

Variable	*B*	*SE*	*Wald* statistic quantity	*p*	*OR*	95%*CI*
Constant	-3.765	0.875	15.359	<0.001	0.022	
Vascular resistance	0.785	0.452	3.467	0.001	1.452	0.725~4.154
Occurrence of angina pectoris 24 hours before the operation	1.033	0.332	6.564	0.011	3.128	1.207~7.365
Diameter of the anterior descending branch≤1.5mm	0.688	0.428	2.211	0.127	2.113	0.663~6.280
Diameter of the distal target vessel >1.5mm	0.402	0.153	5.610	0.015	1.461	1.063~2.142

Table 5
Comparison of cardiac function between the two groups before and after surgery.

Cardiac function	Group	Before the operation	Seven days after the operation	Three months after the operation
LVEF (%)	Mild elevation	53.12±5.16	53.84±5.13	57.48±5.01*
	Severe elevation	53.54±5.31	54.63±5.22	54.32±6.15
LVEDD (cm)	Mild elevation	5.67±0.37	4.73±0.35*	4.74±0.39*
	Severe elevation	5.58±0.59	5.02±0.36	4.78±0.49*

Note: compared with pre-operation * $p < 0.05$

Values expressed as $\overline{x} \pm s$

LVEF left ventricular ejection fraction; LVEDD left ventricular end-diastolic diameter.

ars [8,9] we revised 400 patients; 200 received on-pump CABG and 200 off-pump OPCAB (OPCAB. OPCAB grafting consists of revascularization of bridging blood vessels, which can be improved by distal coronary artery obstruction and stenosis, so myocardial ischemia-reperfusion injury will inevitably occur after the operation [10]. Keeping a clear field of vision for the operation during vascular anastomosis will temporarily block the proximal vessels, resulting in temporary ischemia of the muscles dominated by the distal end of the artery. A significant decrease in blood pressure during the operation can cause arterial vasospasm and then cause myocardial ischemic injury. If the patients do not move in time after sorgery, it is easy to form coronary or bridging vascular thrombosis, resulting in myocardial ischemic injury and a significant increase of myocardial enzymes [11].

CK-MB is a kind of myocardial enzyme mainly present in cardiomyocytes' cytoplasm. As an essential enzyme detection index of myocardial injury, it has been paid more and more attention in clinical practice [12]. The content of CK-MB in serum is minimal. Generally, the cell membrane permeability increases after myocardial injury, and its level in the blood peaks after nine to 30 hours. Therefore, CK-MB detection is often combined with clinical symptoms and cardiac echocardiography to evaluate the severity of myocardial injury [13]the rats received ISO subcutaneously at a dose of 100 mg/kg for three days. In group III, rats received ISO as group II and then GNPs (400 μg/kg/day). In this study, by examining the postoperative CK-MB level, the patients with the highest value ≤ 5.31ng/mL were included in the slightly elevated group (mild elevated group) and those with levels > 5.31ng/mL were classified as the significantly elevated (severe elevated group). Based on the analysis of the data of the two groups, it was found that the peak level of CK-MB appeared 12 hours after the operation, and the level of CK-MB in the severe elevated group was significantly higher than in the mild elevated group at each time point after surgery ($p<0.05$). In addition, the Logistic regression analysis results showed that the occurrence of angina pectoris 24 hours before operation, high vascular resistance, and distal target vessel diameter > 1.5mm were related factors affecting the increase of postoperative CK-MB ($p<0.05$). The related data show that the blood flow of the bridge is positively correlated with the diameter of the distal coronary artery. When there is stenosis or obstruction of the distal vessel, the anastomotic site is not unobstructed and the blood flow is small [14]. The positive correlation between the diameter of the distal target vessel > 1.5mm and the increase of CK-MB may be due to the decrease of blood flow caused by a large vascular plaque load, which in turn affects the increase of CK-MB level [15]each of whom presented with a normal ECG and was subjected to emergency coronary angiography (CAG).

This study showed that the improvement degree and speed of LVEF and LVEDD were different in patients with different levels of CK-MB after OPCAB grafting. The level of LVEF in patients with mild elevation was significantly higher than that before the operation and three months after the operation ($p<0.05$); the level of LVEDD in the mild elevated group was significantly lower than that before the operation at seven days and three months after operation ($p<0.05$); the level of LVEDD in severe elevated group was significantly lower than that before operation and three months after operation ($p<0.05$). This finding suggests that the higher the level of CK-MB after surgery, the longer the duration, the more serious the myocardial injury of patients, and the worse the prognosis.

To sum up, angina pectoris attack, distal target vessel diameter > 1.5mm, and elevated vascular resistance before beating coronary artery bypass grafting can affect the elevation of myocardial enzymes after the operation, and the recovery of myocardial function is slow in patients with signifi-

cantly increased myocardial enzymes, which can be used as an essential biological index to reflect the prognosis of patients.

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Competing Interests

The authors declared that they have no competing interests.

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Authors' Contribution

Conception and design: Z Z: Administrative support; LW: Provision of study materials or patients; ZZ, JW: Collection and assembly of data; NL, YL: Data analysis and interpretation; LS: Manuscript writing; All authors: Final approval of manuscript.

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Clinical value of four-dimensional hysterosalpingo-contrast sonography assisted by intrauterine pressure measurement for tubal patency evaluation.

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Keywords: tubal patency; 4D hysterosalpingo-contrast sonography; intrauterine pressure.

Abstract. We aimed to explore the clinical value of four-dimensional hysterosalpingo-contrast sonography (4D-HyCoSy) assisted by intrauterine pressure measurement for evaluating tubal patency. One hundred and thirty-two patients diagnosed with tubal factor infertility from February 2018 to February 2021 were selected as subjects. With hysterosalpingography diagnosis results as the gold standard, 4D-HyCoSy was conducted for all patients, and the status of the fallopian tubes was classified into patency, occlusion, and partial occlusion. Based on the function of fallopian tubes, 4D-HyCoSy diagnosis results revealed that fallopian tubes showed bilateral patency, incomplete patency (including bilateral partial occlusion, unilateral patency, and unilateral partial occlusion, unilateral patency and unilateral occlusion), unilateral partial occlusion and unilateral occlusion, and bilateral occlusion. The cutoff value of peak intrauterine pressure was determined using the receiver operating characteristic curve (ROC), specificity, and the area under the ROC curve (AUC) between 4D-HyCoSy alone and 4D-HyCoSy assisted by intrauterine pressure measurements. There were significant differences in the peak intrauterine pressure among patients with bilateral patency, incomplete patency, unilateral partial

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occlusion, and unilateral and bilateral occlusions ($p<0.05$). The corresponding cutoff values of peak intrauterine pressure were 24.42, 36.34, and 47.68 kPa; AUC values were 0.812, 0.836, and 0.827, respectively. The FSM model showed that the AUC of 4D-HyCoSy alone, assisted by peak intrauterine pressure was 0.85, with a higher sensitivity (88.13%) than that of 4D-HyCoSy ($p<0.05$). 4D-HyCoSy, assisted by intrauterine pressure measurement, has an excellent value for evaluating tubal patency.

Valor clínico de la histerosalpingo-sonografía 4D por contraste, asistida por medición de la presión intrauterina, para evaluar la permeabilidad tubárica.

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Palabras clave: permeabilidad tubárica; ecografía con contraste de histerosalpingo en cuatro dimensiones; presión intrauterina.

Resumen. Nuestro objetivo fue explorar el valor clínico de la histerosalpingo-sonografía con contraste en cuatro dimensiones (4D-HyCoSy) asistida por la medición de la presión intrauterina para evaluar la permeabilidad tubárica. Se seleccionaron como sujetos un total de 132 pacientes diagnosticadas como infertilidad por factor tubárico desde febrero de 2018 hasta febrero de 2021. Con los resultados del diagnóstico de histerosalpingografía como estándar de oro, se realizó 4D-HyCoSy para todas las pacientes y el estado de las trompas de Falopio se clasificó como permeable, ocluída y parcialmente ocluída. Según la función de las trompas de Falopio, los resultados del diagnóstico 4D-HyCoSy revelaron que las trompas de Falopio mostraban permeabilidad bilateral, permeabilidad incompleta (incluida oclusión parcial bilateral, permeabilidad unilateral y oclusión parcial unilateral, permeabilidad unilateral y oclusión unilateral), oclusión parcial unilateral y oclusión unilateral, y oclusión bilateral. El valor de corte de la presión intrauterina máxima se determinó utilizando la curva característica operativa del receptor (ROC), la especificidad y el área bajo la curva ROC (AUC) entre 4D-HyCoSy sola y 4D-HyCoSy asistida por la medición de la presión intrauterina. Hubo diferencias significativas en la presión intrauterina máxima entre pacientes con permeabilidad bilateral, permeabilidad incompleta, oclusión parcial unilateral y oclusión unilateral y oclusión bilateral ($p<0,05$). Los valores de corte correspondientes de la presión intrauterina máxima fueron 24,42, 36,34 y 47,68 kPa, y los valores de AUC fueron 0,812, 0,836 y 0,827, respectivamente. El modelo FSM mostró que el AUC de 4D-HyCoSy asistida por la presión intrauterina máxima fue de 0,85, con una sensibilidad más alta (88,13%) que la de 4D-HyCoSy ($p<0,05$). 4D-HyCoSy asistido por la medición de la presión intrauterina tiene un valor diagnóstico significativo en la evaluación de la permeabilidad tubárica.

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INTRODUCTION

Infertility is a health concern worldwide. Among the total cases of female infertility, 54.7% of patients suffer from tubal factor infertility, and the cases show a year-by-year uptrend due to increased reproductive infections, sexually transmitted diseases, and endometriosis [1-3]. Therefore, effective diagnostic measures are essential for accurately evaluating the tubal patency of patients. Transvaginal hysterosalpingo-contrast sonography (TV HyCoSy) is a primary noninvasive method to assess the tubal patency of infertile patients. As technologies constantly progress, four-dimensional HyCoSy (4D-HyCoSy) has been widely applied in clinical diagnosis. Patients can still undergo missed diagnosis by this method despite of its advantages such as simple operation, low cost, and low risk [4-8]. In recent years, intrauterine pressure measurement is a new auxiliary means to examine fallopian tube pressure. It can be applied in combination with 4D-HyCoSy to examine the intrauterine pressure at a constant speed, improving the accuracy of the clinical diagnosis and ensuring the safety of patients [9,10]. In this study, the clinical data of 132 patients with tubal infertility were analyzed, and the diagnostic value of 4D-HyCoSy assisted by intrauterine pressure measurement was assessed for tubal patency.

PATIENTS AND METHODS

General data

One hundred and thirty-two outpatient infertile patients treated in our hospital's Department of Obstetrics and Gynecology from February 2018 to February 2021 were selected. They were 23-42 years old (28.69±4.32) and examined at 3-7 d after menstruation, including 42 primary and 90 secondary infertile patients. This study was approved by the Ethics Committee of our hospital and performed following the Declaration of Helsinki. All the patients included in this study and their families were informed of this study and signed an informed consent.

Inclusion criteria

1) Patients with no pregnancy for more than one year, 2) those whose husbands had a normal reproductive function, and those who had sexual harmony with their husband and took no contraceptive measures, 3) those with indications confirmed by HyCoSy, 4) those suspected of primary or secondary tubal infertility, 5) those with negative hysterosalpingography (HSG) results, and 6) those whose family members signed the informed consent after communication.

Exclusion criteria

1) Patients with serious gynecological diseases (vaginitis, pelvic inflammatory disease, adnexitis, etc.), 2) those complicated with malignant tumors, 3) those with ovulation failure caused by congenital malformations or other genetic factors, 4) those with heart, liver or kidney dysfunction, 5) those who were pregnant or suspected of pregnancy, 6) those who suffered from abortion within six months, 7) those who were allergic to contrast media, or 8) those with hysteromyoma >5 cm.

Diagnosis methods

HSG is a safe method with high accuracy for checking tubal patency. This study applied HSG diagnosis results as the gold standard and compared them to 4D-HyCoSy diagnosis results, and statistical analysis was carried out.

Apparatus and reagents

In this study, a Philips EPIQ5 color Doppler ultrasound diagnostic machine (Philips, Netherlands) was adopted for 4D-HyCoSy with a probe frequency of 5-9 MHz under low image quality; the volume angle of 100-120°, the volume frame angle of 179°, direction (Up/Down), the frame frequency of 0.6 and low threshold (20, adjusted according to different images). The contrast agent

used was SonoVue (59 mg/tube, NMPN: J20080052, Bracco, Italy). The antispasmodic and anti-inflammatory agents [gentamicin (80,000 units) + dexamethasone (2.5 mg) + atropine (0.25 mg) + lidocaine (50 mg)] were added into 20 mL of 0.9% normal saline, and mixed evenly to prepare a suspension. Later, 59 mg SonoVue was added into 5 mL of injectable normal saline and mixed evenly to form a microbubble suspension, which was diluted with 5 mL of normal saline before injection.

4D-HyCoSy diagnosis

1) Thirty min before diagnosis, atropine was injected. 2) Upon bladder filling, the patients were placed in the bladder lithotomy position. Then the shape of the patient's uterus and ovary, relative cross-section position, and pelvic cavity effusion were observed. Next, the injection pressure for bilateral ovaries was slightly increased, and the ovarian activity was evaluated. 3) Perineum and vagina were disinfected using complex iodine; a double-cavity Foley catheter was placed into the uterine cavity under the guidance of the abdominal probe, and 1.5-2 mL of normal saline was injected into the balloon using a syringe. 4) After the size and position of the balloon were adjusted, 5 mL of normal saline was slowly injected through the Foley catheter and then pumped back. After that, the tubal patency was preliminarily evaluated according to the resistance and the amount of liquid pumped back. 5) The probe was continuously adjusted to turn on the four-dimensional mode, and the contrast agent was injected slowly. Next, the solvent data were recorded in real-time following the optimization of the sampling frame, and the data of liquid pumped back were recorded. Afterward, the speed and pressure of the injection were adjusted at any time based on patients' adverse reactions. 6) The Foley catheter was removed, and the uterine cavity was observed under the radiography mode.

HSG diagnosis

The patients were placed in the supine bladder lithotomy position, and the first slice was taken. A vaginal speculum was put after disinfection. Then the cervical side wall was fixed with cervical forceps, the second slice was taken after contrast agent iohexol was slowly added, and the shape of the uterus was observed. Subsequently, the angle was adjusted for photography of the third slice, after which the development of fallopian tubes and uteri were observed. If the development effect was unsatisfactory, the medicine could be increased appropriately.

HSG diagnostic criteria were displayed below: 1) Patency: the fallopian tubes on both sides were completely developed, showing a natural shape. The contrast agent overflew normally at the umbrella end and diffused in the pelvic cavity. There was almost no contrast agent left in the fallopian tubes. 2) Partial occlusion: fallopian tubes had poor morphology, and a small amount of contrast agent was left. 3) Occlusion: the contrast agent gathered in the occlusion site and did not enter the pelvic cavity.

Combined diagnosis

The receiver operating characteristic (ROC) curve was plotted to calculate the cutoff value of intrauterine pressure measurement, and the sensitivity, specificity, and area under ROC curve (AUC) of 4D-HyCoSy and combined diagnosis were determined based on 4D-HyCoSy diagnosis results, which coincided with the peak intrauterine pressure interval, with HSG diagnosis results as the gold standard.

Diagnostic criteria of 4D-HyCoSy

1) Patency: there was no resistance when the contrast agent was injected, the fallopian tubes ran smoothly and softly, and a large amount of contrast agent overflew from the umbrella end and diffused in the pelvic cavity. 2) Partial occlusion: there was positive resistance during the injection of contrast

agent, fallopian tubes were stiff and twisted, the thickness of the lumen was uneven, and a small amount of contrast agent overflew from the umbrella end. 3) Occlusion: during the injection of the contrast agent, there was significant resistance, no development in the whole or distal end of fallopian tubes, and no diffusion of the contrast agent in the pelvic cavity.

Observation of adverse reactions

Adverse reactions included adverse drug reactions, which were determined according to SonoVue instructions, and adverse non-drug reactions, such as pain, pale complexion, dizziness, profuse sweating, hypotension, arrhythmia, nausea, vomiting, and fainting.

Statistical analysis

The IBM® SPSS23.0 software was utilized for statistical analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and compared between groups using the *t*-test. Numerical data were expressed as percentages (%) and compared between groups *via* the χ^2 test. Dichotomous logistic regression analysis was conducted to analyze diagnostic factors, and the finite state machine (FSM) diagnostic model was established and verified using the ROC curve. $p<0.05$ represented a statistically significant difference.

RESULTS

Detection results of tubal patency by 4D-HyCoSy

All 132 patients completed the 4D-HyCoSy examination, and their images were clear. Among 264 fallopian tubes, 145 were open, 42 were occluded entirely, and 77 were partially occluded. Among them, there were

- 53 cases of bilateral patency (106 fallopian tubes),
- 13 cases of bilateral occlusion (26 fallopian tubes),
- 20 cases of bilateral partial occlusion (40 fallopian tubes),
- Nine cases of unilateral patency and unilateral occlusion (9/9 fallopian tubes),
- 30 cases of unilateral patency and unilateral partial occlusion (30/30 fallopian tubes), and
- Seven cases of unilateral occlusion and unilateral partial occlusion (7/7 fallopian tubes).

Besides, there were nine cases of arcuate uterus, five incomplete mediastinal uterus cases, three intrauterine adhesions, and two intrauterine polyps (Fig. 1).

4D-HyCoSy and HSG diagnosis results

Within two weeks after the 4D-HyCoSy diagnosis, 132 patients underwent HSG diagnosis. The results showed that among 264 fallopian tubes, 132 were open, 55 were occluded entirely, and 77 were partially occluded. In the 4D-HyCoSy diagnosis, the patency of 212 fallopian tubes conformed to the HSG examination results, with a diagnostic coincidence rate of 86.18% (212/264). With HSG diagnosis results as the gold standard, the diagnostic coincidence rate of tubal patency was the highest [84.83% (123/145)], and that of tubal occlusion and partial occlusion was 80.95% (34/42) and 76.62% (59/77), respectively (Table 1).

Bilateral patency was defined as open fallopian tubes on both sides. Incomplete patency included partially occluded fallopian tubes on both sides, an open fallopian tube on one side, a partially occluded fallopian tube on the other, an open fallopian tube, and an occluded fallopian tube on the other. Unilateral partial occlusion and unilateral occlusion referred to a partially occluded fallopian tube on one side and an occluded fallopian tube on the other side. *Bilateral occlusion* was defined as occluded fallopian tubes on both sides. The diagnostic coincidence rate of the 4D-HyCoSy diagnosis in

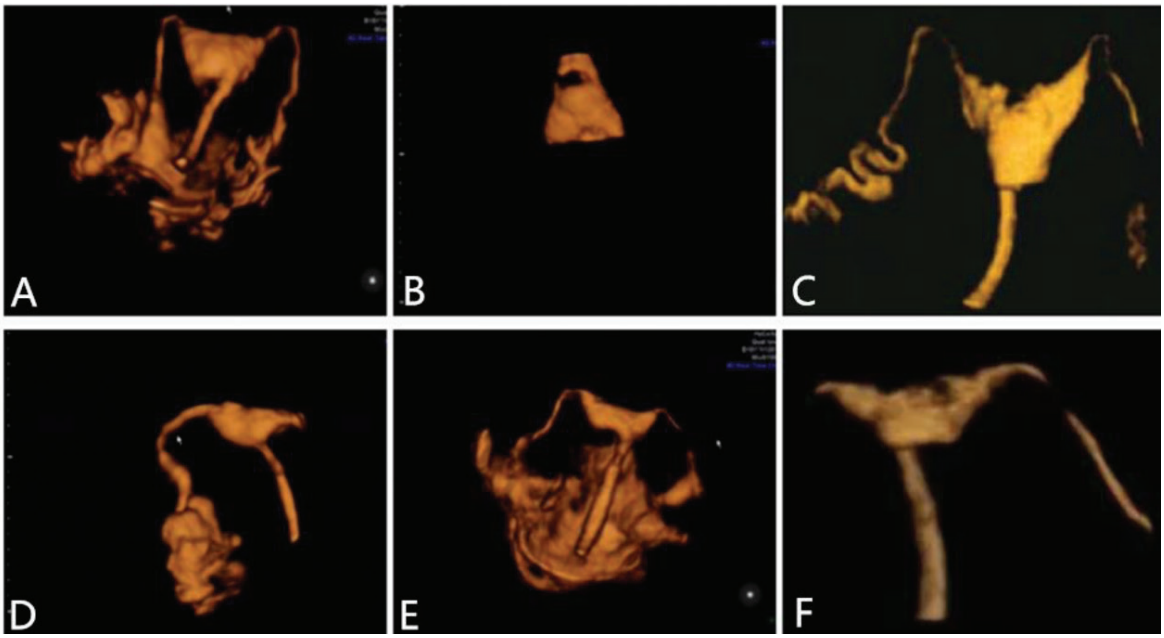

Fig. 1. Fallopian tube images obtained after 4D-HyCoSy diagnosis. A. Bilateral patency, B. Bilateral occlusion, C. Bilateral partial occlusion, D. Unilateral partial occlusion, and unilateral occlusion. E. Unilateral patency and unilateral partial occlusion, and F. Unilateral patency and unilateral occlusion.

Table 1
4D-HyCoSy diagnosis results

4D-HyCoSy	HSG			
	Patency	Partial occlusion	Occlusion	Total
Patency	123 (84.83%)	12 (8.28%)	10 (6.90%)	145 (58.94%)
Partial occlusion	7 (9.09%)	59 (76.62%)	11 (14.29%)	77 (31.30%)
Occlusion	2 (4.76%)	6 (1.43%)	34 (80.95%)	42 (17.07%)
Total	132 (53.66%)	77 (31.30%)	55 (22.36%)	264 (100%)
4D-HyCoSy diagnostic coincidence rate	123/132 (93.18%)	59/77 (76.62%)	34/55 (61.82%)	212/264 (80.30%)

Values n= 132 are expressed in n (%).

132 patients was 87.12% (115/132), with HSG diagnosis results as the gold standard (Table 2).

Intrauterine pressure measurement results

Seventy out of 132 patients underwent intrauterine pressure measurement through the connection with a pressure measurement device before the 4D-HyCoSy diagnosis, and their intrauterine pressure results were obtained. Pairwise comparisons of the pressure values were carried out in six groups. The results revealed no significant differences in the peak intrauterine pressure among patients in the unilateral patency and the unilateral occlusion group, unilateral patency and unilateral partial occlusion group, and the bilateral partial occlusion group ($p<0.05$). The patients

Table 2
Diagnostic results of 4D-HyCoSy [n=132, n (%)]

4D-HyCoSy	HSG				
	Bilateral patency	Partial occlusion	Unilateral partial occlusion and unilateral occlusion	Bilateral occlusion	Total
Bilateral patency	43 (89.58%)	3 (5.00%)	0 (0.00%)	0 (0.00%)	46 (34.85%)
Partial occlusion	5 (1.04%)	50 (84.75%)	0 (0.00%)	1 (6.67%)	60 (45.45%)
Unilateral partial occlusion Unilateral occlusion	0 (0.00%)	7 (11.86%)	3 (33.33%)	3 (20.00%)	13 (9.85%)
Bilateral occlusion	0 (0.00%)	0 (0.00%)	6 (66.67%)	11 (73.33%)	17 (12.88%)
Total	48 (40.15%)	60 (45.45%)	9 (6.82%)	15 (11.36%)	132 (100%)
4D-HyCoSy diagnostic coincidence rate	48 (90.57%)	50 (84.75%)	6 (66.67%)	11 (73.33%)	115 (87.12%)

in these three groups were included in the incomplete patency group (Group A), and the differences between the bilateral patency group (Group B), bilateral occlusion group (Group C), unilateral occlusion, and unilateral occlusion group (Group D) and the other groups were significant ($p<0.05$). Besides, severer occlusion had higher intrauterine pressure (Table 3).

The ROC curve of the peak intrauterine pressure was plotted. The cutoff value between Group B and Group A was 25.42 kPa, with a sensitivity of 81% and a specificity of 85%. AUC was 0.812 (Fig. 2). The cutoff value between Group A and Group D was 36.34 kPa, with a sensitivity of 73% and a specificity of 89%; AUC was 0.836 (Fig. 3). Moreover, the cutoff value between Group D and Group C was 47.68 kPa, with a sensitivity of 79% and a specificity of 87%; AUC was 0.827 (Fig. 4).

Establishment of the combined diagnosis model

Dichotomous logistic regression analysis was conducted for the 4D-HyCoSy diagnosis (A) and intrauterine pressure measurement (B), to evaluate the efficacy of the combined diagnosis. The results demonstrated t a significant difference between the two diagnosis methods ($p<0.05$), so both could be used as predictive diagnostic indicators. According to the regression coefficient of the two variables, the mathematical diagnosis model of FSM was established as follows: FSM =1.083 ln(A) + 1.275 ln(B) - 8.23 (Table 4).

Model validation results

The diagnostic efficiency of the FSM diagnostic model was assessed using the ROC curve. It was discovered that the combined diagnosis model had a high diagnostic value, with an AUC of 0.85. When FSM=11.3, the model had a high sensitivity (89.36%), which meant that only three of 70 patients undergoing combined diagnosis were misdiagnosed due to a FSM<11.3. When the FSM=13.8, the model had a high specificity (92.55%). Besides, the model was still particularly valuable in the diagnosis of other patients. The diagnostic efficiency was compared between the 4D-HyCoSy diagnosis and the combined diagnosis. The ROC curve illustrated that the diagnostic value of the 4D-HyCoSy diagnosis was lower than that of the combined diagnosis. With the maximum value of the Youden index (YI) as a threshold, the AUC of 4D-HyCoSy diagnosis was

Table 3
Intrauterine pressure measurement results.

Group	Radiography results	n (number of fallopian tubes)	Pressure (kPa)	Peak intrauterine pressure (kPa)	Comparison group	*p*
A	Unilateral patency and unilateral occlusion	5 (10)	17.9~42.6	36.42±3.15	B, C, D	<0.05
A	Unilateral patency and unilateral partial occlusion	11 (22)	15.2~34.8	25.47±2.51	B, C, D	<0.05
A	Bilateral partial occlusion	6 (12)	17.2~37.6	32.17±2.74	B, C, D	<0.05
B	Bilateral patency	35 (70)	13.2~28.4	19.43±1.68	A, C, D	<0.001
C	Bilateral occlusion	6 (12)	42.0~63.2	54.35±2.30	A, B, D	<0.001
D	Unilateral partial occlusion and unilateral occlusion	7 (14)	25.7~52.3	43.42±1.70	A, B, C	<0.05

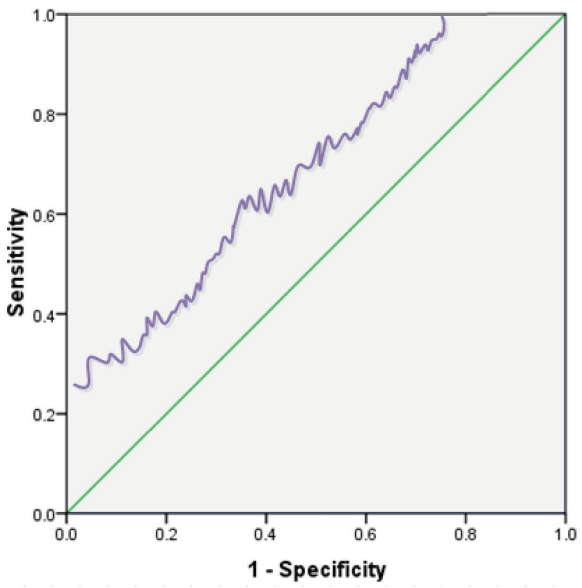

Fig. 2. ROC curves of Group B and Group C.

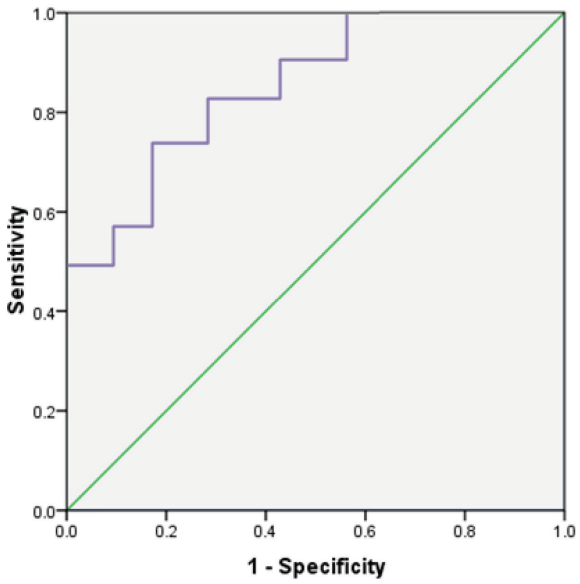

Fig. 3. ROC curves of Group A and Group D.

0.82, with a sensitivity of 78.04%, which was markedly lower than that of the combined diagnosis (sensitivity: 70.13%). However, the specificity displayed no significant difference between the two diagnosis methods (p>0.05) (Fig. 5). Comparative evaluation indicators for diagnostic efficiency are shown in Table 5.

Adverse reactions

Among the 132 patients, the majority had pain during the examination, and the minority suffered from dizziness and nausea without adverse reactions such as arrhythmia, vomiting, hypotension, and fainting. Patients' adverse reactions were all within the tolerable range, and no allergic reactions or vaginal bleeding occurred. In addition, 41 cases (31.06%) had grade 0 adverse reactions, 65 cases (49.24%) had grade I adverse reactions, 19 cases (14.39%) had grade II adverse reactions, and seven cases (5.30%) had grade III adverse reactions (Fig. 6).

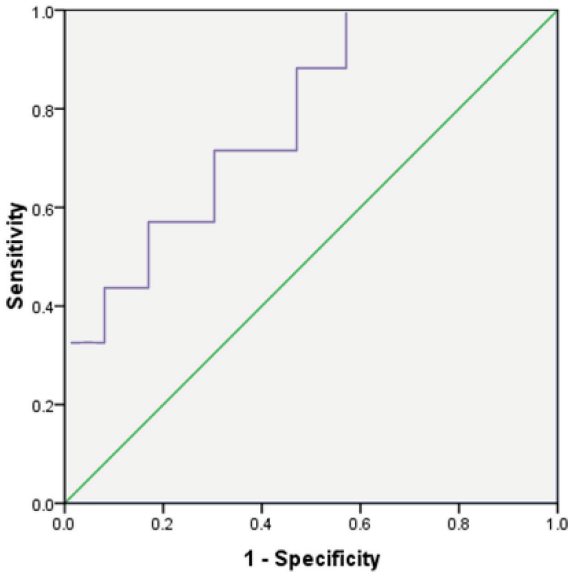

Fig. 4. ROC curves of Group D and Group C.

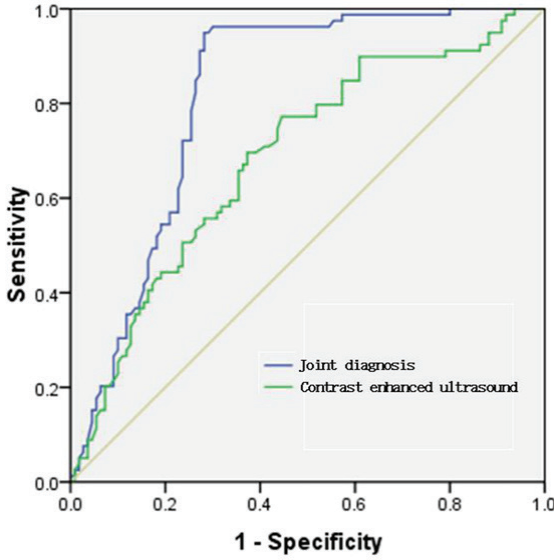

Fig. 5. ROC curves of combined diagnosis and 4D-HyCoSy diagnosis.

Table 4
Results of dichotomous logistic regression analysis.

Indicator	Regression coefficient	Standard error	Wald value	Odds ratio (OR)	95%CI	*p*
4D-HyCoSy	1.083	0.531	4.16	2.953	1.009~12.937	0.017
Peak intrauterine pressure	1.275	0.414	9.485	3.579	1.105~13.564	0.015

Table 5
Evaluation results of combined diagnosis and 4D-HyCoSy diagnosis efficiency.

Indicator	AUC (95% CI)	YI	Threshold value	Sen (%)	Spe (%)	DA (%)	Positive predictive value	Negative predictive value
4D-HyCoSy diagnosis	0.82(0.78-0.88)	0.43	69.57	78.04%	70.13%	78.27%	80.36%	53.14%
Combined diagnosis	0.85(0.81-0.92)	0.56	20.93	88.13%	79.46%	87.97%	94.64%	69.23%

DISCUSSION

Infertility is a common disease in females, severely damaging patients' lives and mental health. It has now become a prevalent disease needing treatment in the medical field. Tubal patency makes the proportion of infertile females exceed 50%, so it is a critical basis for treating infertility to explore efficient methods for diagnosing fallopian tubes at a general degree, contributing to early diagnosis and early treatment [11].

In clinical practice, diagnosis methods commonly used for tubal patency include hydrotubation under the ascites (laparoscopic chromopertubation (LC)), uterine hydrotubation, HSG, hysterolaparoscopy, contrast-enhanced ultrasonography of fallopian tubes and X-ray HSG. Among them, traditional hydrotubation is a blind operation with a low diagnostic accuracy rate. Laparoscopy is an operation causing trauma, which is harmful to patients. LC diagnosis requires general

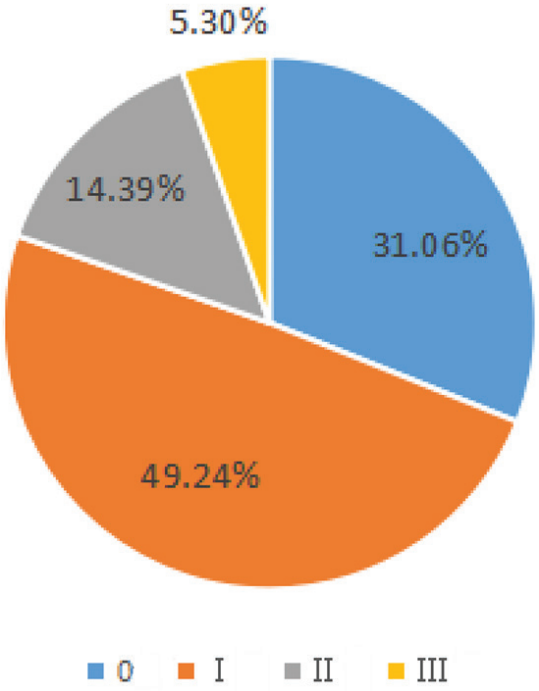

Fig. 6. Adverse reactions of patients.

anesthesia, after which patients have a risk of massive hemorrhage. HSG exhibits high accuracy, but some patients are allergic to iodine. Moreover, X-ray HSG is radioactive to some extent, and also it cannot be applied in patients allergic to iodine [12,13]. Hence, a simple diagnostic method that is easy to operate with low risk and low cost becomes a hot spot in the medical field [14-16]. HyCoSy diagnostic technology is increasingly recognized by doctors and patients since it is safe and non-invasive, with high accuracy, high operability, and good repeatability. The 4D-HyCoSy is a new type of contrast-enhanced ultrasound technology following 2D and 3D, during which the entire fallopian tubes can be visually displayed through imaging, and some fallopian tubes with unique positions or those in twisted and complicated shapes can also be well displayed. Through 4D-HyCoSy diagnosis, there remain cases of missed diagnosis though it is simple and efficient, so there is a necessity to find suitable auxiliary means to improve the accuracy of diagnosis.

In 2016, Kong checked the tubal patency using 4D-HyCoSy assisted by hydrogen peroxide, and its diagnostic coincidence rate (91.8%) was much higher than that of 4D-HyCoSy alone. Nevertheless, during 4D-HyCoSy diagnosis, scanning in a large fan-shaped angle can display the occlusion position of fallopian tubes and the variations of shape, and the injection pressure can be appropriately increased according to the occlusion of fallopian tubes for dredging the slightly adhesive fallopian tubes. Therefore, determining pressure using the traditional hand-pushing method is greatly affected by subjective factors, so a more intuitive auxiliary means is needed for evaluation. In the present study, 132 patients with tubal factor infertility were selected, and the tubal patency was detected by 4D-HyCoSy. Through comparison, it was found that the patency of 212 fallopian tubes conformed to HSG diagnosis results (gold standard), with a diagnostic coincidence rate of 86.18%. Besides, the fractional analysis based on the function of fallopian tubes revealed that the diagnostic coincidence rate of 4D-HyCoSy was 87.12%. Besides, the intrauterine pressure measurement demonstrated that the pressure was lower when the fallopian tubes were open, and the more severe the occlusion, the higher the pressure.

Subsequently, the cutoff value in each group was determined using the ROC curve. It was discovered that the cutoff value among bilateral patency, incomplete patency, unilateral partial occlusion, unilateral occlusion, and bilateral occlusion was 25.42 kPa, 36.34 kPa, and 47.86 kPa, respectively, and the AUC was 0.812, 0.836 and 0.827 respectively. Therefore, intrauterine pressure measurement can be applied as an effective means to assist the 4D-HyCoSy diagnosis to improve the accuracy of diagnosis. Furthermore, to investigate the clinical value of 4D-HyCoSy assisted by intrauterine pressure measurement in the evaluation of tubal patency, dichotomous logistic regression analysis was conducted for diagnosis factors, and the FSM diagnostic

model was established, which was verified by the ROC curve. The results showed that the AUC of combined diagnosis was 0.85, with a higher sensitivity (88.13%) than that of 4D-HyCoSy alone ($p<0.05$) and a specificity (79.46%) showing no significant difference in comparison with that of 4D-HyCoSy alone, confirming the extremely high diagnostic value of 4D-HyCoSy assisted by intrauterine pressure measurement in the evaluation of tubal patency.

In summary, 4D-HyCoSy, assisted by intrauterine pressure measurement, has high diagnostic value in evaluating tubal patency. Regardless, this study had a small sample size so the results may be biased. In the future, we will conduct multicenter studies with larger sample sizes to validate our findings.

Conflict of interest

The authors declare that they have no competing interests.

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Authors contribution

The first two authors contributed equally to this study.

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Association of formation of urinary calculi with blood lipid levels.

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Keywords: urinary calculi; blood lipids; correlation.

Abstract. We aimed to analyze the composition of urinary calculi and its correlations with blood lipids such as triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). Three hundred patients with urinary calculi treated from January 2020 to July 2021 were selected retrospectively into a urinary calculi group, while three hundred healthy individuals who received physical examination in our hospital during the same period were enrolled in a control group. Using the Spearman correlation analysis, we investigated the correlation between the composition of urinary calculi and dyslipidemia and explored the factors affecting urinary calculi through multivariate logistic regression analysis. The serum levels of TG and TC were significantly higher ($p<0.05$), the serum HDL-C level was significantly lower ($p<0.05$), while the serum LDL-C level displayed no significant difference ($p>0.05$) in the urinary calculi group compared with those in the control group. The proportion of uric acid calculi was significantly higher in urinary calculi patients with dyslipidemia than that in those with normal blood lipids ($p<0.05$). However, no significant difference was observed in the proportions of infectious calculi and calcium calculi between urinary calculi patients with dyslipidemia and those with normal blood lipids ($p>0.05$). Dyslipidemia was positively correlated with uric acid calculi ($p<0.05$) but not associated with infectious calculi or calcium calculi ($p>0.05$). TG was a risk factor for urinary calculi ($p<0.05$). The formation of urinary calculi is closely associated with blood lipid levels. Dyslipidemia, especially hypertriglyceridemia, can easily induce the formation of uric acid calculi.

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Asociación de formación de cálculos urinarios con niveles de lípidos en sangre.

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Palabras clave: cálculos urinarios, lípidos en sangre, correlación.

Resumen. Nuestro objetivo fue analizar la composición de los cálculos urinarios y sus correlaciones con los lípidos sanguíneos, como los triglicéridos (TG), el colesterol total (CT), el colesterol de lipoproteínas de baja densidad (LDL-C) y el colesterol de lipoproteínas de alta densidad (HDL-C). Trescientos pacientes con cálculos urinarios tratados desde enero de 2020 hasta julio de 2021 fueron seleccionados retrospectivamente e incluidos en el grupo de cálculos urinarios, mientras que trescientas personas sanas que recibieron un examen físico en nuestro hospital durante el mismo período se inscribieron en el grupo control. La correlación entre la composición de los cálculos urinarios y la dislipidemia se investigó mediante un análisis de correlación de Spearman, y los factores que afectan a los cálculos urinarios se exploraron mediante un análisis de regresión logística multivariable. Los niveles séricos de TG y TC fueron significativamente más altos ($p<0,05$), el nivel sérico de HDL-C fue significativamente más bajo ($p<0,05$), mientras que el nivel sérico de LDL-C no mostró diferencias significativas ($p>0,05$) en el grupo los cálculos urinarios en comparación con los del grupo control. La proporción de cálculos de ácido úrico fue significativamente mayor en los pacientes con cálculos urinarios y dislipidemia que en aquellos con lípidos sanguíneos normales ($p<0,05$). Sin embargo, no se observaron diferencias significativas en las proporciones de cálculos infecciosos y cálculos de calcio entre los pacientes con cálculos urinarios con dislipidemia y aquellos con lípidos sanguíneos normales ($p>0,05$). La dislipidemia se correlacionó positivamente con cálculos de ácido úrico ($p<0,05$), pero no se asoció con cálculos infecciosos o cálculos de calcio ($p>0,05$). TG fue un factor de riesgo para cálculos urinarios ($p<0,05$). La formación de cálculos urinarios está estrechamente relacionada con los niveles de lípidos en sangre. La dislipidemia, especialmente la hipertrigliceridemia, puede inducir fácilmente la formación de cálculos de ácido úrico.

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INTRODUCTION

Urinary calculi is one of the common urinary system diseases, with an annually steadily increasing prevalence rate, especially in young people, and the first attack rate is the highest among people aged 20-30 years old [1,2]. From a global view, urinary calculus presents a high prevalence rate in countries such as the United States, China, Thailand, and the United Kingdom, but is rare in Central America, South America, and Africa. Epidemiological data have manifested that the incidence rate of urinary calculi is 1-5% in China, and about 25% of such patients need to be hospitalized [3,4]. As

people's working rhythm speeds up, their diet structure changes and their living standards improve, the incidence rate of urinary calculi is climbing each year. The formation of urinary calculi is exceptionally complicated and closely associated with abnormal lipid metabolism, insulin resistance, hyperglycemia, hypertension, obesity, etc.[5]. Blood lipids are essential substances for the primary metabolism of living cells, which widely exist in human bodies and can take part in energy metabolism [6]. According to a study [7], abnormal lipid metabolism is essential in forming urinary calculi. However, the correlations between blood lipids and urinary calculi remain unclear. Therefore, this study analyzed the composition of urinary calculi, and the correlations of urinary calculi with blood lipids were investigated to provide a theoretical basis for the clinical prevention and treatment of urinary calculi.

PATIENTS AND METHODS

General data

Three hundred patients with urinary calculi treated in our hospital from January 2020 to July 2021 were selected retrospectively into a urinary calculi group, while three hundred healthy individuals who received physical examination in our hospital during the same period were enrolled in a control group.

Inclusion criteria: 1) patients diagnosed as urinary calculi by B-ultrasound, kidney ureter bladder X-ray, intravenous pyelography, or computed tomography urography, 2) those whose medical history was complete, 3) those without blood diseases, 4) those without autoimmune diseases, 5) those with no cardiovascular diseases, and 6) those who were informed of this study and signed the informed consent. Exclusion criteria: 1) patients complicated with dysfunction of vital organs such as heart, liver, or lung, 2) those pregnant or breastfeeding, 3) those complicated with a malignant tumor, hypertension, or diabetes mellitus, 4) those with abnormal blood coagulation, 5) those with hyperparathyroidism, or 6) those with cognitive dysfunction.

Methods

Age, gender, BMI, smoking history, drinking history, and other general data of all the subjects were recorded after admission. Five ml of fasting peripheral venous blood was collected in the morning and placed in an aseptic vacuum blood collection tube. After centrifugation (Beijing Era Beili Centrifuge Co., Ltd., model: DT4-6D) at 3,000 rpm for 15 min to separate the serum, the serum levels of low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and total cholesterol (TC) were measured using an automatic biochemistry analyzer (Beijing Beiruida Pharmaceutical Technology Co., Ltd., model: SR95-SUNMATIK-9050). The criteria for dyslipidemia were those referred to in the *Chinese Guidelines for the Prevention and Treatment of Dyslipidemia in Adults (2016 Revision)*[8]. Urinary calculi samples were collected on the day of operation, which were washed with distilled water and dried naturally. Later, the composition of urinary calculi was analyzed using a LUMOS stand-alone FT-IR spectroscopy analyzer (Bruker, Germany).

Statistical analysis

The IBM SPSS 26.0 software was utilized for statistical analysis. The numerical data were expressed as n (%) and compared by the χ^2 test between groups. The measurement data were expressed as ($\bar{\chi} \pm s$) and compared by t-test between groups. Spearman's analysis was employed to analyze the correlation between urinary calculi composition and dyslipidemia. Besides, multivariate logistic regression analysis was adopted to explore the factors affecting urinary calculi. $p<0.05$ indicated that the difference was statistically significant.

RESULTS

Baseline clinical data

In the urinary calculi group, there were 190 males and 110 females, aged 18-67 years old, with an average age of 45.56±5.23 years old and a body mass index (BMI) of 24.51±3.34 kg/m^2. Two hundred of the patients had a smoking history, while 210 of them had a drinking history. In terms of urinary calculi type, there were 100 cases of kidney calculi, 190 cases of ureteral calculi, and ten cases of bladder calculi. In the control group, there were 189 males and 111 females, aged 18-68 years old, with a mean age of 45.51±5.45 years old and a BMI of 24.57±3.17 kg/m^2. Among them, 202 patients had a smoking history, while 209 had a drinking history. No significant differences were found in the age, gender, smoking history, BMI, drinking history and other general data between the two groups ($p>0.05$).

Composition of urinary calculi

The composition analysis results of the 300 patients with urinary calculi indicated 260 cases of calcium calculi (including 102 cases of calcium oxalate - calcium oxalate monohydrate/calcium oxalate dihydrate), 32 cases of calcium oxalate + calcium phosphate, 54 cases of calcium oxalate + hydroxyapatite, 54 cases of calcium oxalate + uric acid, and 18 cases of calcium oxalate + uric acid + calcium phosphate, 51 cases of infectious calculi (including 23 cases of hydroxyapatite + calcium oxalate, 14 cases of magnesium ammonium phosphate + calcium oxalate, four cases of hydroxyapatite + magnesium ammonium phosphate, two cases of ammonium urate, and eight cases of magnesium ammonium phosphate + uric acid), and 28 cases of uric acid calculi (including ten cases of uric acid calculi and 18 cases of uric acid + magnesium ammonium phosphate).

Serum levels of TG, HDL-C, TC and LDL-C

The serum levels of TG and TC were significantly higher, while the serum HDL-C levels were significantly lower in urinary calculus group than those in control group, showing statistically significant differences ($p<0.05$). However, the serum LDL-C levels in the urinary calculus group displayed no statistically significant differences in comparison with those in control group ($p>0.05$) (Fig. 1).

Multivariate logistic regression analysis results of factors affecting formation of urinary calculi

Multivariate logistic regression analysis was performed by incorporating the factors with significant differences above, revealing that TG was a risk factor for the formation of urinary calculi ($p<0.05$) (Table 1).

Composition of calculi in urinary calculi patients with normal blood lipids and dyslipidemia

The proportion of uric acid calculi in urinary calculi patients with dyslipidemia was significantly higher than in those with normal blood lipids, showing a statistically significant difference ($p<0.05$). However, there were no statistically significant differences in the proportions of infectious calculi and calcium calculi between urinary calculi patients with dyslipidemia and those with normal blood lipids ($p>0.05$) (Table 2).

Correlation between the composition of urinary calculi and dyslipidemia

Dyslipidemia was positively correlated with uric acid calculi ($p<0.05$), but not associated with infectious calculi or calcium calculi ($p>0.05$) (Table 3).

DISCUSSION

The reasons for the formation of urinary calculi are complicated, and are associated with various factors. According to a study[9], hyperglycemia, hypertension and abnormal lipid metabolism are risk factors for the formation of urinary calculi, and abnormal lipid metabolism is an important contributor to

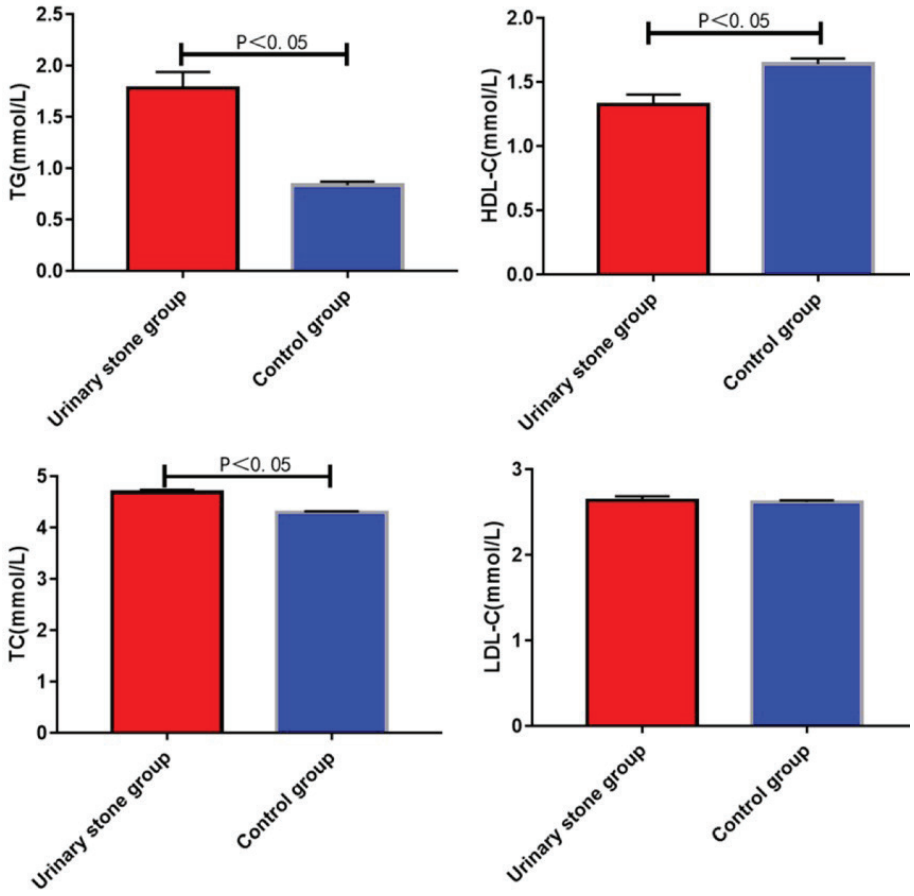

Fig. 1. Serum levels of TG, HDL-C, TC and LDL-C.

Table 1
Composition of calculi in urinary calculi patients with normal blood lipids and dyslipidemia (n(%)).

Group	n	Infectious calculi	Calcium calculi	Uric acid calculi
Normal blood lipids	210	39 (18.57)	180 (85.71)	8 (3.81)
Dyslipidemia	90	12 (13.33)	80 (88.89)	20 (22.22)
χ^2		1.225	0.550	25.241
P		0.268	0.459	<0.001

Tabla 2
Correlation between composition of urinary calculi and dyslipidemia.

Composition of urinary calculi	Dyslipidemia	
	r	p
Infectious calculi	0.082	0.647
Calcium calculi	0.056	0.834
Uric acid calculi	0.678	<0.001

Table 3
Logistic regression analysis results of factors affecting urinary calculi.

Variable	Partial regression coefficient	Standard error	Wald	P	95% CI	OR
Gender	-0.242	0.179	1.562	0.983	0.431-0.902	0.872
Age	0.243	0.154	1.872	0.871	0.672-1.325	1.128
BMI	-0.319	0.147	4.234	0.643	0.542-0.927	0.743
Smoking history	0.156	0.182	1.784	0.743	0.783-1.221	1.209
Drinking history	0.257	0.211	1.673	0.683	1.092-2.532	1.392
TG	0.811	0.305	12.298	0.004	1.547-5.092	2.345
LDL-C	0.041	0.098	0.345	0.823	0.811-1.243	1.112
HDL-C	0.209	0.174	1.205	0.645	0.745-1.562	1.532
TC	0.227	0.172	1.782	0.711	0.943-2.093	1.342

the formation of urinary calculi, which can increase the risk of urinary calculi by 25-30%. A previous study [10] suggested that elevated blood lipid levels will induce rising blood viscosity, leading to atherosclerosis (AS), affecting renal blood flow, and thereby facilitating the deposition of lithogenic substances in the blood to form calculi. The results of this study manifested that the serum levels of TG and TC were remarkably higher. In contrast, serum HDL-C level was dramatically lower in the urinary calculus group than those in the control group. Still, the serum LDL-C levels were not significantly different between the two groups, which was consistent with the study results of Besiroglu *et al.* [11]. These findings suggest that the formation of urinary calculi is associated with dyslipidemia. Moreover, the results of multivariate logistic regression analysis demonstrated that TG was a risk factor for urinary calculi, implying that dyslipidemia, especially hypertriglyceridemia, is more prone to the formation of urinary calculi. Hence, controlling blood lipids has important clinical significance for the prevention of urinary calculi.

For the 300 urinary calculi patients enrolled in this study, the high incidence age was 40-50 years old, with more males than females (the male-to-female ratio was 1.73). By analyzing the composition of urinary calculi, it was found that there were 260 cases of calcium calculi, 51 cases of infectious calculi, and 28 cases of uric acid calculi. It can be inferred that calcium calculi were the major components of calculi in the 300 patients. It is probably because the dietary structure of residents in this area is dominated by seafood, meat, and other high-protein and high-cholesterol foods. Excessive intake of such foods can result in the elevation of blood lipids and increased endogenous acid metabolites in urine, which boosts the excretion of urinary calcium, calcium oxalate, and uric acid, reduces citrate excretion, and lowers the reabsorption of calcium in urine, thereby inducing more calcium oxalate calculi. Among the 300 patients with urinary calculi in this study, the proportion of patients with infectious calculi was next only to those with calcium calculi; most were female. Infectious calculi are the results of infection caused by urease-producing bacteria in urine, and the major components include magnesium ammonium phosphate, apatite and so on [12]. It has been pointed out in a study [13] that urinary tract infection is one of the most common pathogenic factors of female urinary calculi, and the incidence rate of upper urinary tract infectious calculi in female is three times that in men. In the present study, uric acid calculi were

the least common among the 300 patients with urinary calculi. Uric acid is not only the major composition of uric acid calculi, but also involved in the formation of calculi composed of other elements. Hyperuricemia-induced calculi are co-formed by a variety of components under different internal environments [14]. It has been revealed in a study [15] that patients with uric acid calculi have higher incidence rates of diabetes mellitus, poor glucose tolerance, and hypertriglyceridemia than normal people. The results of this study also demonstrated that the proportion of uric acid calculi in urinary calculi patients with dyslipidemia was dramatically higher than that in those with normal blood lipids. Still, there was no significant difference in the proportions of infectious calculi and calcium calculi between urinary calculi patients with dyslipidemia and those with normal blood lipids.

This suggested that dyslipidemia can easily lead to the formation of urinary calculi, especially uric acid calculi, which was further confirmed by the Spearman correlation analysis in this study. It is because elevated blood lipid levels can lead to lipid deposition, which damages the tubular excretory function, thus resulting in rising uric acid levels. Meanwhile, increased uric acid levels reduce lipoprotein esterase activity and TG decomposition. As a result, the blood TG level rises. In addition, high uric acid can cause increased excretion of urine, rising uric acid concentration in urine, and decreased urine PH, so it is more likely to induce the formation of uric acid calculi under the action of multiple factors [16-18]. Uric acid can reduce the protective activity of inhibitors against calcium oxalate crystal formation in urine, allowing the formation of calcium oxalate crystals and aggregation into stones. Therefore, hyperuricemia is related to uric acid-induced heterogeneous nucleation of calcium oxalate crystals [19].

Hence, in clinical treatment of urinary calculi, besides calculus treatment, attention should be paid to regulating the metabolism level in the body, effective control of blood lipids, reasonable diet, and more exercise, so as to lower the risk of recurrent urinary calculi.

In summary, the formation of urinary calculi is closely associated with blood lipid levels. Dyslipidemia, especially hypertriglyceridemia, can easily lead to the formation of uric acid calculi. Controlling blood lipids has important clinical significance for the prevention of urinary calculi. Regardless, this study is limited. This is a single-center retrospective study with a small sample size, so the results may be biased. In the future, we will perform multicenter prospective studies with larger sample sizes to clarify the specific mechanism.

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Conflicts of interest

The authors reported no potential conflict of interest.

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Authors' contribution

LT and HY designed the study; YQ, MY and WG conceived and supervised the study; QH and YS performed and analyzed the experiments; QW drafted the paper. All authors read and approved the final manuscript.

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Modified High-Intensity Interval Training and its effects on immunometabolic regulation in sedentary young adults with overweight and obesity.

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Keywords: obesity; physical activity; lymphocytes; metabolic syndrome; visceral fat.

Abstract. Sedentary lifestyles can contribute to obesity and other diseases; while chronic low-grade inflammation associated with obesity can lead to metabolic alterations. As physical activity is an alternative to decrease excess weight and its related comorbidities, High-Intensity Interval Training (HIIT) has recently emerged as effective in regulating whole-body metabolism and inflammatory processes in people with excess weight. The objective was to compare the effects of a modified HIIT program on peripheral blood leukocytes (PBL), metabolic profile, insulin resistance (IR), and body composition (BC) in sedentary adults with excess weight. PBL, biochemical variables, IR, and BC were analyzed in 37 participants, 23 sedentary young adults (17 with overweight and six with obesity), before and after eight weeks of a modified HIIT program and compared with those of 14 healthy-weight participants**.** The results showed that after HIIT, total lymphocytes, TCD3+, and TCD8+ lymphocytes decreased; granulocytes and naïve TCD3+ cells increased in patients. Regarding partial correlations, we found that changes (Δ) in TCD8+ lymphocytes correlated positively with glucose and LDL-c, while naïve TCD3+ cells correlated with total cholesterol and LDL-c. Δ in TCD4+CD45RA+ cells correlated negatively with Δ in subcutaneous fat tissue and body fat mass. This study reports that sedentary young adults who completed the modified HIIT program showed lymphocyte levels similar to those in healthy-weight individuals and positive changes in the study variables. Such changes suggest immunometabolic regulation through the implementation of HIIT in participants with overweight and obesity.

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Entrenamiento modificado de intervalos de alta intensidad y sus efectos sobre la regulación inmunometabólica en adultos jóvenes sedentarios con sobrepeso y obesidad.

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Palabras clave: obesidad; actividad física; leucocitos; síndrome metabólico; grasa visceral.

Resumen. El sedentarismo puede contribuir a obesidad y otras enfermedades; la inflamación crónica de bajo grado asociada a la obesidad puede llevar a alteraciones metabólicas. La actividad física es una alternativa para disminuir el exceso de peso y sus comorbilidades asociadas, donde el Entrenamiento de Intervalos de Alta Intensidad (HIIT, por sus siglas en inglés) ha emergido como un regulador del metabolismo del cuerpo y del proceso inflamatorio en personas con exceso de peso. El objetivo de esta investigación fue analizar los efectos del programa de HIIT modificado sobre leucocitos en sangre periférica (LSP), perfil metabólico, resistencia a la insulina y composición corporal (CC) en 37 participantes, 23 con exceso de peso (17 con sobrepeso y 6 con obesidad), después de ocho semanas del entrenamiento y compararlos con 14 participantes con peso saludable. Se encontró que los linfocitos totales, linfocitos TCD3+ y TCD8+ disminuyeron; los granulocitos y las células TCD3+ vírgenes aumentaron. En cuanto a las correlaciones parciales, encontramos que los cambios (Δ) en los linfocitos TCD8+ se correlacionaron positivamente con la glucosa y LDL-c, mientras que las células TCD3+ vírgenes correlacionaron con colesterol total y LDL-c. Δ en células TCD4+CD45RA+ correlacionaron negativamente con Δ en tejido adiposo subcutáneo y masa grasa corporal. Estos resultados mostraron que los adultos sedentarios que completaron el entrenamiento presentaron niveles de linfocitos similares a los de los participantes con un peso saludable y cambios positivos en las variables de estudio. Tales cambios sugieren una regulación inmunometabólica en los participantes con sobrepeso y obesidad.

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INTRODUCTION

About one-third of the world population aged 15 years and older do not engage in sufficient physical activity. Consequently, health risks associated with a sedentary lifestyle are on the rise. Sedentary lifestyles and behaviors have several negative effects on the human body including, 'risks of metabolic disorders such as diabetes mellitus, hypertension, and dyslipidemia' [1].

Obesity has been considered a chronic low-grade inflammatory process characterized by an increase in the baseline number of leukocytes and an imbalance between pro- and anti-inflammatory cytokines in circulation [2-4]. Such a condition is associated with insulin resistance (IR) [5], which is characterized by impaired insulin function in metabolically essential tissues, affecting lipid homeostasis and glucose and contributing to metabolic disorders [6,7].

It has been proposed that weight gain and its related comorbidities can be reversed through caloric restriction and increased physical activity [8]. In addition, it has been found that relative health benefits can emerge even in those cases in which there is no change in total sedentary time but intermittent physical activities are included [1]. In this respect, it has been observed that regular exercise is an effective measure against chronic diseases as it improves the inflammatory profile [9].

Recently, modern lifestyles have increasingly promoted sedentary behavior also among young adults. Against this background, High-Intensity Interval Training (HIIT) has emerged as an alternative that 'burns many calories in a short time, so busy people can be more inclined to incorporate intermittent bursts of exercise into their schedule' [10]. HIIT effectively regulates whole-body metabolism [11,12], enhances insulin signaling, and improves anthropometric measures and body composition (BC) [13-14]. In addition, HIIT has been associated with immunomodulatory effects with potential anti-inflammatory benefits [4].

Although positive effects of HIIT have been reported concerning obesity, little is known about the relationship between different variables like body composition, metabolic profile, lymphocyte subpopulations, and inflammatory response. Because the problem of obesity has grown in recent years and it is presented as a complex condition to treat and because it implies lifestyle changes, it is necessary to propose strategies to the population that, in the short term, provide them with some benefit, which is observable as physical activity. Therefore, this study aimed to investigate the effects of a modified HIIT program over eight weeks on the variables mentioned above, peripheral blood cells, metabolic profile, IR, anthropometric measures, and BC in apparently healthy sedentary patients with overweight or obesity, with or without metabolic syndrome (MS).

METHODS

Study design

A longitudinal clinical-trial study was performed with sedentary young adults in Mexico City, Mexico. The study was conducted following the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Metropolitan Autonomous University of Xochimilco (Agreement 13/16,8.1). Participants provided written informed consent before enrolling in the study.

The study population was selected according to the following criteria:

Inclusion criteria: adults aged 18-40 years apparently healthy with overweight (OW) or obesity (Ob) who did not engage in regular and sufficient physical activities.

Exclusion criteria: individuals with any infection, pregnant women, individuals with diabetes mellitus type 1 or type 2 (DM2), or autoimmune, hepatic, renal, endocrine, cancer, or heart disease, who were taking any medication and/or participating in regular exercise training, and who did not present a medical certificate to engage in regular exercise.

The program included no dietary or alimentary behavior modification to minimize the influence of additional factors. All the variables listed below were assessed before and after two months of modified HIIT workouts. Measures were taken after at least 48 h of the last exercise session to avoid data on the acute effects of exercise. All the variables listed below were evaluated at the beginning and after two months (eight weeks) under a modification of the HIIT training.

Se dentary behavior

Sedentary behavior was evaluated using an International physical activity questionnaire (IPAQ)-short form (7-day physical activity). All participants were required to complete the questionnaire in advance of the study. An IPAQ activity level of <600 MET/week was defined as sedentary behavior.

Clinical measurements

Anthropometric measurements were performed following the standardized protocol of the International Society for the Advancement of Kinanthropometry (ISAK)[15] (Although the person who made the measurements for the study is not ISAK certified, it was standardized by someone who is ISAK certified). A SECA 213 stadiometer set at 0.1 cm of precision was used to measure height. Weight was measured using InBody720 equipment (Biospace, Inc. Los Angeles, CA, USA), and waist circumference (WC) was measured employing SECA brand 201 (Chino, CA, USA) fiberglass tape. Nutritional status was assessed through body mass index (BMI) according to the World Health Organization (WHO) [16] criteria for adults.

A dual-energy X-ray absorptiometry (Hologic Discovery Wi. Hologic, Inc. Bedford, MA, USA) was used to measure body composition, obtaining data on skeletal muscle mass (SMM, in kg), body fat mass (BFM, in kg) and the percentage of body fat (BF%). A multi-frequency impedance body composition analyzer (InBody 720, equipment) was utilized to obtain visceral adipose tissue (VAT) in square meters, in which visceral obesity (increased VAT) was diagnosed in persons with ≥100 cm^2 of fat and normal VAT in persons with <100 cm^2 of fat).

Lipid and glucose measurements

For biochemical tests, 5 mL of a venous blood sample was collected using an automated clinical chemistry equipment KONTROLab, model Ikem (KONTROLab Co., Ltd. Roma, Italia), to obtain: triglycerides (TG); high-density lipoprotein-cholesterol (HDL-c), fasting glycemia (Glu), and total cholesterol (TCho). Low-density lipoprotein-cholesterol (LDL-c) was calculated based on the Friedewald formula. Samples were obtained after a 12-h fasting period. Blood pressure measurements were performed according to the guidelines of the Mexican Official Norm (NOM-030-SSA2-1999). The presence of MS was determined according to the Cholesterol Education National Program (ATP III) definition and modified according to the Hispanic population [17]. These guidelines suggest that MS is present when an association of at least three of the following factors is found: TG ≥150 mg/dL; HDL-c <40 mg/dL; blood pressure ≥130/85 mmHg or a previous diagnosis; Glu ≥100 mg/dL, and a WC in women of ≥80 cm and in men ≥90 cm.

Insulin resistance

The METabolic Scale for Insulin Resistance (METS-IR) tool was employed to measure the IR parameter. This consists of an indirect method for the detection of insulin action that takes into account blood concentrations of TG, HDL-c, glucose, and BMI as follows: (Ln((2*G0)+TG0)*IMC)/(Ln(HDL-c)); G0: fasting glucose, and TG0: fasting triglycerides [18].

Measurement of lymphocyte subpopulations

A 5 mL venous blood sample was collected in tubes containing K_2 EDTA (BD Biosciences, San Jose, CA, USA). Cells were stained with conjugated commercial antibodies (BD Biosciences, San Jose, CA, USA), and the combinations employed were the following: FITC-anti-IgG1/PE-anti-IgG2; FITC-anti-CD45/Pe-anti-CD14; FITC-anti-CD3/PE-anti-CD16+CD56/PerCP-anti-CD19; FITC-anti-CD4/PE-anti-CD62/APC -anti-CD3; FITC-anti-CD8/PE-anti-CD28/APC-anti-CD3, and FITC-anti-CD45RA/PE-anti-CD45RO/PerCP-anti-CD4/APC-anti-CD3. Peripheral blood samples were stained with conjugated antibodies for 20 min in the dark, then 3 mL of lysis buffer solution was added. Samples were washed (PBS), fixed with 1% p-formaldehyde, and analyzed within 24 h of staining [4, 19].Cell analysis was performed using a FACSCanto II Cytometer with FACSDiva software (BD Biosciences, San Jose, CA, USA). For each sample, 10,000 cells were counted.

Intervention

HIIT group participants were supervised along three HIIT exercise sessions per

week for eight weeks. Each training session consisted of 25 min of effective exercise, and each minute was divided into 30-s intervals of activity, followed by a 30-s of active rest (Active rests refers to the participants did not stop moving altogether; they continued jogging; it was rest from the exercises involved in the session, such as squats or some other exercise). Effective exercise time and intervals increased according to the participants' capacity. Eventually, the exercise session consisted of 45 min of active exercise, while each minute consisted of 50-s intervals of activity, followed by 10-s of active rest. On the recommendation of the Centers for Disease Control and Prevention, the American College of Sports Medicine, and the American Heart Association [20], sessions were organized into three parts.: warm-up (5-9 min), aerobic part/resistance (HIIT) (25-45 min), and cooling/flexibility (8-12 min).The activity intensity was measured according to the Perceived Exertion Rating Scale (RPE), managed at moderate intensity (between 5 and 6) [21]. As HIIT works with high-intensity intervals and since we worked with sedentary subjects with overweight and obesity, a modification to this training system was introduced in order to work at moderate intensity. In addition, the Karvonen formula (Functional Capacity Evaluation [FCE]= {(FM-FR)*PI} + FR) was adapted, and the percentage of intensity (PI) at which participants worked was obtained by taking into account heart rate (FCE), resting frequency (RF), and maximal frequency (MF) during the training sessions) [22]. For the latter, the FC was taken at each session before and after the training.

The participants' adherence to the study was calculated as the percentage of complete sessions attended in relation to the total number of training sessions proposed in the present study.

Statistical analysis

The Shapiro–Wilk test was utilized to determine whether the variables presented had a Gaussian distribution. Logarithmic transformation was performed to calculate the normality in variables without Gaussian distribution. The results are presented as means ± standard deviations (SD) and medians, employing an interquartile range (IQR, 25-75). Paired Student *t*-tests were employed to compare pre-and post-exercise data. The One-way analysis of variance (ANOVA) and Bonferroni post-hoc tests were applied to determine differences between >two groups. Data were adjusted for gender and age. Bilateral partial correlation analyses adjusted for gender, age, and BMI were performed to estimate the correlation of the Δ of the lymphocyte subpopulations with the Δ of the anthropometric, BC, and biochemical variables. Then, step-wise forward linear regression models utilizing the Δ of lymphocyte subpopulations as dependent variables to evaluate the association with Δ of anthropometric, BC, and biochemical variables adjusted for gender, age, and BMI were performed. A $p<0.05$ was considered significant, using the IBM SPSS ver. 21 statistical software program.

RESULTS

Forty overweight or obese persons were accepted to participate in the training sessions; however, several individuals withdrew from the exercise (n=11). In addition, six individuals did not attend their second evaluation. Thus, the study group consisted of 23 individuals. Remarking this way, the problem that sedentary people have of adhering to a routine.

Thirty-seven people participated, and two groups were formed: 1) the control group, consisting of 14 participants with normal BMI, normal weight (NW), and VAT, without MS, matched by gender and age with the study group, who did not perform training, and 2) the study group, consisting of 23 persons with OW and Ob who underwent the training. All participants included in the study attended at least 80% of the classes

conducted. The average age of participants was 26.4±6.5 years, and 73% were female.

Baseline characteristics

Thirty-seven percent of participants (n=14) had normal BMI, 46% (n=17) people with OW, and 16% (n=6) had Ob. A prevalence of 26% (n=6) of MS was found in the study group. It was also observed that individuals with MS were mostly people with visceral obesity.

Laboratory analysis, anthropometric measurements, and BC

Regarding biochemical variables, it was observed that persons with obesity had the highest TG values compared to individuals with NW and diastolic pressure in relation to individuals with OW. It was also found that HDL-c was lower in individuals with OW than in those with NW. Concerning IR, it was found that METS-IR increased as the BMI did; therefore, individuals with NW presented the lowest values and persons with Ob the highest values; these differences were significant (Table 1).

Concerning anthropometric measurements and BC, it was found that participants with NW had the lowest values in WC, ST, BFM, and VAT compared to individuals with OW or Ob (Table 1).

Lymphocyte subpopulations

It was found that total lymphocytes (TL) increased according to BMI. On the other hand, granulocytes were lower in persons with NW when compared with individuals with OW or Ob; all of these differences were statistically significant. No significant differences were observed when analyzing the memory and naïve cells according to nutritional status (Table 2).

Intervention

Overall, participants worked at 54 ± 10.6% of their maximal level according to the formula of Karvonen. In addition, the intensity percentage was increased as the training weeks progressed, finding a significant difference between weeks 1 and 6 (46±13 vs. 61.9±10%, p<0.05).

Laboratory analysis, anthropometric measurements, and BC

In individuals with OW or Ob, no significant positive changes were found in biochemical indicators after training by group (Table 1). However, when each participant was analyzed, it was found that TG decreased in 39% of the participants by 25±14.7%, Glu in 61% by 22±10%, TCho in 74% of individuals by 24±10%, LDL-c in 74% by 37±16%, and HDL-c increased in 52% of persons by 20±10% (Fig. 1).Regarding anthropometric measurements and BC after training, persons with OW presented a statistically significant decrease in WC (Table 1). Also, when each participant was analyzed, it was found that VAT decreased in 57% of the participants by 5.3±4.6cm^2, ST in 57% by 1.9±2.2%, BFM in 61% of individuals by 1.7±1.5kg, and SMM increased in 30% by 1.3±1.6kg (Fig. 2). In addition, it was observed that one person with Ob passed to OW and two with OW passed to NW; furthermore, two persons with increased VAT passed to normality.

Lymphocyte subpopulations

After training, TL and TCD8+ lymphocytes decreased in the study group. Also, granulocytes and näive TCD3+ cells increased.

When the subpopulations were analyzed according to BMI, TL, TCD3+, and TCD8+ decreased in individuals with OW, approaching the percentages of persons with NW. In addition, an increase of TCD3+CD45RA+ and monocytes was found in persons with OW. All of these differences were statistically significant (Table 2). According to MS, we found that TCD4+ lymphocytes decreased (55.6±13.2 vs. 29±13%; p<0.0001) after the training activity. In individuals without MS, the monocytes increased (6.6±3.5 vs. 8.2±2.4%; p<0.0001), while TCD8+ lymphocytes decreased (39±8.5 vs. 29.8±11.8%; p<0.05).

Table 1
Biochemical indicators, anthropometric measurements, and body composition according to Body Mass Index before and after two months of High-Intensity Integral Training.

Variable (n=37)	Normal (n=14)	Overweight (n=17)			Obesity (n=6)			p (ANOVA)	
		B	A	p#	B	A	p#	p	p (post hoc)*&
TG (mg/dL)	80 ±26.5	119.8±52.6*	150.5±70.8	0.088	163.1±42.3*	187.3±58.9	0.188	0.002	0.002
c-HDL (mg/dL)	51.9 ±13.6	39.2* ±8.1	38.2 ±8.4	0.607	43.2 ±13.3	40.5 ±3.9	0.477	0.011	0.009
Glu (mg/dL)	81.1 (77.6-85.7)	79.4 (70.2-94.3)	83.8 (57.6-95.9)	0.597	81.2 (72.9-103.8)	78.7 (72.2-88.4)	0.226	0.754	
c-LDL (mg/dL)	80.3 (64.9-93.6)	91.6 (60.9-130.3)	63.6 (46.4-88.7)	0.124	80.2 (46.7-144.1)	83.9 (48.1-99)	0.818	0.631	
TCho (mg/dL)	146.9 (133.8-166.8)	161.7 (128.4-193.9)	140.2 (114-173.5)	0.608	165.3 (115.5-228.1)	156.9 (130.8-182.5)	0.757	0.628	
SBP (mmHg)	105.5 ± 8.6	106.2 ±10.8	107 ±6.8	0.819	116.3 ±15.6	111.6 ±11.6	0.309	0.144	
DBP (mmHg)	69.5 ±7	69.5 ±7.8	73.5 ±9.9	0.151	79 ±10.3&	76.6 ±5.1	0.675	0.049	0.059
METS-IR	31.8 ±4.2	42.4 ±4.9*	43 ±43	0.494	52 ±6.6*&	51.6 ±6.4	0.712	0.000	0.001
Weight (kg)	60.9 ±9.9	76.3 ±10*	75.9 ±10.4	0.328	87.1 ±17.5*	86.7 ±18.2	0.436	0.000	0.000
BMI (kg/m^2)	22.5 ±1.7	27.3 ±1.3*	27.2 ±1.6	0.304	33.2 ±2.3*&	32.5 ±2.5	0.242	0.000	0.000
WC (cm)	81.2 ±7.4	90.9 ±6.4*	88.7 ±6.5#	0.001	102.7 ±10.5*&	99.7 ±11.2	0.187	0.000	0.000
SMM (kg)	23.8 (18.5-26.2)	26.3 (23.1-30.1)	26.6 (24.4-29.6)	0.949	23 (21.8-37)	23.6 (22-37.6)	0.131	0.150	
ST (%)	27.7 ±6	35.5 ±5.9*	35 ±5.6	0.473	43.5 ±6.4*&	42.3 ±6.2	0.187	0.000	0.000
BFM (kg)	16.8 ±3.2	27 ±5.3*	27.2 ±6.8	0.822	37.1 ±3.8*&	36 ±4.7	0.258	0.000	0.000
VAT (cm^2)	67.1 ±19	99.5 ±18*	98.5 ±18.8	0.500	139.9 ±28.6*&	138.5 ±33	0.722	0.000	0.000

Data are presented in media ± SD or median and interquartile range (IQR). p (post hoc): p adjusted with the Bonferroni test. # Statistically significant difference between before and after. * Statistically significant difference vs normal. & Statistically significant difference vs overweighs ($p<0.05$). B: before; A: after; TG: triglycerides; c-HDL: high-density lipoprotein cholesterol, Glu: glucose; c-LDL: Low-density cholesterol; TCho: total cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; WC: waist circumference; SMM: skeletal muscle mass; ST: subcutaneous adipose tissue; BFM: body fat mass; VAT: visceral adipose tissue.

Table 2
Leukocyte distribution before and after High-Intensity Integral Training according to Body Mass Index

Variable (n=34) (%)	Normal (n=11)	Overweight (n=17)			Obesity (n=6)			
		B	A	p#	B	A	p#	P*&
Total lymphocytes	29.3 ±10.9	40.3 ±9.6*	32.2 ±8.5#	0.048	53.5 ±9*&	33.1±7.5#	0.029	0.000
Monocytes	7.8 ±2.4	7.1 ±3.1	8.4 ±2.2#	0.044	5.6 ±2.5	5.9 ±1.3	0.893	0.256
Granulocytes	62.6±11.1	52.4 ±8.5*	59.1±8.2	0.064	40.9±8.7*	60.7 ±6.6#	0.014	0.000
Lymphocytes B	7.5 (5-12.6)	7.4 (5.4-10.5)	9.2 (6.1-1.7)	0.148	11.6 (7.7-18.8)	15.2 (9.9-18.6)	0.293	0.202
Lymphocytes NK	19.5 (12.8-32.7)	14.3 (10.6-19.9)	16.6 (14.2-24)	0.171	13 (9.4-19.8)	20 (16.2-26.7)	0.199	0.430
TCD3+	68.4 ±10.6	76.5 ±8.1	68.9±10.5#	0.043	71.2±10.9	62 ±9.3	0.244	0.112
TCD4+	56.9 (46-60.7)	52.2 ±7.7	43.5 ±16.5	0.139	51.4±13.7	38.4 ±19.5	0.568	.763
TCD8+	29.8 ±14.9	38.7 ±7.8	29.6±12.1#	0.002	34 ±7.4	24.1±11.1	0.147	0.198
TCD3+CD45RA+	54.2 ±13	48.5 ±11.6	53.4 ±8.7#	0.050	44.5±18.7	59 ±15.6	0.330	0.456
TCD3+CD45RO+	32.1 ±9.4	31.6 ±8.7	31.4 ±5.8	0.792	39.7±14.9	26.1±9.5	0.230	0.358
TCD3+CD45RO+ CD45RA+	15.7 ±6	16.7 ±5.9	12.7 ±6.6	0.454	14.6 ±5.8	11.6 ±6.7	0.558	0.828
TCD4+CD45RA+	36.4 ±14.7	20.3 ±7.2	35 ±17.3	0.438	33.9 ±5.1	52.5 ±15.2	0.160	0.521
TCD4+CD54RO+	43.2 ±13.4	48.1 ±8.8	53.1 ±20.6	0.712	43 ±20.6	36 ±12.7	0.661	0.796
TCD4+ CD45RO+CD45RA+	17.6 ±5.8	16.7 ±6.1	11.5 ±3.8	0.250	24.4±15.4	11.8 ±5.7	0.444	0.395

Data are presented in means ± SD or median and interquartile interval (IIC).* Statistically significant difference vs normal. & Statistically significant difference vs. overweight. # Statistically significant difference before vs. after ($p<0.05$). B: before; A: after; Lymph B: Lymphocytes B; Lymph NK: Lymphocytes natural killer.

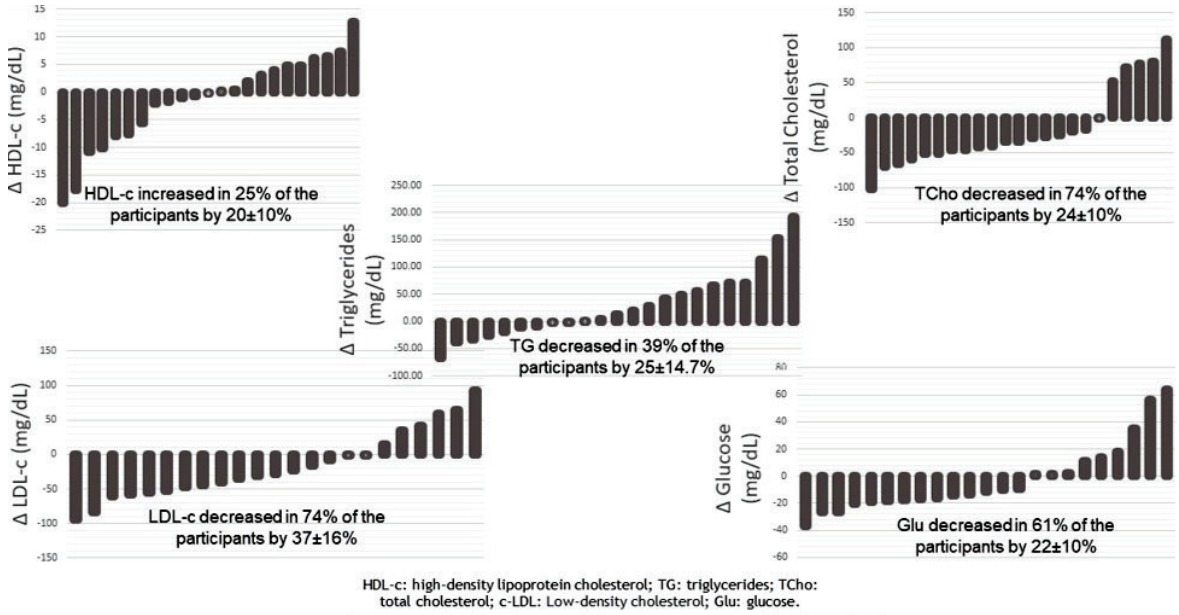

Fig. 1. Distribution and improvement of biochemical indicators after training.

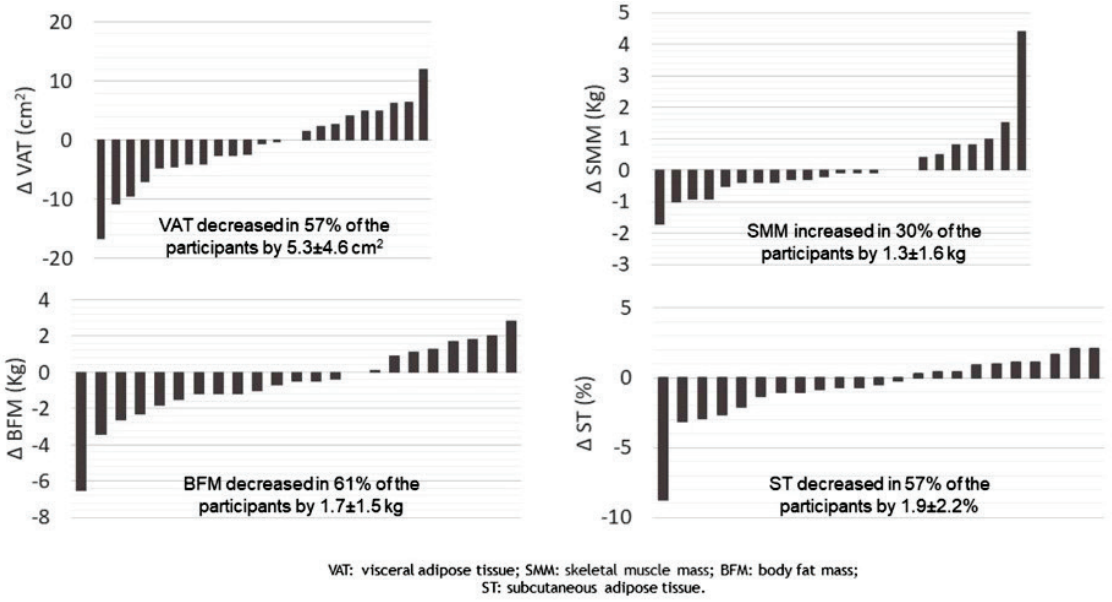

Fig. 2. Distribution and improvement of anthropometric measurements and body composition after training.

Partial correlations were made of the observed variations in leukocyte cells after the intervention concerning the changes (Δ) in the different variables, adjusting the analysis by gender, age, and BMI. We found that Δ at the peripheral level of the granulocytes and TCD8+ lymphocytes correlated negatively with Δ in WC; additionally, the Δ of TCD8+ lymphocytes correlated positively with glucose and LDL-c. The Δ of the total näive cells correlated positively with TCho and LDL-c, and the Δ of the positive double cells of TCD3+ (TCD3+CD45RA+CD45RO+) positively correlated with the Δ in MERS-IR. Last, the Δ of the näive cells of TCD4+ lymphocytes correlated negatively with the Δ

percentage and kilograms of fat, all of these differences statistically significant (Table 3). No correlation was observed between exercise intensity and change in lymphocyte subpopulations.

Furthermore, we decided to perform a linear regression adjusted for gender, age, and BMI among the changes in lymphocyte subpopulations statistically correlated with the changes in the different variables. A positive correlation was found between the changes of TCD8+ lymphocytes and TCD3+CD45RA+ lymphocytes with changes in LDL-c, where a decrease of 1 mg/dl of this cholesterol was associated with a decrease of 3% (range, 0.8-4.5%) of TCD8+ lymphocytes and an increase of 6% (range, 2.8-9.6%) of TCD3+CD45RA+ lymphocytes. Also, TCD3+CD45RA+ lymphocytes correlated positively with TCho, where a decrease of 1 mg/dL of this type of cholesterol was associated with an increase of 7% (range, 1-11%) of TCD3+CD45RA+ lymphocytes. Finally, TCD3+CD45RA+CD45RO+ lymphocytes correlated positively with METS-IR, where a decrease of 1 unit of METS-IR was associated with a decrease of 1% (range, 0.5-2%) of TCD3+CD45RA+CD45RO+ lymphocytes (Fig. 3).

DISCUSSION

Obesity is caused by a genetic predisposition and environmental and lifestyle factors, including physical inactivity and poor eating habits [23]. Physical inactivity and obesity are associated with visceral fat accumulation, leading to chronic low-grade inflammation and the pathogenesis of IR, MS, DM2, cardiovascular diseases, and cancer [24,25]. Total sedentary time and moderate-to-vigorous physical activity have been reported to be negatively correlated. Waist circumference, body mass index, triglyceride level, and plasma glucose level have also been reported to decrease while the number of breaks in sedentary time increased [1]. On that basis, the present study aimed at determining the impact of a modified HIIT on metabolic, an-

Table 3
Partial correlation between changes in leukocyte cells and changes in the anthropometric, biochemical, and body composition variables.

Leukocyte cells	Variables	p	P
Δ of Total lymphocytes	Δ of WC	0.491	0.053
Δ of granulocytes	Δ of WC	-0.514	0.041*
Δ of lymphocytes TCD8+	Δ of WC	-0.612	0.007*
	Δ of Glu	0.474	0.047*
	Δ of DBP	0.458	0.056
	Δ of c-LDL	0.617	0.006*
	Δ of Total Cho.	0.460	0.055
Δ of TCD3+CD45RA+	Δ of c-LDL	0.959	0.010*
	Δ of Total Cho.	0.941	0.017*
Δ of TCD3+CD45RA+CD45RO+	Δ of METS-IR	0.962	0.009*
Δ of TCD4+CD45RA+	Δ of ST	-0.950	0.050*
	Δ of BFM	-0.974	0.026*
	Δ of c-HDL	0.938	0.062

WC: waist circumference; Glu: glucose; DBP: diastolic blood pressure; c-LDL: Low-density cholesterol; Total Cho: total cholesterol; ST: subcutaneous adipose tissue; BFM: body fat mass; c-HDL: high-density lipoprotein cholesterol. p: p value adjusted by gender, age, and BMI ($p<0.05$).

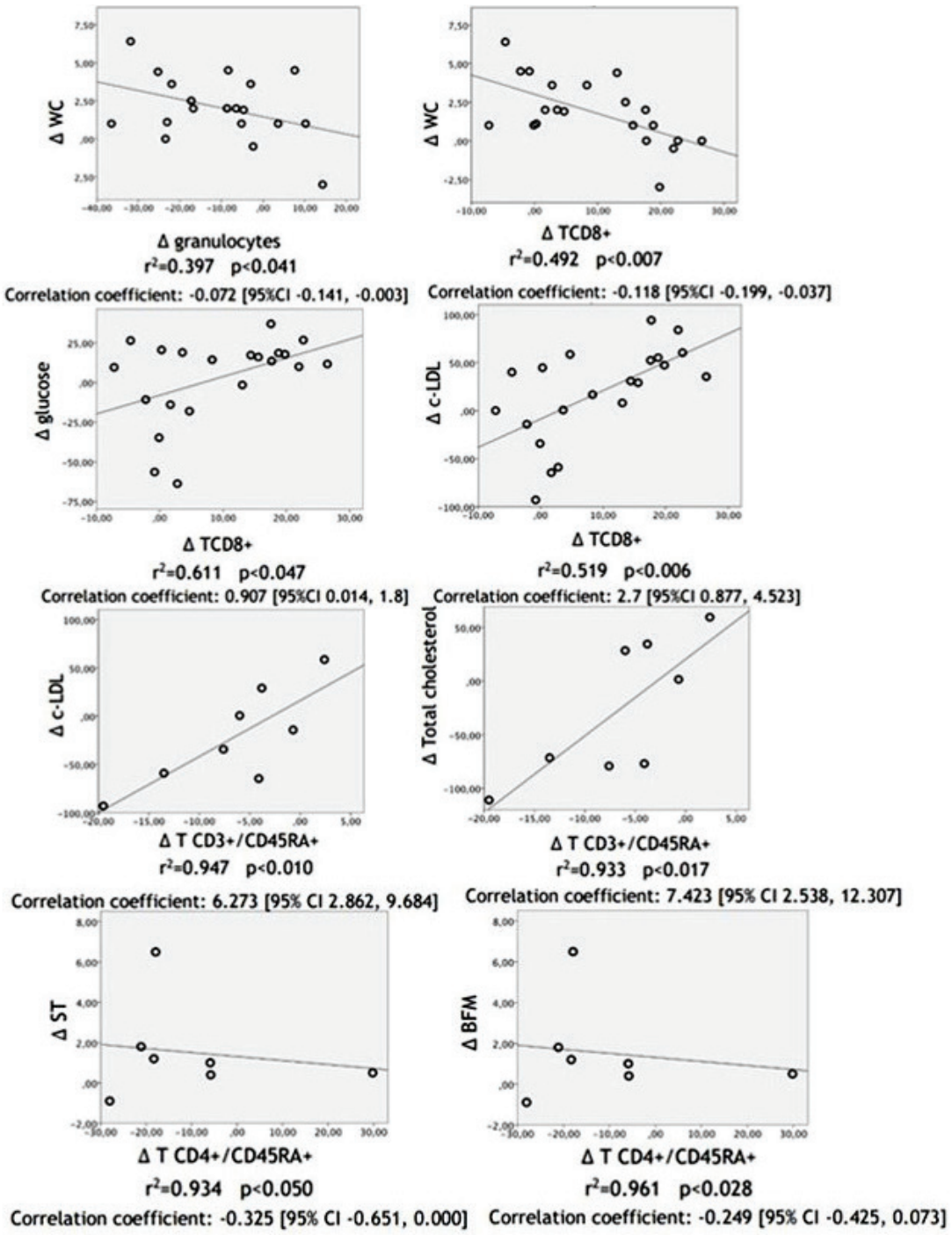

Fig. 3. Linear regression between changes in leucocytes cells and changes of the different variables.

thropometric, BC, and PBL measures in sedentary patients with OW and Ob.

HIIT has been proposed as a better-suited activity for people with OW and Ob than traditional continuous exercise [4]. Some studies have reported a reduction in fasting glucose, insulin, diastolic blood pressure (DBP), and systolic blood pressure (SBP) and improvements in insulin sensitivity after 16, 14, 12, or 2 weeks of HIIT [3, 26-29]. In the present study, about 74% of participants showed improvements in TG, Glu, TCho, LDL-c, and HDL-c levels after training. Concerning BC, other studies that implemented HIIT in persons with OW or Ob for two and 12 weeks, found a reduction in weight, WC, fat mass, and BMI [27, 29]. In this study, a significant statistical reduction was observed only in WC in persons with OW, as well as about 50% of participants showed decreased VAT (5.3±4.6cm^2), ST (1.9±2.2%), and BFM (1.7±1.5kg), and increased SMM (1.3±1.6kg).

Although participants were untrained people, they reached moderate intensity during weeks 6 and 7 (62% of their intensity percentage). The intensity percentage increased as weeks progressed, observing a significant difference between week 1 and week 6 ($p<0.05$).

On the other hand, physical activity has been often recognized as a powerful countermeasure to inflammation [30]. A study carried out with untrained young adults who participated in HIIT for three days found that the percentages of TCD4+, TCD8+, and CD19+ lymphocytes increased significantly after training [31]. Another study carried out over two weeks with inactive persons with OW or Ob revealed that training did not affect the blood concentration of total lymphocytes (TL), monocytes, and neutrophils [27]. Both data do not coincide with those reported in the present study, as it was found that in persons with OW, TL, TCD3+, and TCD8+ decreased (averages were similar to those in persons with NW). On the other hand, in individuals with Ob, TL decreased, and granulocytes increased statistically, getting closer to those values in persons with NW. With these results, it can be observed that only TL had the same behavior both in individuals with OW and in those with Ob. These data suggest differences in the mobilization of leukocytes at the peripheral blood level according to the nutritional status after physical activity.

In addition, it has been reported that T lymphocytes and TCD8+ increase with obesity [32, 33] and that they infiltrate adipose tissue and promote the classical pro-inflammatory activity of M1 macrophages and the production of pro-inflammatory cytokines. These phenomena can trigger a metabolic imbalance, such as an increase in Glu, TCho, LDL-c, and TG and a decrease in HDL-c, among others [34-36]. The finding in the present study of diminished TL, TCD3+, and TCD8+ might indicate a decrease of the inflammatory process at the peripheral blood level, improving the metabolic profile, because the improvement was found in the different metabolic variables in a little more than 70% of the participants, in addition, in different correlations and linear regressions, it was found a relationship between improvements in lymphocyte subpopulations and both biochemical and anthropometric variables.

Furthermore, in other studies, it has been observed that monocytes, B cells, NK cells, and T cells (CD4+ and CD8+) are found in higher proportions in people with obesity and provide a link between systemic inflammation and IR [37-40], which would guide us to reiterate that having found some of these cells decreased after physical activity means an improvement of the inflammatory process that is associated with IR and metabolic alterations, representing a better metabolic state for the participants.

On the other hand, in persons with obesity, immunosenescent behavior has been reported, similar to that appearing in the elderly. This has been denominated as "premature immunosenescence", an imbalance between senescent, naïve, and mem-

ory cells that renders the individual with obesity more susceptible to the disease [19, 43]. The specific increase in memory cells in obese patients could somehow reflect what some authors have pointed out about the capacity for proliferation and activation of memory cells, revealing the high degree of chronic adaptive immune activation [41,42], which has been associated, as has already been mentioned, with metabolic alterations in these patients.

In this sense, it has been observed that exercise may counteract immunosenescence and its associated diseases by limiting the accumulation of senescent T cells and repopulating the blood with naïve T cells [30], mainly through promoting the expansion of the naïve cell repertoire as a consequence of the apoptosis of senescent T cells [44-47], this apoptotic process is thought to induce hematopoietic stem cells production in the bloodstream, which may move to the thymus and stimulate the development of naïve T cells [30]. In the present study, in addition to finding an increase in naïve cells after training (TCD3+ ($p<0.05$) in persons with OW), a correlation between such an increase and the reduction of total lymphocytes, IR and LDL- cholesterol was observed. These results suggest that this training could promote immunometabolic regulation in these patients. Furthermore, it has been found that senescent T lymphocytes are related to high percentages of body fat [44, 46, 48]. It would be valuable and interesting to investigate the presence of senescent T cells in persons with obesity.

Strengths, limitations and conclusions

Exercise is one of the therapies that accompany the treatment of obesity; However, on some occasions, depending on the period and the routine that is studied, it has been observed that "there is no change", but with this work, we can realize that the physical activity (modified HIIT with moderate intensity, in this case) carried out, is influencing the inflammatory process that triggers obesity and, in turn, these changes were related to immunometabolic improvements, thus highlighting the relevance of this study.

Some of the study's strengths were that it was possible to implement an exercise routine in a sedentary population; immunometabolic changes were obtained in only eight weeks of exercise. Also, the presence of comorbidities (for example, dyslipidemias, hypertriglyceridemias, hyperglycemia, MS, among others) was detected and treated in 25% of the participants with exercise; All international standards were followed to evaluate the selected variables.

Certain limitations of the present study were: The lack of analysis of other inflammation markers that could give us more information about the inflammatory process before and after routine physical activity and the voluntary desertion of some patients due to the problem of adherence to a routine in sedentary people.

In conclusion, this study reports that an eight-week modified HIIT program brought about positive changes in peripheral lymphocyte subpopulations in sedentary individuals with OW or Ob, as it observed a reduction in TL and TCD8+ and an increase in naïve cells, bringing the values closer to those in persons with NW. These changes correlated with healthier metabolic variables. Also, differential leukocyte changes were observed according to BMI. These results represent novel knowledge about the positive effects of HIIT, not only regulating whole-body metabolism but also regulating immunomodulatory effects with anti-inflammatory benefits. All these results reinforce the benefits of HIIT as an exercise strategy to promote the regulation of immunometabolism.

As shown in this study, exercising is essential, and obese patients should be made aware that they should make changes in their lifestyle in general, particularly in terms of having a more active life. For this reason, it is essential to continue conducting studies of this nature.

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Conflict of interest

None

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COVID-19 and bacterial superinfections: clinical and microbiological profiles, and determinants of mortality in a reference center in Quito, Ecuador.

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Keywords: sepsis; COVID-19; mortality; antibacterials.

Abstract. The massive prescription of antimicrobials accelerated the generation of multi-resistant bacteria during the SARS-CoV-2 pandemic. This work aims to present the epidemiological, clinical, and microbiological profiles of a series of patients with bacterial superinfections hospitalized in a COVID-19 reference center. We conducted a retrospective observational study in adult COVID-19 patients hospitalized between January and December 2021 who presented with bacterial superinfections. Mortality at discharge was the variable outcome. The median age of the 240 patients included in the study was 55 years, and the male sex predominated at 68.75%. The median stay of hospitalization was 24 days. Superinfections occurred in 55% of patients with mechanical ventilation. The most frequent bacteria were KPC-producing *Klebsiella pneumoniae complex* (24.17%), ESBL-producing *Klebsiella pneumoniae complex* (17.92%), and carbapenem-resistant *Pseudomonas aeruginosa* (13.75%). The most used empirical and targeted antibiotic schemes consisted of the association of carbapenem, glycopeptides, and aminoglycosides (56.09 and 38.55%, respectively). In the multivariate analysis, older age (p= 0.006, OR 1.03, 95% CI: 1.01-1.06), central venous catheter-related bacteremia (CLBSI) (p= 0.028, OR 1.94, 95%CI: 1.07-3.49), and the use of colistin associated with other antibiotics as targeted therapy (p: 0.028, OR 12, 95%CI: 1.30-110.52), were independent predictors of mortality. In this series, we found that in patients with COVID-19 and bacterial superinfection, age, CLBSI, and colistin use were independent predictors of non-survival. The most frequently isolated microorganisms were ESBL- and KPC-producing enterobacterales and non-fermenting Gram-negative bacilli resistant to carbapenems.

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COVID-19 y sobreinfección bacteriana: perfil clínico, microbiológico y determinantes de mortalidad en un centro de referencia en Quito, Ecuador.

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Palabras clave: septicemia; COVID-19; mortalidad; antibacterianos.

Resumen. En la pandemia por SARS-CoV-2, la prescripción masiva de antimicrobianos aceleró la generación de bacterias multirresistentes. El objetivo de este trabajo fue presentar el perfil epidemiológico, clínico y microbiológico de una serie de pacientes con sobreinfección bacteriana, hospitalizados en un centro de referencia COVID-19. Se realizó un estudio observacional retrospectivo, en pacientes adultos hospitalizados entre enero y diciembre de 2021 con COVID-19, que presentaron sobreinfecciones bacterianas. La mortalidad al egreso fue la variable desenlace. En 240 pacientes, la mediana de edad fue 55 años y predominó el sexo masculino 68,75%. La mediana de hospitalización, fue 24 días. El 55% de las sobreinfecciones se presentó en pacientes con ventilación mécanica. Las bacterias más frecuentes, fueron *Klebsiella pneumoniae complex* productora KPC (24,17%), *Klebsiella pneumoniaecomplex* productora ESBL (17,92%) y *Pseudomonas aeruginosa* resistente a carbapenémicos (13,75%). Los esquemas antibióticos empíricos y dirigidos más utilizados constaron de la asociación de carbapenémico, glicopéptido y aminoglucósido (56,09 y 38,55% respectivamante). En el análisis multivariado la mayor edad (p= 0,006 OR 1,03 IC95%: 1,01-1,06); la bacteriemia relacionada a catéter venoso central (CLBSI) (p: 0,028 OR 1,94 IC95%: 1,07-3,49) y el uso de colistina asociado a otros antibióticos como terapia dirigida (p= 0,028 OR 12 IC95%: 1,30-110,52), fueron predictores independientes de mortalidad. En esta serie encontramos que en pacientes con COVID-19 y sobreinfección bacteriana, la edad, la CLBSI y el uso de colistina, fueron predictores independientes de no supervivencia. Los microorganismos más frecuentemente aislados fueron los enterobacterales productores de ESBL y KPC y los bacilos Gram negativos no fermentadores resistentes a carbapenémicos.

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INTRODUCTION

COVID-19 is an emerging viral disease initially reported in Wuhan-China at the end of 2019, rapidly expanding and, in months, collapsed global health systems [1]. Regardless of the speed with which the causative agent, a beta coronavirus, was identified and sequenced and what this meant for developing the first vaccines, compassionate drug use and the abuse of antibiotics were common global scenarios [2,3].

The prescription and self-medication of antimicrobials, many of them for exclusive and restricted hospital use, seeking to reduce the mortality and morbidity associated with this disease, accelerated the selective appearance of multi-resistant bacteria [4].

These bacteria generated significant phenotypic and antibiotype changes in global hospital epidemiology, which propelled a revolution in the massive use of empirical and targeted (directed) antibiotic therapy, using multiple schemes and with unusual antibiotics such as polymyxins and carbapenems on a large scale [5]. In addition, given the high rates of multi-resistance, new broad-spectrum antibiotics were required. However, they were not available in the lists of essential drugs in the public system and were particularly expensive. This kind of antibiotic therapy made the availability of the drugs the exception and not the rule in low-income centers.

With the advent and mass use of vaccines and the use of drugs such as steroids, specific antivirals, and monoclonal antibodies that have demonstrated their efficacy in extensive clinical studies, COVID-19 is on the brink of becoming endemic, and its morbidity and mortality are minor, despite the appearance of more effective variants in its transmission [6].

Falcone *et al*. [7] have reported the results from a nationwide multicenter study in Italy, performed with 1276 patients with bacteremia due to Gram-negative bacilli, in which they observed a marked expansion of resistance to carbapenems and other molecules, new resistance mechanisms, and associated high mortality.

This work aims to present the epidemiological, clinical, and microbiological profiles of a series of patients with bacterial superinfections hospitalized in a center that exclusively treated COVID-19 in Quito, Ecuador.

MATERIALS AND METHODS

Location

The study was performed in a second-level hospital, a regional reference center for COVID-19, located in Quito, Ecuador's capital (2850 meters above sea level), in the province of Pichincha (with a total population of 3,340,039).

Study design

We conducted an analytical retrospective observational study. The universe to be considered consisted of all adult patients hospitalized from January 1, 2021, to December 31, 2021, with a diagnosis of COVID-19. Anonymized data were collected from the consultation database of the Infectologist in charge of infection control and from the electronic clinical records of adult patients admitted to the different areas of the hospital with a diagnosis of COVID-19.

Population and sample size

The universe to be considered consisted of all adult patients hospitalized from January 2021 to December 2021 with a diagnosis of COVID-19. All adult patients diagnosed with healthcare-associated infections (superinfections) were included in the study. A total of 240 patients met the inclusion criteria.

Inclusion criteria

Patients admitted to the health center with a diagnosis of COVID-19 confirmed by rt-PCR and who presented during their hospital stay any healthcare-associated infection (superinfection) determined by the clinical judgment of the treating physician and the infectious disease specialist, whose evaluation was requested. This request was based on findings from the physical examination, laboratory tests, and imaging studies and, with microbiological confirmation, by isolating pathogenic microorganisms in samples taken appropriately.

Exclusion criteria

Patients admitted to our hospital without a confirmed diagnosis of COVID-19 were not included in the study, nor were those who did not present superadded infections during their hospital stay, patients with known coinfections on admission, or transferred with infections acquired in other hospitals. Neither were included those patients with negative microbiological results, whose

cultures were not taken, or whose reports suggested sample contamination.

Data Collection

The clinical and epidemiological variables obtained and analyzed from electronic medical records were age, gender, comorbidity, diagnosis of associated bacterial infections, antibiotic treatment schemes received (empirical and culture-directed), hospital stay, and outcome. Severity indicator biomarkers were also collected: complete blood count and glucose, creatinine, D-dimer, ferritin, lactate dehydrogenase, and C-reactive protein levels.

The information on the cultures taken, the microbiological isolation, and susceptibility profiles were obtained directly from the reports of the Microbiology area of the Hospital's Clinical Laboratory.

Statistical analysis

The analyses were performed with the IBM SPSS version 23 statistical package. Descriptive statistics was used, employing tables and graphs, representing the qualitative variables in absolute and relative values, whereas measures of central tendencies and variabilities were used for the quantitative variables.

In inferential statistics, bivariate analyses were performed to determine the variables to be considered in the multivariate analysis. With this purpose, the Chi-square test or Fisher's exact statistic were applied for the qualitative variables, while for the quantitative ones, the Mann-Whitney test was used when data did not comply with normality. Multivariate logistic regression analysis was used to relate the variables with mortality. Statistical significance was established as $p<0.05$.

RESULTS

Two hundred forty patients with a median age of 55 and a predominance of men (68.75%) were analyzed. The median hospital stay was 24 days. When comparing age by the discharge condition, significant differences were observed with $p= 0.002$, with medians of 52.5 years in survivors vs. 58.5 years in non-survivors. The hospital stay presented significant differences by discharge condition with $p<0.001$, with the median stay being 29 days in survivors vs. 16 days in non-survivors (Table 1).

Table 2 reports the analytical and cytometric values, which indicate that although the elevation of biomarkers characteristic of severe forms of the disease is striking, such as the white count, C-reactive protein, D-dimer, and ferritin, there were no significant differences when compared to the condition at discharge.

Regarding associated infections, ventilator-associated pneumonia (VAP) was observed more frequently in 55% of cases,

Table 1
Relationship between clinical characteristics and discharge condition.

Clinical Characteristics	Total	Discharge Condition		p
		Survivor	Non-survivor	
Age (median (IQR))*	55 (45-64)	52.5 (42.75-62)	58.5 (48.75-67.25)	0.002
Sex (n (%))**				
Female	75 (31.25)	45 (30.82)	30 (31.91)	0.858
Male	165 (68.75)	101 (69.18)	64 (68.09)	
Days of stay (median (IQR))*	24 (16-35)	29 (21-41.5)	16 (12-22.5)	<0.001

* Mann-Whitney test, ** Chi-square test.

Table 2
Relationship between analytical parameters and cytometry by discharge condition.

Cytometry and Analytical Parameters	Total	Discharge Condition		p
		Survivor	Non-Survivor	
	Median (IQR)	Median (IQR)	Median (IQR)	
Leukocytes (cells x mm^3)	11510 (8210-14770)	11940 (8120-14910)	10710 (8400-14590)	0.6531
Neutrophils (cells x mm^3)	9640 (6840-13320)	9920 (6620-13190)	9100 (7200-13340)	0.8527
Lymphocytes (cells x mm^3)	750 (520-1130)	760 (540-1160)	710 (470-1030)	0.2554
NLR (Neutr-Lymph ratio)	12.85 (7.46-21.09)	13.11 (7.13-20.52)	12.36 (7.48-22.4)	0.6191
Neutrophils (%)	88 (82-91)	88 (81-91)	88 (83-92)	0.4382
Lymphocytes (%)	7 (4-11)	7 (4-11)	7 (4-11)	0.6428
Hemoglobin (g/dL)	15.5 (14.2-17)	15.55 (14.1-17)	15.45 (14.3-17.2)	0.8504
Hematocrit (%)	46 (42-50)	47 (42-51)	0.46 (42-50)	0.5625
Platelets (cells x mm^3)	253500 (204250-331750)	271000 (206750-342500)	238500 (198500-312750)	0.0732
MPV (fL)	8.8 (8.3-9.6)	8.7 (8.3-9.55)	8.9 (8.3-9.63)	0.4118
Glucose (mg/dL)	134 (107-168)	133 (106-171.2)	137 (109-164.25)	0.6469
Creatinine (mg/dL)	0.87 (0.68-1.09)	0.86 (0.66-1.08)	0.89 (0.75-1.09)	0.3726
D Dímer (ng/mL)	1027.25 (653.28-1741.28)	1100.6 (653.4-1860.1)	972.5 (640.3-1672.1)	0.4093
Ferritin (ng/mL)	1087.2 (604.6-1639.7)	1026.7 (595.98-1649.75)	1111 (649.25-1543.55)	0.6414
LDH (UI/L)	855 (627-1045.75)	838 (627-1078)	864 (629-982)	0.5934
PCR (mg/dL)	15.39 (9.19-23.25)	16.42 (8.3-24.37)	14.67 (10.23-21.22)	0.7600

Mann-Whitney test.

followed by catheter-associated urinary infections (CAUTI) at 43.75%, central line-associated bloodstream infection (CLBSI) 31. 25%, and colonization at 15.42%, among others. The CLBSI presented significant differences by discharge condition with a p of 0.030; the proportions were 26.03% in survivors vs. 39.36% in non-survivors (Table 3).

Among the most frequent bacteria causing superadded infections in our study, KPC-type carbapenem-producing Klebsiella pneumoniae complex was observed in 24.17% of the cases, followed by Klebsiella pneumoniae producing extended-spectrum β-lactamase (ESBL) in 17.92%, and carbapenem-resistant Pseudomonas aeruginosa in 13.75%, among others. Serratia marcescens presented significant differences by discharge condition with a p of 0.013, the proportion of this bacterium being 6.16% in survivors vs. 0% in non-survivors (Table 4).

Among the most used antibiotic schemes, meropenem + vancomycin + amikacin was observed in 56.09% of the empirical scheme and 38.55% of directed schemes. In the directed scheme, significant differences were observed by discharge condition with a p = 0.036, specifically for colistin + others, whose proportions were 16.95% in survivors vs. 41.67% in non-survivors (Table 5).

Multivariate logistic regression analysis was performed to determine the relationship between age, CLBSI, and antibiotic scheme with mortality, observing the following: age was related to mortality with p = 0.006, whereas due to the one-year increase in patients, the risk of mortality of patients was increased by 3%.

Table 3
Relationship between associated infections and discharge condition.

Associated Infections	Total	Discharge Condition		p
		Survivor	Non-Survivor	
	n (%)	n (%)	n (%)	
VAP	132 (55)	80 (54,79)	52 (55,32)	0,936
CAUTI	105 (43,75)	68 (46,58)	37 (39,36)	0,272
CLBSI	75 (31,25)	38 (26,03)	37 (39,36)	0,030*
Colonization	37 (15,42)	27 (18,49)	10 (10,64)	0,100
SSTI	5 (2,08)	2 (1,37)	3 (3,19)	0,383
SSI	5 (2,08)	4 (2,74)	1 (1,06)	0,651
ENT	1 (0,42)	1 (0,68)	0 (0)	1,000

* significant difference, Chi-square test or Fisher's exact test. VAP: ventilator-associated pneumonia; CAUTI: catheter-associated urinary infections; CLBSI: central line-associated bloodstream infection; SSTI: skin and soft tissue infections; SSI: surgical site infection; ENT: ear, nose, and throat infections (otitis, retro tonsillar abscesses, etc.).

Table 4
Relationship between the type of bacteria and discharge condition.

Bacteria	Total	Discharge Condition		p
		Survivor	Non-Survivor	
	n (%)	n (%)	n (%)	
E. coli	32 (13.33)	21 (14.38)	11 (11.7)	0.698
E. coli ESBLs	24 (10)	17 (11.64)	7 (7.45)	0.290
Enterobacter cloacae complex	5 (2.08)	3 (2.05)	2 (2.13)	1.000
Enterococcus faecalis	9 (3.75)	5 (3.42)	4 (4.26)	0.740
K. oxytoca	5 (2.08)	3 (2.05)	2 (2.13)	1.000
K. oxytoca ESBLs	5 (2.08)	4 (2.74)	1 (1.06)	0.651
K. pneumoniae complex	14 (5.83)	10 (6.85)	4 (4.26)	0.403
K. pneumoniae complex ESBLs	43 (17.92)	22 (15.07)	21 (22.34)	0.152
K. pneumoniae complex KPC	58 (24.17)	40 (27.4)	18 (19.15)	0.145
Morganella morganii	6 (2.5)	3 (2.05)	3 (3.19)	0.681
Proteus mirabilis	5 (2.1)	2 (1.4)	3 (3.2)	0.383
Proteus mirabilis ESBLs	7 (2.92)	3 (2.05)	4 (4.26)	0.437
P. aeruginosa	19 (7.92)	11 (7.53)	8 (8.51)	0.784
P. aeruginosa CR	33 (13.75)	18 (12.33)	15 (15.96)	0.426
Serratia marcescens	9 (3.75)	9 (6.16)	0 (0)	0.013*
S. aureus complex	19 (7.92)	12 (8.22)	7 (7.45)	1.000
S. aureus complex MR	11 (4.58)	5 (3.42)	6 (6.38)	0.348
S. epidermidis	17 (7.08)	8 (5.48)	9 (9.57)	0.303
S. hominis MR	6 (2.5)	4 (2.74)	2 (2.13)	1.000
Streptococcus pneumoniae	5 (2.16)	4 (2.86)	1 (1.1)	0.651

*significant difference, Chi-square test or Fisher's exact test. ESBLs: extended-spectrum beta-lactamase; KPC: KPC-type carbapenemase; MR: methicillin-resistant; CR: carbapenemase-resistant.

Table 5
Relationship between antibiotic scheme and discharge condition.

Antibiotic Scheme	Total	Discharge Condition		p
		Survivor	Non-survivor	
	n (%)	n (%)	n (%)	
Empirical				
Aminopenicillin + others	41 (17,83)	29 (20,57)	12 (13,48)	0,151
Colistin + others	28 (12,17)	21 (14,89)	7 (7,87)	
Meropenem + vancomycin + Amikacin	129 (56,09)	73 (51,77)	56 (62,92)	
Piperacillin/tazobactam + others	32 (13,91)	18 (12,77)	14 (15,73)	
Directed				
Aminopenicillin + others	13 (15,66)	12 (20,34)	1 (4,17)	0,036*
Colistin + others	20 (24,1)	10 (16,95)a	10 (41,67)b	
Meropenem + vancomycin + Amikacin	32 (38,55)	22 (37,29)	10 (41,67)	
Piperacillin/tazobactam + others	18 (21,69)	15 (25,42)	3 (12,5)	

*significant difference; different superscripts indicate antibiotics that differ by discharge condition, Chi-square test.

CLBSI infections were related to mortality with a p of 0.028, whereas patients with CLBSI infections were 1.94 times more likely not to survive (Table 6).

Among the directed (targeted) antibiotic schemes, it was observed that Colistin + others were related to mortality with a p = 0.028, where patients treated with these antibiotics presented a 12 times greater probability of not surviving compared to those who received aminopenicillins + others (Table 7).

The enzymatic resistance of these bacteria was studied. 40% of the *K. pneumoniae complex* and *E. coli* isolates were ESBL producers. In addition, one out of every five *K. pneumoniae complex* isolates was a KPC producer. The resistance observed to carbapenems by P. aeruginosa is very high: resistance to carbapenems was found in seven out of ten isolates. *S. aureus complex* and *S. epidermidis* showed 30 and 80% resistance to methicillin, respectively (Table 8).

DISCUSSION

This study, conducted on 240 hospitalized patients with COVID-19 who presented with bacterial superinfection, has shown that older male patients with bacteremia who received treatment with antibiotic regimens containing colistin were associated with lower survival.

Of these factors, age, bloodstream infection, and colistin use were independent factors for mortality when adjusted in a multivariate model. Furthermore, it was also observed that VAP was the most frequent site of superinfection; and that enzymatic resistance due to ESBL and KPC-type serine betalactamases was frequent in enterobacteria. The most used empirical scheme was composed of carbapenems, aminoglycosides, and glycopeptides (meropenem, amikacin, and vancomycin), and the presence of carbapenem-resistant P. aeruginosa was relevant.

Patients with severe COVID-19 admitted to a hospital are at greater risk of developing infections during their hospital stay [8–11,] and this risk increases as their hospital stay is prolonged [12]. This increased risk of superinfection is due to greater exposure of patients to immunosuppressive treatments (especially steroids) and also for reasons specific to health centers, of a structural

Table 6
Multivariate analysis for mortality based on age, CLBSI, and empirical antibiotic scheme.

Variables	B	p	OR	OR (95% CI)	
				Lower	Upper
AGE	0.03	0.006*	1.03**	1.01	1.06
CLBSI	0.66	0.028*	1.94**	1.07	3.49
Aminopenicillin + others (reference)					
Colistin + others	-0.15	0.797	0.86	0.28	2.68
Meropenem + Vancomycin + Amikacin	0.59	0.136	1.81	0.83	3.94
Piperacillin/tazobactam + others	0.55	0.278	1.74	0.64	4.71

Note: *significant variables, **factor associated with mortality, based on logistic regression: OR (95% CI): Odds ratio (95% Confidence interval). CLBSI: central venous catheter-related bacteremia.

Table 7
Multivariate analysis for mortality based on a culture-directed antibiotic regimen.

Directed Scheme	B	p	OR	OR (95% CI)	
				Li	LS
Aminopenicillin + others (reference)				1.3	
Colistin + others	2.48	0.028*	12**	0	110.52
Meropenem + Vancomycin + Amikacin	1.70	0.126	5.45	0.62	47.90
Piperacillin/tazobactam + others	0.88	0.472	2.40	0.22	26.12

Note: *significant variable, ** mortality associated factor, based on logistic regression. OR (95% CI): Odds ratio (95% Confidence interval).

type (opening of new ICU beds in other areas of the hospital), organizational (hiring or transferring personnel without prior training in the management of critical patients) and functional (changes in patient care standards, and prolonged use of personal protective equipment). In addition, these patients require vascular access, urinary devices, and invasive mechanical ventilation due to their condition, increasing the risk of infections. These hospital infections are more complex than usual because they are associated with bacteria with resistance to antibiotics[13].

The documented evidence demonstrates that male patients with COVID-19 are at greater risk of presenting more severe forms of COVID-19, with prolonged hospitalizations and more extended use of invasive devices, which increases the risk of superinfection,[5] according to the findings of our study. The mean age in the sample was 55 years, with age ranges between 50 and 60 years, similar to the data reported by other authors [5,10,14]. In addition, this data was associated with higher mortality: the increase in one year of age increases mortality risk by 3%, agreeing with what has been reported in the literature [5,7,10].

The average hospital stay was 24 days, less in the non-survivor group, with a statistically significant difference. As a possible hypothesis, it is proposed that they were patients with more severe diseases that coursed with more torpid and abrupt evolutions, con-

Table 8
Isolated bacteria and enzymatic resistance.

Bacterial species	Enzyme production	%
Klebsiella pneumoniae complex	ESBL/KPC	39.10%/ 20.96%
Pseudomonas aeruginosa	CR	70.42%
Escherichia coli	ESBL	40.57%
Staphylococcus aureus complex	MRSA	31.11%
Staphylococcus epidermidis	MR	81.25%
Proteus mirabilis	ESBL	68.75%
Enterobacter aerogenes	AmpC	10.00%
Klebsiella oxytoca	ESBL/ KPC	12.50%/ 12.50%
Enterobacter cloacae complex	ESBL	16.60%

ESBL: extended-spectrum beta-lactamase, KPC: KPC-type carbapenemase, MR: methicillin-resistant, CR: carbapenemase-resistant.

sistent with what has been reported by some authors [10,12,15,16].

The laboratory data (Table 2) show that the two groups (survivors and deceased) generally presented similar patterns of acute inflammatory reaction, state of hyperinflammation, and hypercoagulability, with no statistically significant differences. It is suggested that both groups were patients admitted with severe disease with systemic involvement. From the point of view of the severity of the condition caused by COVID-19, they were similar groups.

VAP was the most common infection reported in this investigation, representing 55% of infections. In other studies, it has been reported that VAP represented between 40% and 60% of documented superinfections in patients with COVID-19. The highest predisposition to these processes has been associated with prolonged ventilation, prone position, use of corticosteroids, lung damage, and episodes of cross-contamination, most likely due to the prolonged use of personal protective equipment. In addition, the same COVID-19 infection can cause inflammation of the pulmonary vascular endothelium and subsequent thrombosis, creating an environment conducive to bacterial growth [17–19].

The incidence of CLBSI tripled during the pandemic [20]. In this study, patients with this type of infection were 1.94 times more likely not to survive. Other authors have reported higher mortality from CLBSI in patients with COVID-19 (between 40 and 54%) than from this type of infection in pre-pandemic or non-COVID-19 patients (between 24 and 33%) [20–22]. This higher mortality may be due to the use of these devices for a longer time, the prone position that limited the care of the line, and the breach of asepsis and antisepsis when manipulating the catheter in the harsh conditions faced during the health emergency, generating bacteremia more frequently with a more significant inoculum.

K. pneumoniae complex was the most frequently recovered bacterium in cultures, followed by *P. aeruginosa* and *E. coli*. Generally, the reports in the literature vary widely because of local microbiology. However, most of the studies at a global and regional level, carried out during the pandemic, agree with this research, showing that these bacteria are the most frequent [5,8,15-17,23].

In our study series, a high percentage of Enterobacterales were ESBL producers, and one in five *K. pneumoniae complex* isolates were KPC-type carbapenemase producers, consistent with other reports in Latin America during the pandemic [24]. Most recovered *Pseudomonas* cultures reported resistance to carbapenems (70%). These findings are worrying due to the impact on public health and the limitation of the therapeutic arsenal against such microorganisms, especially in countries such as Ecuador [25].

In non-randomized clinical studies, using colistin as monotherapy or combination therapy has mixed results; however, it presents higher mortality in patients who previ-

ously received carbapenems [26–28]. In the IDSA guidelines, the use of colistin as targeted therapy is discouraged, giving preference to monotherapy with new antibiotics such as ceftazidime-avibactam [29]. Patients who received colistin (combined with other antibiotics) were associated with higher mortality in our series. We attributed this finding to prior treatment with carbapenems and subsequent resistance to them, the side effects of colistin and associated antibiotics, and the fact that they presented a more severe disease. Moreover, this higher mortality could occur because colistin was indicated as a desperate measure since there were no better therapeutic options available in the hospital or because its susceptibility to the microorganism could not be confirmed since the sensitivity cut-off points had not been established with an antibiogram for the said antibiotic, against certain bacteria and/or in some tissues.

On the other hand, it should be considered that the hospital in this study is located almost 3000 meters above sea level. Although the effect of high altitude (≥1500m) and its potential association with mortality from COVID-19 is still controversial, there are studies in this direction[30,31]; therefore, more research is required in this regard and its impact on the high mortality of severely ill patients with bacterial superinfections.

This work has significant limitations. It is monocentric and retrospective, with the biases inherent in this design; furthermore, there was no control group. There could also be a possible underdiagnosis of fungal infections, which were not reported in this series and constitute a clinical challenge, especially in the context of patients infected with SARS-CoV-2. Finally, the health center needed molecular biology studies to determine resistance genes. Nevertheless, in the case of the *K. pneumoniae complex* isolates, in those cases where the automated system reported strains probably producing KPC, lateral flow tests were carried out, and the strains were sent to the national reference laboratory in Ecuador: Instituto Nacional de Investigación en Salud Pública "Dr. Leopoldo Izquieta Pérez" (INSPI). Molecular biology studies were performed at the institution for confirmation, and the hospital was notified.

In the present retrospective study, we found that in patients with COVID-19 and bacterial superinfection, age, central venous catheter-associated bacteremia, and colistin use were independent predictors of mortality. The most frequently isolated microorganisms were ESBL- and KPC-producing Enterobacterales and non-fermenting Gram-negative bacilli resistant to carbapenems.

These findings are consistent with what has been reported in world and regional series and emphasize the urgent need to establish programs for the rational use of antibiotics, especially in countries with a limited therapeutic arsenal.

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Conflict of interest

There is no conflict of interest.

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Effect of anakinra, tocilizumab, and the combination thereof on bladder ischemia-reperfusion damage in albino Wistar-type rats.

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Keywords: anakinra; anakinra and tocilizumab combination; bladder; ischemia-reperfusion damage; rats; tocilizumab.

Abstract. Several studies have reported that oxidative stress, and proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-one beta (IL-1β), and interleukin-six (IL-6) are the main factors underlying bladder ischemia-reperfusion (I/R) damage. Anakinra and tocilizumab are known to be antioxidants and proinflammatory cytokine inhibitors. Our study aims to investigate if anakinra, tocilizumab, and the combination (ATC) thereof have a protective effect against oxidative and inflammatory bladder damage induced through the I/R procedure in rats, and evaluate by comparing these compounds. Male rats were divided into five groups: bladder sham-operation applied group (SG); bladder only I/R applied group (IRG); anakinra+bladder I/R applied group (AIR); tocilizumab+bladder I/R applied group (TIR); and ATC+bladder I/R applied group (ATIR). An atraumatic clamp was placed on the abdominal aorta of animals in all groups (except SG), and one hour of ischemia followed by two hours of reperfusion was performed. Our biochemical findings showed that anakinra and tocilizumab significantly inhibited the increase of oxidant malondialdehyde (MDA) and the decrease of antioxidants such as total glutathione (tGSH), superoxide dismutase (SOD), and catalase (CAT) in bladder tissue by I/R, both at the same levels. Furthermore, anakinra and tocilizumab significantly suppressed the I/R-associated increase of TNF-α, IL-1β, and IL-6 in bladder tissue. ATC was the one that best prevented the I/R-related increase in MDA, TNF-α, IL-1β, and IL-6 and the decrease in tGSH, SOD, and CAT in the bladder tissue. ATC was more beneficial than anakinra or tocilizumab alone in treating bladder I/R damage.

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Efecto de la anakinra, el tocilizumab y la combinación de ambos sobre el daño por isquemia-reperfusión vesical en ratas albinas tipo Wistar.

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Palabras clave: anakinra; combinación de anakinra y tocilizumab; vejiga; daño por isquemia-reperfusión; ratas; tocilizumab.

Resumen. Varios estudios han demostrado que el estrés oxidativo, y las citocinas proinflamatorias tales como el factor de necrosis tumoral alfa (TNF-α), la interleucina uno beta (IL-1β) y la interleucina seis (IL-6) son los principales factores subyacentes al daño por isquemia-reperfusión (I/R) vesical. Se sabe que la anakinra y el tocilizumab son antioxidante e inhibidores de las citocina proinflamatorias. Nuestro estudio pretende investigar si la anakinra, el tocilizumab y la combinación (ATC) de ambos tienen un efecto protector contra el daño oxidativo e inflamatorio de la vejiga inducido mediante el procedimiento de I/R en ratas, y evaluarlo mediante la comparación de estos compuestos. Se dividieron a ratas macho en cinco grupos: un grupo sometido a la operación simulada (SG); un grupo al cual solo se aplicó I/R a la vejiga (IRG); un grupo al cual se aplica el procedimiento I/R a la vejiga + anakinra (AIR); un grupo en el que se aplica el procedimiento I/R a la vejiga + tocilizumab (TIR); y un grupo en el que se aplica el procedimiento I/R a la vejiga + ATC (ATIR). Se colocó una pinza atraumática en la aorta abdominal de los animales de todos los grupos (excepto SG), y se realizó una hora de isquemia seguida de dos horas de reperfusión. Nuestros hallazgos bioquímicos mostraron que la anakinra y el tocilizumab inhibieron significativamente el aumento del malondialdehído oxidante (MDA) y la disminución de antioxidantes como el glutatión total (tGSH), la superóxido dismutasa (SOD) y la catalasa (CAT) en el tejido de la vejiga por I/R, ambos a los mismos niveles. Además, la anakinra y el tocilizumab suprimieron significativamente el aumento de TNF-α, IL-1β e IL-6 asociado a la I/R en el tejido vesical. La combinación ATC fue la que mejor previno el aumento de MDA, TNF-α, IL-1β e IL-6 relacionado con la I/R y la disminución de tGSH, SOD y CAT en el tejido vesical. La ATC resultó más beneficiosa que la anakinra o el tocilizumab solos en el tratamiento del daño por I/R de la vejiga.

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INTRODUCTION

As known, the bladder's function is to store urine and empty it at an appropriate time. An adequate amount of blood flow, oxygen, and nutrient support is needed to maintain this function at a normal level [1]. In disorders such as urinary retention, atherosclerosis, vasospasm, embolization, and thrombosis, the bladder cannot be supplied adequately with blood, and ischemia develops [2]. Clinical and experimental studies have shown that reperfusion contributes to the impairment of bladder function in case of re-blooding of the ischemic bladder [3,4]. It has been reported in the literature that

the factors underlying bladder I/R damage are reactive oxygen species (ROSs) [5]. It has been reported that I/R procedure-induced increase in ROSs production in the bladder leads to accelerated lipid peroxidation (LPO) [6]. In addition, there are also studies linking bladder I/R damage with an increase in proinflammatory cytokines [5]. In particular, proinflammatory cytokines such as interleukin one beta (IL-1β) and interleukin six (IL-6) are thought to be the main factors in the pathogenesis of bladder I/R damage [7,8]. This information obtained from the literature suggests that IL-1β and IL-6 cytokine antagonists with antioxidant effects may be helpful in the treatment of bladder I/R.

Anakinra, which we will investigate its effect against bladder I/R damage in our study, is a recombinant antagonist of the IL-1β receptor [9]. Anakinra is known as an anti-inflammatory agent [10]. Since it blocks both IL-1α and IL-1β receptors, it is used in treating various inflammatory diseases [11]. Anakinra has been shown to protect testicular tissue from I/R damage by inhibiting the increased production of IL-1β and malondialdehyde (MDA), a toxic product of LPO [12]. In addition, it has been reported that anakinra protects ovarian tissue from oxidative and inflammatory damage of I/R [13].

Tocilizumab, which we plan to investigate its effect against bladder I/R damage, is a monoclonal antibody drug that is an IL-6 receptor antagonist [14]. Tocilizumab has been approved for the pediatric treatment of rheumatoid arthritis and polyarticular and systemic juvenile idiopathic arthritis [15]. Erdem KTO et al. reported that tocilizumab protects kidney tissue from inflammatory and oxidative damage of I/R by inhibiting the increase of IL-6 and other cytokines [16]. All this information obtained from the literature shows that anakinra and tocilizumab may be effective in treating bladder I/R damage. In particular, it suggests that the combination of anakinra and tocilizumab (ATC) may be more effective in the treatment of bladder I/R damage. There was no information in the literature investigating the effects of anakinra, tocilizumab, and ATC against bladder I/R damage. Therefore, our study aims to investigate whether anakinra, tocilizumab, and ATC have a protective effect against I/R-induced oxidative and inflammatory bladder damage in rats, and evaluate by comparing both compounds.

MATERIALS AND METHODS

Animals

A total of 30 albino Wistar-type male rats weighing between 270-290 g were utilized in the experiment. Erzincan Binali Yildirim University Experimental Animals Application and Research Center provided all animals. The animals were fed with animal food in groups at average room temperature (22°C) and hosted in 12 hours of light, and 12 hours of darkness environment, under appropriate conditions before the experiment. The experiments were conducted following the Turkey Regulation of Animal Research Ethics. In addition, this study was carried out under the principles of the Declaration of Helsinki. The protocols and procedures were approved by the local Animal Experimentation Ethics Committee of Erzincan Binali Yildirim University (Meeting Date: 26.01.2023; Meeting No: 2023/01; Decision No: 01).

Chemicals

A Pfizer Turkey representative provided ketamine used in this experiment while anakinra was obtained from Sobi-Sweden, and a Roche Mustahzarları Turkey representative provided tocilizumab (80 mg/4 mL concentrated solution for infusion).

Experimental Groups

Animals were divided into five groups: sham-operation applied group (SG); bladder only I/R applied group (IRG); anakinra+bladder I/R applied group (AIR); tocilizumab+bladder I/R applied group (TIR); and ATC+bladder I/R applied group (ATIR).

Anesthesia procedure

Surgical procedures were carried out under sterile conditions by intraperitoneal (IP) ketamine administration at a dose of 60 mg/kg. After the ketamine injection, the rats were kept waiting for the appearance of the appropriate anesthesia period during which the surgical procedure would be performed. When the animals remained immobile in the supine position was considered a suitable anesthesia period for surgical intervention [17].

Experimental procedure

One hour before anesthesia, anakinra was injected IP at a dose of 50 mg/kg in the AIR group of animals (n=6), and tocilizumab at a dose of 8 mg/kg in the TIR group (n=6). The ATIR (n=6) group of animals was administered 50 mg/kg anakinra + 8 mg/kg tocilizumab IP. The SG (n=6), and IRG (n=6) groups of animals were given distilled water as a solvent by the same route. One hour following the administration of drugs and distilled water, laparotomy was carried out with a 2.5 cm long midline incision applied to the abdomen of the rats under sterile conditions. Thereupon, an atraumatic clamp was placed on the abdominal aorta of the animals in all groups (except the SG group), and ischemia was induced for one hour, followed by two hours of reperfusion. At the end of this period, all animal groups were sacrificed with high doses (120 mg/kg) of ketamine anesthesia. Bladder tissues were removed from the sacrificed animals and were analyzed biochemically. The biochemical experimental results obtained from all groups were evaluated by comparing the groups.

Biochemical analysis

Preparation of samples

Tissue samples were washed with physiological saline and placed in Petri dishes. The tissues were pulverized by grinding in the presence of liquid nitrogen. In addition, tissue samples were homogenized. The supernatants were used for MDA, tGSH, SOD, CAT, and protein analyses.

Determination of MDA, tGSH, SOD, CAT, and protein

Determination of MDA, tGSH, and SOD in bladder tissues was carried out by enzyme-linked immunosorbent assay (ELISA) kits which are produced for experimental animals, and each analysis was performed according to the kit instructions (MDA: Catalog no. 10009055; tGSH: Catalog no. 703002; SOD: Catalog no. 706002; Cayman Chemical Company). CAT determination was performed in line with the method proposed by Goth [18]. Protein determination was determined spectrophotometrically at 595 nm following the Bradford method [19].

TNF-α, IL-1β, and IL-6 analysis

We measured the weight of the samples. After that, all the tissues were cut, rapidly frozen with liquid nitrogen, and homogenized by pestle and mortar; we maintained samples at 2-8°C after melting. We added PBS (pH 7.4), 1/10 (w/v), and then vortex for 10 seconds, centrifuged 20 min at 10000 x g, and collected the supernatants carefully. Tumor necrosis factor alpha (TNF-α; ng/L) was assayed using a TNF-α ELISA rat kit (Cat no: YHB1098Ra, Shanghai LZ, Shanghai, China), interleukin one beta (IL-1β; pg/L) was assayed using an IL-1β ELISA rat kit (Cat no: YHB0616Ra, Shanghai LZ, Shanghai, China), and interleukin-6 (IL-6; ng/L) was assayed using a commercial kit supplied by Eastbiopharm Co Ltd (Hangzhou, China).

Statistical analysis

The experiment results were expressed as means ± standard errors of the mean (Mean±SEM). The statistical analyses were performed with the SPSS Statistics Program for Windows (IBM Corp., released 2013, Version 22.0, Armonk, NY, USA). The normality of the distribution for continuous variables in the biochemical test results was checked by the Shapiro–Wilk test. The significance of differences between groups was determined using one-way ANOVA, as the distribution was normal. Levene's test was performed to

determine whether the homogeneity of variances was achieved. Afterward, Tukey HSD (honestly significant difference) or Games-Howell was applied as a posthoc test depending on the assumption whether the variances were homogeneous or not. The probability value of $p<0.05$ was regarded to indicate statistical significance.

RESULTS

MDA analysis results of bladder tissue

As shown in Fig. 1A, MDA levels in the bladder tissue of the I/R procedure-applied group were higher than in the bladder tissue of the sham operation-applied group. The difference in the levels of MDA in the bladder tissue of the sham-operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.007$), tocilizumab ($p=0.003$), and ATC ($p<0.001$) significantly suppressed the increase in MDA levels induced by the I/R procedure in bladder tissue. Anakinra ($p=0.002$) and tocilizumab ($p=0.001$) were found to approximate the MDA levels in the bladder tissue to the control group values. The closest MDA value to the control group that underwent sham operation was found in the ATIR group ($p=0.014$).

tGSH analysis results of bladder tissue

The tGSH levels in the bladder tissue of the I/R procedure-applied group were found to be lower than that in the bladder tissue of the sham operation-applied group (Fig. 1B). The difference in the levels of tGSH in the bladder tissue of the sham-operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.004$), tocilizumab ($p=0.003$), and ATC ($p<0.001$) significantly suppressed the decrease in tGSH levels induced by the I/R procedure in bladder tissue. A statistically significant difference was found in the tGSH levels in the bladder tissue of the anakinra ($p<0.001$) and tocilizumab ($p<0.001$)-treated groups compared to the values of the control group that underwent sham operation. The closest tGSH value to the control group that underwent sham operation was found in the ATIR group ($p=0.015$).

SOD analysis results of bladder tissue

As shown in Fig. 1C, SOD activity in the bladder tissue of the I/R procedure-applied group was lower than that in the bladder tissue of the sham operation-applied group. The difference in activity of the SOD in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.012$), tocilizumab ($p=0.011$), and ATC ($p<0.001$) significantly suppressed the decrease in SOD activity caused by the I/R procedure in bladder tissue. A statistically significant difference was found in the SOD activities in the bladder tissue of the anakinra ($p<0.001$) and tocilizumab ($p<0.001$) applied groups compared to the control group values that underwent sham operation. The closest SOD activity to the control group that underwent sham operation was found in the ATIR group ($p=0.043$).

CAT analysis results of bladder tissue

The CAT activity in the I/R procedure applied group's bladder tissue was lower than that in the bladder tissue of the sham operation applied group (Fig. 1D). The difference in activity of the CAT in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.009$), tocilizumab ($p=0.002$), and ATC ($p<0.001$) significantly suppressed the decrease in CAT activity in bladder tissue caused by the I/R procedure. A statistically significant difference was found in the CAT activities in the bladder tissue of the anakinra ($p<0.001$) and tocilizumab ($p<0.001$) applied groups compared to the control group values that underwent sham operation. The closest CAT activity to the control group that underwent sham operation was found in the ATIR group ($p=0.015$).

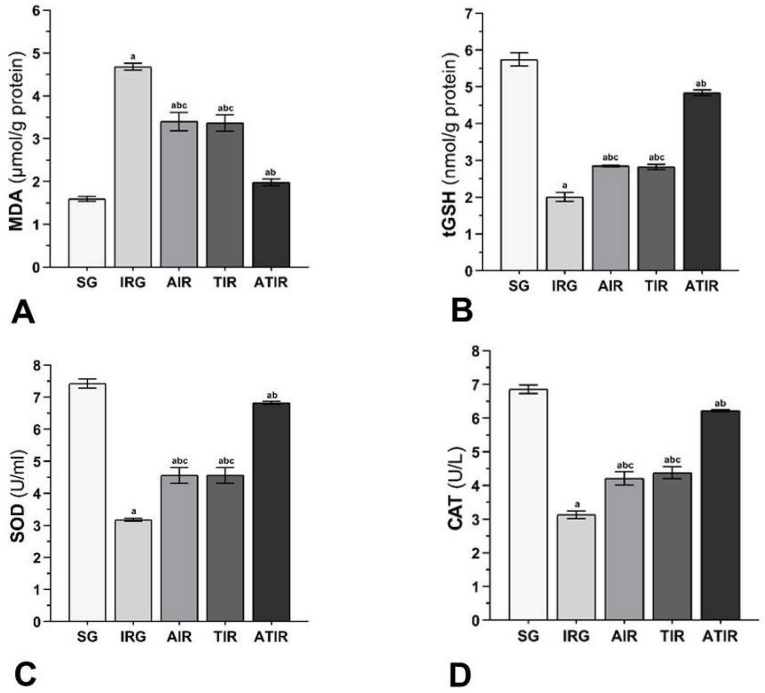
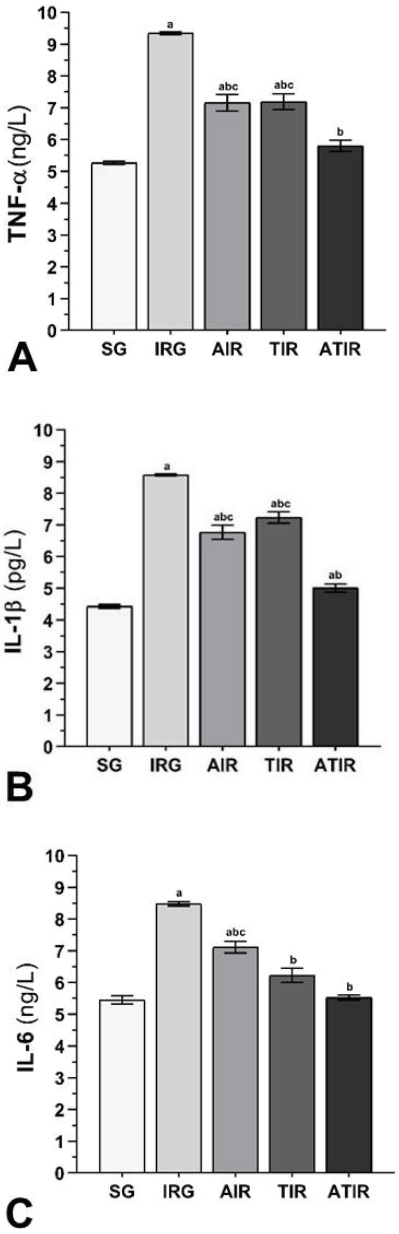


Fig. 1. Oxidative stress in bladder tissues of experimental groups. **A)** Malondialdehyde (MDA) levels: Bars are means ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. **B)** Total glutathione (tGSH) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.001$ when drug treatment groups were alone compared with the ATIR combined treatment group. **C)** Superoxide dismutase (SOD) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. n=6 per group. **D)** Catalase (CAT) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. Group. n=6 per group. SG: sham-operation group; IRG: ischemia-reperfusion group; AIR: anakinra+ischemia-reperfusion group; TIR: tocilizumab+ischemia-reperfusion group; ATIR: anakinra+tocilizumab+ischemia-reperfusion group.

TNF-α analysis results of bladder tissue

As shown in Fig. 2A, TNF-α levels in the bladder tissue of the I/R procedure applied group were higher than in the bladder tissue of the sham operation applied group. The difference in the levels of TNF-α in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.002$), tocilizumab ($p=0.002$), and ATC ($p<0.001$) significantly suppressed the increase in TNF-α levels caused by the I/R procedure in bladder tissue. Anakinra ($p=0.003$) and tocilizumab ($p=0.003$) were found to approximate TNF-α levels in the bladder tissue to the control group values. There was no significant difference in TNF-α levels between the control group that underwent the sham operation and the group that applied ATC ($p=0.140$).



IL-1β analysis results of bladder tissue

IL-1β level in the bladder tissue of the I/R procedure applied group was found to be higher than the bladder tissue of the sham operation applied group (Fig. 2B). The difference in the levels of IL-1β in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.002$), tocilizumab ($p=0.003$), and ATC ($p<0.001$) significantly suppressed the increase in IL-1β levels caused by the I/R procedure in bladder tissue. Anakinra ($p=0.001$) and tocilizumab ($p<0.001$) were found to approximate IL-1β levels in the bladder tissue to the control group values. The closest IL-1β value to the control group that underwent sham operation was found in the ATIR group ($p=0.028$).

IL-6 analysis results of bladder tissue

As shown in Fig. 2C, IL-6 levels in the bladder tissue of the I/R procedure applied group were higher than in the bladder tissue of the sham operation applied group. The difference in the levels of IL-6 in the bladder tissue of the sham operation-applied group and the I/R procedure-applied group was statistically significant ($p<0.001$). Anakinra ($p=0.002$), tocilizumab ($p=0.001$), and ATC ($p<0.001$) significantly suppressed the increase in IL-6 levels caused by I/R in bladder tissue. A significant difference was found between the IL-6 levels in the bladder tissue

Fig. 2. Cytokine expressions in bladder tissues of experimental groups. **A)** Tumor necrosis factor-alpha (TNF-α) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. **B)** Interleukin- 1 beta (IL-1β) levels: Bars are mean ± SEM. [a] means $p<0.05$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. **C)** Interleukin- 6 (IL-6) levels: Bars are mean ± SEM. [a] means $p<0.001$ when all groups were compared with the SG group. [b] means $p<0.05$ when the other drug treatment groups were compared with the IRG group. [c] means $p<0.05$ when drug treatment groups were alone compared with the ATIR combined treatment group. n=6 per group. SG: sham-operation group; IRG: ischemia-reperfusion group; AIR: anakinra+ischemia-reperfusion group; TIR: tocilizumab+ischemia-reperfusion group; ATIR: anakinra+tocilizumab+ischemia-reperfusion group.

of the anakinra-applied group and the control group values ($p<0.001$). The difference between the IL-6 levels in the bladder tissue of the tocilizumab-applied group and the control group was statistically insignificant ($p=0.102$). The closest IL-6 value to the control group who underwent sham operation was found in the ATIR group ($p=0.986$).

DISCUSSION

This study investigated the effects of anakinra, tocilizumab, and ATC on experimentally induced bladder I/R injury in rats. It has been reported in the literature that during the bladder I/R process, the increased production of ROSs disrupts the antioxidant balance and ultimately leads to oxidative stress [5,6]. Oxidative stress is known to first affect lipids in the cell membrane. When ROSs induce the cell membrane's LPO reaction, MDA emerges as an end product [6]. MDA resulting from LPO is also itself toxic and may cause further destruction by disrupting the structure and functions of the membrane [20]. This series of events occurring in bladder cells is known to be one of the most accused factors in the formation of bladder damage [5]. Therefore, MDA, a toxic product of LPO and an essential indicator of oxidative stress, was measured in our study. It has been reported that the MDA level is significantly increased in bladder I/R damage models, and this increase in MDA level is consistent with a significant decrease in the contractile ability of the bladder [5,21,22]. The fact that the MDA level was high in the bladder I/R group in our study findings shows that our experimental results coincide with the literature information.

However, our experimental I/R model found that anakinra, tocilizumab, and ATC significantly suppressed the increase in MDA level caused by the I/R procedure. At the same time, it was detected that anakinra and tocilizumab showed a synergistic effect together, reducing the severity of the LPO reaction at the highest level and bringing MDA levels closer to the values of the control group. There was no information in the literature showing the effect of anakinra and tocilizumab on bladder I/R damage. However, previous studies reported that anakinra significantly suppressed the increase of MDA level in intestinal tissue and tocilizumab in renal tissue by I/R and showed a protective effect [16,23].

It is known from the literature that the impaired redox balance is closely associated with the I/R event [5,24]. As such, our study investigated the effects of anakinra, tocilizumab, and ATC on tGSH, SOD, and CAT levels in the bladder tissue of rats applied with the I/R procedure. The cited parameters are significant indicators of antioxidant capacity and are known to protect tissues against oxidative stress [25]. Our results show that a significant decrease was detected in tGSH, SOD, and CAT levels in parallel with the increasing MDA concentration following the I/R procedure. Our study's stated results are similar to previous studies showing a decrease in antioxidant enzymes following an increase in LPO in experimentally created bladder I/R [24,26,27]. ATC, whose effect we investigated on oxidative damage, prevented the reduction of tGSH, SOD, and CAT in I/R-induced bladder tissue more significantly than anakinra and tocilizumab administered alone. Even though there are no studies in the literature examining the effects of anakinra and tocilizumab on antioxidant enzyme levels in rats with formed experimental bladder I/R, it has been reported that they significantly suppress the decrease in antioxidant enzyme levels in intestinal and renal I/R damage, respectively [16,23]. Our findings indicate that anakinra and tocilizumab support antioxidant defense mechanisms by showing additive, synergistic effects in bladder tissue.

Many experimental studies have shown that ROSs increase proinflammatory cytokine production in bladder I/R-damaged cells [7,8]. TNF-α, IL-1β, and IL-6 are the most emphasized cytokines in bladder inflamma-

tory response [5,7,28]. As can be understood from our findings, it was detected that there was a significant increase in TNF-α, IL-1β, and IL-6 levels in the bladder tissue of rats after the I/R procedure compared to the control group. Our findings correspond with previous studies such as Shin et al., Kanno *et al.*, and Altunkaynak *et al.* [5,7,28]. In our study, we found that anakinra and tocilizumab alone and ATC significantly inhibited the increase of TNF-α, IL-1β, and IL-6 in bladder tissue, and this effect was more significant in the ATC group. In the available literature, we found no information on the effect of anakinra and tocilizumab on the inflammation in I/R-induced bladder tissue. However, Butler *et al.* reported that the anakinra suppressed inflammation by regulating neuropeptide levels in an experimental cystitis model and protected the bladder by reducing the number of bacteria and neutrophils in the urine [29]. However, in the literature, anakinra and tocilizumab have significantly inhibited inflammation caused by testicular, ovarian, and renal I/R damage [12,13,16].

In conclusion, the I/R procedure has led to oxidative stress and inflammation in bladder tissue. Anakinra and tocilizumab alone suppressed I/R-induced oxidative and inflammatory bladder damage to almost the same extent. ATC was the best suppressor of I/R-induced bladder oxidative and inflammatory damage. This effect appeared due to anakinra and tocilizumab's additive, synergistic effect. Our results suggest that ATC may be more useful than anakinra and tocilizumab alone in treating bladder I/R damage. We think that histopathologic studies may be helpful in the detailed elucidation of this issue.

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Conflict of interest

None

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Authors' participation

Concept and design of the study: SB, BS, DA and HS. Acquisition of data: BS, RM, BY, BC and TAC. Data analysis and interpretation: SB, BS, RM, BC, DA, TAC and HS. Writing the manuscript: SB, DA, TAC and HS. Critical revision of the manuscript: RM, BY, BC, DA and HS. Approval of the final version: SB, BS, RM, BY, BC, DA, TAC, and HS. Statistical advice: BY and DA. Ethical or administrative advice: HS.

All authors of this paper have read and approved the final version of the submitted manuscript.

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Transición epitelio – mesenquima y cáncer.

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Palabras clave: cáncer; epitelio; caderina; plasticidad; transición epitelio-mesenquima.

Resumen. La migración e invasión de células cancerosas son componentes claves de la enfermedad metastásica, que es la principal causa de muerte en pacientes con cáncer. La transición epitelio-mesenquima (TEM) y la transición mesenquima-epitelio (TME) son una vía implicada en la metástasis del cáncer. Este proceso comprende la degradación de las uniones célula-célula y célula-matríz extracelular, y la subsecuente pérdida de la regulación de proteínas de unión como la caderina-E, por lo que las células experimentan una reorganización del citoesqueleto. Estas alteraciones están asociadas con un cambio de la forma celular, de una morfología epitelial a una mesenquimatosa. La comprensión de la base molecular y celular de la TEM y de la TME, proporciona conocimientos fundamentales sobre la etiología del cáncer, que pueden conducir a nuevas estrategias terapéuticas. En esta revisión, discutimos algunos de los mecanismos reguladores y el papel patológico de la plasticidad epitelio-mesénquima, con un enfoque en los conocimientos sobre la complejidad y la dinámica de este fenómeno en el cáncer.

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Epithelial-mesenchymal transition and cancer.

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Keywords: cancer; epithelium; cadherin; plasticity; epithelial-mesenchymal transition.

Abstract. Cancer cell migration and invasion are critical components of metastatic disease, the leading cause of death in cancer patients. The epithelium-mesenchyme-transition (EMT) and mesenchyme-epithelium-transition (MET) are pathways involved in cancer metastasis. This process involves the degradation of cell-cell and cell-extracellular matrix junctions and the subsequent loss of regulation of binding proteins such as E-cadherin. Cells undergo a reorganization of the cytoskeleton. These alterations are associated with a change in cell shape from epithelial to mesenchymal morphology. Understanding EMT and MET's molecular and cellular basis provides fundamental insights into cancer etiology and may lead to new therapeutic strategies. In this review, we discuss some of the regulatory mechanisms and pathological role of epithelial-mesenchymal plasticity, focusing on the knowledge about the complexity and dynamics of this phenomenon in cancer.

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INTRODUCCION

El cáncer constituye un problema de salud mundial debido a un aumento de la población, al envejecimiento, la adopción de un estilo de vida en el que existen factores de riesgo como el hábito de fumar, el sedentarismo, una alimentación deficiente, la obesidad, etc. Tradicionalmente, se consideraba el cáncer como un proceso exclusivo del genotipo celular como único causante. Actualmente, se abarca como un sinergismo entre el genotipo celular, y el microambiente tumoral. Estudiar este proceso complejo como un todo es difícil, mientras que hacerlo por etapas más sencillas y estudiarlas por separado, permite obtener resultados parciales, que en conjunto facilitan el estudio del proceso. Actualmente, la tendencia se centra en adquirir la capacidad de proporcionar un tratamiento adecuado a cada paciente, lo que se denomina una medicina de precisión, asumiendo que la biología que tiene cada tumor es única para cada caso. Esto permitirá desarrollar fármacos dirigidos a blancos específicos en las células tumorales, evitando dañar las células no tumorales, y disminuyendo sus efectos secundarios [1]. La progresión tumoral es un proceso complejo en el que están implicadas múltiples alteraciones genéticas que conducen a las células normales a malignizarse, a través de un proceso de transformación progresivo [2]. Se han descrito ocho alteraciones fisiológicas características de las células tumorales, comunes en la mayoría de los tumores, que son: 1) autosuficiencia de las señales de crecimiento, 2) insensibilidad a las señales inhibidoras de crecimiento, 3) evasión de la muerte celular programada o apoptosis, 4) potencial replicativo ilimitado, 5) capacidad de angiogénesis, 6) capacidad invasiva y metastática, 7) reprogramación del metabolismo energético, y 8) evasión del sistema inmune [3].

Células epiteliales: El tejido epitelial recubre las superficies externas e internas del organismo y está formado por una o varias capas de células unidas entre sí. Presenta una estructura característica formada por capas de células epiteliales unidas entre sí mediante contactos celulares, entre los que se incluyen: uniones estrechas, uniones adherentes, uniones tipo comunicantes y desmosomas. Así mismo, las células se contactan con la membrana basal, a través de enlaces célula-sustrato, que las separa de las células del tejido conectivo denominado estroma. La polaridad ápico-basal es característica de las células epiteliales, ya que presentan su superficie apical hacia el lumen, y su superficie basal hacia la membrana basal. Esta polarización conlleva una localización asimétrica del núcleo celular sobre la superficie basal, y una mayor superficie apical donde están presentes proteínas de superficie celular, mediante las cuales se establecen los diferentes tipos de uniones con células epiteliales adyacentes [4].

Los carcinomas. Son los más comunes de todos los cánceres, y tienen su origen en células epiteliales. Presentan una gran complejidad estructural, donde se observan diferentes tipos celulares, incluyendo células normales o no neoplásicas, que constituyen el estroma y que representan alrededor del 90% de la masa tumoral, y las células tumorales o transformadas. Los tipos celulares que forman el estroma son fibroblastos, células endoteliales, células de músculo liso, adipocitos, macrófagos y linfocitos, entre otras. La comunicación entre los diferentes tipos celulares que forman el estroma y el epitelio se define como heterotípica, que es fundamental para mantener la arquitectura y estructura del tejido. Las células estromales y epiteliales colaboran en la formación de la matríz extracelular (MEC) [5].

Invasión y metástasis en carcinomas. Los carcinomas comienzan en la zona epitelial en contacto estrecho con la membrana basal, y se consideran benignos mientras que las células que lo forman no atraviesen la membrana basal. En algunos casos, los carcinomas adquieren la capacidad de romper la membrana basal, invadir el estroma y metastatizar; es a partir de este punto que se denominan malignos, y se considera a este tumor original, como un tumor primario. Para que ocurra la invasión local del estroma, se requiere la secreción de proteasas que degradan la MEC para generar espacios a través de los cuales pueden migrar las células tumorales. En algunos tipos de cánceres, las propias células tumorales secretan sus propias proteasas, mientras que, en otros tipos de tumores, son las células estromales las que secretan dichas proteasas [6].

Para que un tumor primario crezca, necesita que se desarrolle una red de vasos linfáticos y sanguíneos para cubrir las necesidades metabólicas de las células, como nutrientes y oxígeno. Esta nueva formación de vasos se denomina linfangiogénesis y angiogénesis, respectivamente, y la entrada de las células a los vasos linfáticos y sanguíneos ocurre mediante un proceso denominado intravasación, accediendo así al torrente circulatorio y permitiendo la diseminación de las células tumorales [7]. En algunas ocasiones, estas células tumorales presentes en el sistema circulatorio, pueden penetrar en un tejido distal al tumor primario mediante un proceso denominado extravasación, y dar lugar a micrometástasis. Las células que forman parte de la micrometástasis pueden crecer, dividirse y formar una macrometástasis para alcanzar el estadio de colonización, y dar lugar a lo que se denomina tumor secundario [8]. La probabilidad de que una célula tumoral complete todos los pasos descritos, desde la ruptura de la membrana basal, invasión hasta la formación de metástasis, es muy baja. Los primeros pasos son ejecutados con elevada eficiencia por las células metastáticas, no así los pasos finales que implican la colonización. Aún así, el hecho de que células neoplásicas circulen por el torrente circulatorio corporal se considera un factor de mal pronóstico para el paciente. Las metástasis son responsables de alrededor del

90% de las muertes por cáncer, por eso es importante un diagnóstico precoz, así como el estudio de nuevas terapias que bloqueen los estadios tempranos del proceso de invasión y metástasis [9].

Transición epitelio-mesénquima: fisiológica y patológica

La progresión tumoral de carcinomas se caracteriza por la capacidad de invasión por parte de las células tumorales epiteliales que sufren múltiples alteraciones al someterse a un proceso denominado "Transición epitelio-mesénquima (TEM)", y que tiene lugar en estadios tempranos de la invasión y la metástasis. Durante la TEM en cáncer, las células epiteliales pierden su fenotipo epitelial y polarizado [10,11]. Este proceso está acompañado de una reorganización del citoesqueleto celular y de una pérdida de adhesión célula-célula y célula-sustrato, que dan lugar a una morfología mesenquimal, que proporciona a las células una capacidad invasiva y de metástasis, pudiendo migrar por el torrente sanguíneo a órganos distales diferentes al tumor primario [12,13].

La TEM fue inicialmente descrita por Elisabeth Hay, durante el desarrollo embrionario, como un paso clave para la formación de órganos y tejidos [14]. La TEM se ha observado en los procesos de regeneración tisular, fibrosis, y cáncer. El proceso de TEM se ha descrito en tres marcos biológicos muy diferentes, donde la generación de células mesenquimales es común para todos ellos, mientras que el proceso biológico a través del cual se adquiere el fenotipo mesenquimal es muy diferente. Se conocen tres tipos de TEM propuestos en función del proceso biológico: Tipo I: asociada con la implantación, la formación embrionaria y el desarrollo de órganos. Todos estos procesos precisan generar diferentes tipos celulares, que comparten fenotipos mesenquimales comunes. Tipo II: asociada con la cicatrización de heridas, regeneración tisular, inflamación y fibrosis, este proceso genera fibroblastos y otros tipos celulares relacionados para regenerar el tejido dañado después de un traumatismo o una inflamación. Tipo III: asociada a la conversión de células epiteliales en células mesenquimales, que pueden migrar e invadir tejidos, favoreciendo la progresión tumoral [11].

Transición epitelio-mesénquima (TEM) y cáncer

La adquisición de capacidades propias de células mesenquimales por parte de las células epiteliales, implica una progresión maligna de las mismas a través de un proceso biológico en el que se suprimen los marcadores celulares epiteliales, y donde las células pierden su polaridad, y las uniones célula-célula. Esto provoca cambios en la morfología celular y en el citoesqueleto, lo que hace que las células pierdan el fenotipo epitelial y adquieran una morfología similar a fibroblastos, adquiriendo capacidades de migración e invasión celular [15]. Estos cambios están acompañados por la pérdida de marcadores epiteliales en las células, tales como caderina-E, zonula occludens-1 (ZO-1), citoqueratinas (proteína fibrosa de los filamentos intermedios del citoesqueleto), así como por un incremento de los marcadores mesenquimales que incluyen caderina-N, vimentina, actina de músculo liso, y proteína-1 específica de fibroblastos, además de la producción de componentes de matríz extracelular como colágeno tipo I y fibronectina (glucoproteina) [16] (ver Fig. 1). La desestabilización del complejo caderina-E/catenina, provoca una acumulación citoplasmática de catenina, que es capaz de translocarse al núcleo donde actúa como activador de Tcf-1/LEF (factores de transcripción involucrados en la vía de señalización Wnt), y por consiguiente comienzan a expresarse genes que codifican proteínas con un importante papel oncogénico, tales como ciclina D1(proteínas que controlan la progresión de la fase G1 a la fase S) y c-Myc (protooncogen), originando la inducción del proceso TEM [17]. No está claramente definido el total de señales que contribuye al proceso de TEM asociado al

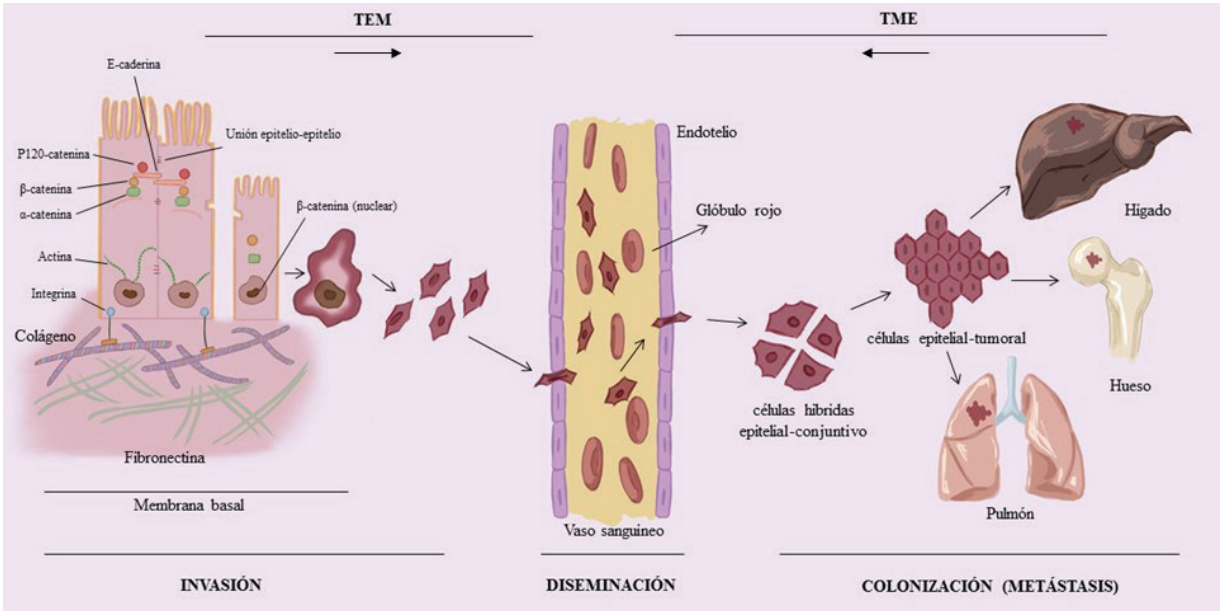

Fig. 1. Plasticidad tumoral. Estado de la transición epitelio-mesenquima (TEM) y del proceso inverso transición mesnquima-epitelio (TME). (Diseño Gabriel Sojo, 2023).

cáncer. Una hipótesis plantea que mediante las comunicaciones heterotípicas originadas por las células del estroma asociados al tumor, se inducen las alteraciones genéticas y epigeneticas, que sufren las células tumorales durante el proceso de formación del tumor primario. Además, las células estromales también pueden secretar factores de crecimiento, tales como el factor de crecimiento hepático (HGF) [18], el factor de crecimiento epidérmico (EGF) [19], el factor de crecimiento derivado de plaquetas (PDGF) [20], y el factor de crecimiento transformante beta (TGβ) [21], que pueden inducir la expresión de los factores de transcripción Snail, Slug, ZEB-1, ZEB-2 y Twist [22,23]. Estos factores de transcripción actúan como represores transcripcionales de la caderina-E, originando por consecuencia, la pérdida de uniones entre las células epiteliales y de la polaridad apical. Durante la progresión tumoral la perdida de la caderina-E va acompañada de la expresión de las caderinas mesenquimales caderina-N, caderina-P, caderina-6, caderina-11 (glucoproteína transmembrana)[24]. En cáncer de próstata y de mama la sobreexpresión de caderina-N promueve la motilidad y la migración celular [25]. En el cáncer colorrectal se ha observado un significativo incremento de la expresión de caderina-N comparado al tejido adyacente sano, y se ha relacionado con una baja diferenciación tumoral, y una baja supervivencia [26]. El intercambio de caderina-E por caderina-N en cáncer epitelial de vejiga se ha propuesto como un factor de malignidad que identifica a las células tumorales epiteliales de vejiga con un fenotipo invasivo [27]. Así mismo, la caderina-P y la caderina-11 se expresan en cáncer de mama invasivo, mientras que la cadherina-6 se encuentra sobreexpresada en carcinoma renal [28].

Plasticidad epitelial: implicaciones clínicas

La TEM es un proceso transitorio y reversible, es decir, las células epiteliales que han adquirido capacidades y fenotipos mesenquimales pueden revertir el proceso, y volver a su estado epitelial original diferenciado, a través del procedimiento denominado Transición mesénquima-epitelio (TME), que es transitorio y reversible, y que recibe el nombre de plasticidad epitelial [29,30] (Fig. 1). Clínicamen-

te, el proceso de TEM tiene lugar en las células del carcinoma primario, probablemente en estadios tempranos de la invasión y la metástasis. La TEM en la progresión tumoral está en discusión, porque la identificación y detección de células tumorales circulantes (CTCs), que sufren un proceso de TEM en el torrente sanguíneo de pacientes oncológicos es muy difícil, debido a que se encuentran presentes en muy bajo número. Las CTCs son células tumorales que se desprenden del tumor primario y viajan por el torrente sanguíneo hasta alcanzar otras partes del cuerpo originando metástasis tumorales [31]. Las metástasis tumorales son las causantes de la mayoría de las muertes por cáncer. El proceso biológico de la metástasis es muy poco eficaz, ya que menos del 0.01% de las células presentes en el tumor primario es capaz de acceder al torrente sanguíneo y dar lugar a las metástasis [32]. Se considera que las metástasis clínicas ocurren incluso antes de detectar el tumor primario, lo que sugiere que el proceso de TEM ha tenido lugar antes de que el tumor crezca en el órgano primario [33]. Terapéuticamente, hay diferentes estrategias para poder impedir la formación de metástasis a través de la acción sobre la TEM: a saber 1) Inhibir la inducción de la TEM, 2) Promover la TME, 3) eliminar o inhibir funcionalmente a las células tumorales mesenquimales [34]. En cáncer de ovario, una de las primeras estrategias para bloquear la metástasis fue inhibir el proceso de TEM para prevenir la diseminación de células tumorales desde el tumor primario [35]. Se ha observado que la inducción de la TEM conduce a la colonización, crecimiento y formación de tumores secundarios y por consiguiente a la micrometástasis [36]; por lo que en principio, el objetivo fue bloquear aquellas células que se diseminaban y así evitar su desplazamiento y el inicio de la metástasis. Al iniciarse el bloqueo muchas de estas células, ya estarían trasladándose a los tejidos blanco, producto de su liberación del tumor primario en las etapas tempranas del desarrollo tumoral. Por tal motivo, el tumor primario no sería el objetivo sino el nicho metastásico, evitando el alojamiento, división y formación de nuevos tumores al bloquear las señales de crecimiento que evitaría la colonización a otros tejidos [13].

Uniones adherentes: caderinas-E

La caderina-E, es la mejor caracterizada de las uniones adherentes en células epiteliales de mamíferos. La caderina-E es un supresor de tumores, y su pérdida de expresión es un marcador de TEM, y está asociada a estadios tempranos de invasión y metástasis, a un menor grado de diferenciación, y a un mal pronóstico clínico [37]. La pérdida de regulación de la caderina-E en las uniones adherentes es importante, ya que marca los estadios tempranos de cáncer antes que ocurra la metástasis. Es importante destacar que las células epiteliales deben mantenerse unidas entre sí para estructurar el epitelio que formará parte de un tejido polarizado e íntegro en su estructura [37].

Estructura de la caderina-E

La caderina-E es el prototipo mejor caracterizado de la familia de las células epiteliales. Se expresa en todos los epitelios presentes en mamíferos, y funcionalmente, se ha relacionado con el mantenimiento de la polaridad ápico-basal y la preservación de la supervivencia, y del control de la proliferación celular. Esta proteína está codificada por el gen cadherin 1 (*CDH1*) presente en el brazo largo del cromosoma 16. El dominio citoplasmático se divide en 1) dominio de juxtamembrana, y 2) dominio de unión a catenina [38]. Se ha propuesto que la regulación del mantenimiento de caderina-E en las uniones célula-célula, se realiza a través del bloqueo de la interacción de miosina II, proteína motora dependiente de actina, con caderina-E evitando de esta manera su disgregación [39].

Inhibidores de microtúbulos y transición epitelio-mesénquima

El citoesqueleto celular está formado por el citoesqueleto de actina, los filamentos intermedios y la red de microtúbulos, y juega un papel fundamental en el proceso

de TEM. La reorganización del citoesqueleto de actina durante la progresión tumoral, es el paso clave en la adquisición de capacidades migratorias e invasivas de las células tumorales que promueven la metástasis celular. Se ha observado una sustitución de la citoqueratina presente en los filamentos intermedios por vimentina, durante la TEM [40]. Los microtúbulos proporcionan la fuerza motora necesaria para la migración celular durante la progresión tumoral, los cuales se distribuyen uniformemente en el citoplasma de las células epiteliales no transformadas, mientras que las protusiones de la membrana celular, debido a TEM son estructuras asociadas a microtúbulos [41]. Dada la función de los microtúbulos en el crecimiento y la proliferación celular, son considerados blancos para el empleo de compuestos que inhiben la polimerización de los microtúbulos en el tratamiento de pacientes con cáncer [42]. Los compuestos antimitóticos que se fijan a microtúbulos se clasifican en dos grupos: 1) agentes estabilizadores de microtúbulos, y 2) agentes desestabilizadores de microtúbulos que actúan inhibiendo la polimerización, como la vinflunina y el nocodazole. Por otra parte, el compuesto quinolina-6-il oxyacetamidas, identificado inicialmente como un fungicida frente a un amplio rango de fitopatógenos, tamnién actúa como un desestabilizador de microtúbulos, con propiedades antiproliferativas en células tumorales de pulmón y ovario resistentes al tratamiento con otras drogas antineoplásicas [43,44]. Las drogas que inhiben la actividad de los microtubulos puede tener un efecto potencial sobre la adhesión celular [45].

Regulación de la caderina-E

En la mayoría de los cánceres humanos de origen epitelial, se ha demostrado a menudo la pérdida de caderina-E [46]. Los niveles de caderina-E para mantener la adhesión celular dependen del balance entre la síntesis y la degradacion [47]. Existe una correlacion inversa entre los niveles de caderina-E, el estadio del tumor y la tasa de mortalidad, aunque en algunos tumores diferenciados se expresa la caderina-E [48]. La restitución de los complejos formados por la interacción de caderina-E con catetina-β, catetina-∑ (proteína intracitoplasmatica), y p120, induce a una reversión del fenotipo mesenquimal hacia el fenotipo epitelial de células tumorales en cultivo celular [49].

Los reguladores de caderina-E son:

a. **Reguladores genéticos o epigenéticos**. En tumores de mama y ovario se han identificado alteraciones genéticas del gen *CDH1*, que codifica para caderina-E, que causan la pérdida de su funcionalidad [50]. La región del promotor de *CDH1* que corresponde a las islas CG, sufre una metilación anormal o hipermetilación, ocasionando pérdida de la expresión de caderina-E [51].
b. **Reguladores transcripcionales** La acción de diferentes represores transcripcionales de caderina-E durante la TEM, como Snail, Slug, Twist, ZEB-1, y ZEB-2, da origen a la pérdida de expresión de la caderina-E [52].
c. **Reguladores post-traduccionales**. La regulación post-traduccional se realiza a través de la fosforilación, la glicosilación o la proteolisis. La fosforilación de los resíduos de tirosina de la caderina-E, está mediada por el EGF y el factor de crecimiento de fibroblastos (FGF), responsable de la fosforilación de caderina-E, y relacionado con la pérdida de caderina-E [53]. La proteína caseína quinasa 1 (CK1) es un regulador negativo de la caderina-E que participa en la fosforilación de resíduos en su dominio citoplasmático [54]. Diferentes metaloproteasas de la matríz (MMP), como MPP2, MPP9 y MPP14, participan en la degradación de la caderina-E. La caderina-E está representada como una molécula soluble de 80 kDa, que se encuentra en células tumorales en cultivo y en las biopsias tumorales [55]. En el cáncer nasofaríngeo la sobreexpresión de MMP-2 inhibe la adhesión célula-célula,

promoviendo la TEM e invasión tumoral, con una baja regulación de caderina-E y alta regulación de caderina-N, fibronectina y Slug [56].

Migración de las células mesenquimáticas

Una vez que ha ocurrido la TEM, las células mesenquimáticas invaden la membrana basal y sintetizan matríz de fibronectina, que proporciona una vía para la migración de estas células mesenquimales durante la TEM, manteniendo el fenotipo mesenquimal [57]. La presencia de fibronectina en la MEC aumenta la rígidez del sustrato, [58] controlado por las señales SMAD (factor de transcripción) y JNK (quinasa) activadas por TGF-β (factor de crecimiento β) [59]. Una mayor cantidad de fibronectina junto con Ras (reguladoras de los procesos de transducción) activado en las células epiteliales, provoca el reemplazo de las integrinas a_6b_3 por las a_5b_1 que mejoran la capacidad migratoria de las células, aumentando su adhesión celular a la fibronectina [60]. El aumento de colágeno tipo I y fibronectina en la MEC, también se correlaciona con el cambio de las integrinas a un estado de unión a ligandos de alta afinidad, aumentando la actividad de señalización a través de FAK (proteína de anclaje) e ILK (integrina), promoviendo la TEM [61]. La diversidad en las afinidades de unión de diferentes integrinas, permite a las células responder a una amplia gama de elementos extracelulares, y mediar en diferentes cascadas de señalización en respuesta a un entorno de matríz cambiante. A medida que cambia la composición de la MEC, también lo hace la abundancia de integrinas en la superficie celular, lo que promueve la progresión de la TEM bajo el control del entorno pericelular. Las integrinas a_Vb_3 facilitan la fosforilación de TGF-βRII mediada por Src (proto-oncogen), originando un sitio de acoplamiento para ShcA (receptor nuclear) y GRB2 (proteína de receptor de crecimiento), que envían señales a través de la vía p38 MAPK (proteínas quinasa) para inducir TEM [62]. La importancia del colágeno tipo I en la TEM es evidente en varios sistemas celulares. El colágeno tipo I se asocia con la TEM en los carcinomas de pulmón, mama y páncreas, lo que destaca la importancia del entorno de la matríz en la metástasis [63]. La inducción de TEM depende de la interacción entre las fibras de colágeno tipo I y la integrina a_2b_1, lo que desencadena una cascada intracelular [64]. El colágeno de tipo I provoca la fosforilación de IkB (inhibidor de kB) dependiente de ILK para aumentar la abundancia de NF-κB (factor de transcripción) de localización nuclear, que promueve la expresión de *SNAI1* y *LEF1* (proteína en células B y T) para inducir TEM [65]. El aumento de colágeno tipo I activa las vías de JNK, donde se ha demostrado que la inhibición farmacológica de la señalización de JNK anula la migración mediada por colágeno tipo I, y la metástasis de las células en cáncer de mama [66,67].

Heterogeneidad y resistencia

La heterogeneidad biológica que se manifiesta a través de mecanismos tanto genéticos como no genéticos, contribuye a las diferencias fenotípicas entre las diferentes subpoblaciones de células cancerosas que residen en tumores individuales. Se reconoce la capacidad de las células cancerosas para adquirir estados fenotípicos alternativos, sin cambios mutacionales en su genoma. El concepto de células madres cancerosas (CMC) postula la presencia de poblaciones menores de CMC que tienen la capacidad de sembrar nuevos tumores. Existen mecanismos reguladores epigenéticos que pueden contribuir a la diversidad fenotípica de distintas subpoblaciones de células cancerosas dentro de un tumor. Esto se basa en el concepto de que células cancerosas fenotípicamente distintas que residen dentro de la misma masa tumoral están organizadas en jerarquías, semejante a la jerarquía de células madres del tejido no neoplásico. En los tumores las células con fenotipo CMC deberían en principio, renovarse por sí mismas, para generar nuevas CMC que se diferencien en una descenden-

cia menos tumorigénica y no autorrenovadora; es decir, las no CMC que forman la mayor parte del tumor. El concepto de CMC tal vez define la eficacia a menudo limitada de las terapias convencionales contra el cáncer, debido a que se centran en la población mayor de células no CMC dentro de los tumores individuales y no eliminando específicamente las CMC. Las evidencias experimentales han demostrado que las CMC son más resistentes que las no CMC. Esto demuestra la capacidad de las CMC para ser precursoras de nuevas masas tumorales, lo que trae como consecuencia la recaída clínica. En los cánceres epiteliales, estos cambios adaptativos pueden involucrar, al menos en parte TEM y el proceso inverso TME. La TEM puede desencadenar la reversión a un fenotipo similar a CMC, [68,69] lo que proporciona una asociación entre TEM, CMC y resistencia a los fármacos que origina cambios fenotípicos sin que ocurran cambios genéticos. Se ha observado una relación directa entre TEM y resistencia a quimioterapia, además de la importancia del proceso de TEM en la invasión y la metástasis. Existen múltiples estudios clínicos que ensayan compuestos farmacológicos cuyo blanco terapeútico son genes implicados en el proceso de plasticidad epitelial. La detección de altos niveles de Zeb-1 y Twist, represores transcripcionales de caderina-E, y por consiguiente reducción de caderina-E y otros marcadores epiteliales que se relaciona con la resistencia al tratamiento con diferentes drogas, como son 5-fluorouracilo, gemcitabina o cisplatino [70]. La disminución en la expresión de caderina-E se utiliza en el ámbito clínico como factor del mal pronóstico, ya que se relaciona con una baja diferenciación epitelial de las células tumorales, y se asocia a procesos de invasión y metástasis que implican baja supervivencia en diferentes tipos de cáncer tales como el cáncer de mama o cáncer colorrectal [71]. En el cáncer colorrectal (CCR) una de las neoplasias malignas más agresivas, la LACTB (serina proteasa mitocondrial que actua como regulador del metabolismo de los lípidos mitocondriales) funciona como un supresor de tumores. Se ha demostrado que LACTB puede inhibir la TEM y la proliferación del cáncer de mama. LACTB (serina proteasa) regula el nivel de PIK3R3 (fosfatidil-inositol 3-quinasa) para promover la autofagia e inhibir la TEM y la proliferación. Estos efectos se logran en parte a través de la vía de señalización PI3K /AKT (oncogen de retrovirus murino)/ mTOR (proteína quinasa) en el CCR [72].

Mecanismo de resistencia a fármacos inducida por TEM

Durante mucho tiempo se ha tratado de establecer una relación entre TEM y la resistencia a los medicamentos, pero hasta el presente no se ha dilucidado un mecanismo concreto. Probables evidencias se han obtenido de los trabajos con las CMC, con los cuales se tienen perspectivas sobre el mecanismo de resistencia a los fármacos en células sometidas a TEM. Las CMC son una subpoblación de células que forma parte de la masa tumoral responsable de la tumorigénesis [73]. Se han encontrado similitudes en las vías de señalización de Wnt, Hedgehog y Notch (vías de señalización que regulan la proliferación, diferenciación y migración) en el fenotipo activado TEM y CMC. Estas vías son críticas para el mantenimiento y renovación de CMC [74]. Esto sugiere que las células sometidas a TEM presentan propiedades parecidas a las células madres al compartir vías de señalización de fenotipo resistente a fármacos [75,76]. La terapia del cáncer está asociada con la quimioresistencia y la recurrencia después de la quimioterapia [77,78]. Esta resistencia se asocia a su vez, con la presencia, dentro de los tumores, de poblaciones agresivas de células cancerosas que desarrollaron mecanismos de quimioresistencia que conducen a una disminución de las tasas de supervivencia de pacientes tratados por cáncer [79]. En varios estudios, se han identificado estas poblaciones de cánceres agresivos como CMC [80,81]. La quimioresistencia media-

da por CMC se basa en varios mecanismos celulares:

1. Baja tasa de proliferación. La quimioterapia se dirige a las células altamente proliferativas, a través del daño en el ADN e inhibición de la división mitótica; su acción es limitada cuando se aplica a células cancerosas lentas y que no se dividen, como las CMC [82,83]. Algunos estudios han demostrado que las CMC de diferente origen tisular, podrían resistir el efecto de una amplia gama de fármacos como la doxorrubicina, temozolomida, cisplatino, paclitaxel, etopósido y metotrexato [84]. Por lo tanto, el desarrollo de terapias que se dirijan a las células inactivas o de división lenta como CMC es esencial para prevenir cánceres recurrentes.

2. Expresión de transportadores de "casette" de unión a ATP (ABC). Esta gran superfamilia de proteínas de membrana, funciona en el transporte transmembrana de varios sustratos mediante la conversión de la energía obtenida de la hidrólisis del ATP [85]. Aunque, el tipo de transportadores ABC varía de un tipo de cáncer a otro, las CMC expresan altos niveles de estos transportadores, lo que contribuye a un mayor transporte de compuestos quimioterapéuticos fuera de las células, contribuyendo así a la quimiorresistencia de las CMC. Por ejemplo, el transportador ABCC1 conocido como MRP1 (proteína de resistencia a múltiples fármacos 1), expresado por CMC en glioblastoma, está involucrado en la salida de una variedad de compuestos terapéuticos que incluyen: metotrexato, edatrexato, ZD1694, doxorrubicina, daunorrubicina, epirrubicina, idarrubicina, etopósido, vincristina, vinblastina, paclitaxel, irinotecán, SN-38, flutamida e hidroxiflutamida [86]. Por otra parte, el transportador MDR1 (proteína de resistencia a múltiples drogas 1), expresado igualmente por CMC' se encuentra en cánceres de ovario y de mama, leucemia mieloide aguda (LMA), glioblastoma y carcinoma de células renales, proporciona multirresistencia a fármacos como las antraciclinas, actinomicina D, colchicina, etopósido, tenipósido, metotrexato, mitomicina C, mitoxantrona, paclitaxel, docetaxel, vincristina y vinblastina [87]. Hay otros transportadores ABC con capacidad como ABCA1 (transportador de casette de unión al ATP A1), que se expresa en las CMC de ovario y que transporta cisplatino [88]. En las células sometidas a TEM con sobreexpresión de los transportadores ABC y mostraron un fenotipo de resistencia a fármacos similares a las CMC. Saxena y col. en 2011, demostraron que los promotores de los transportadores ABC, contienen varios sitios de unión para TEM-TF. La sobreexpresión de TEM-TF como Twist, Snail y FOXC2 (factor de transcripción), aumentó la actividad promotora y la expresión de transportadores ABC en células de cáncer de mama. Estas células mostraron una resistencia diez veces mayor al tratamiento con doxorrubicina comparadas con el control [89].

3. Aumento de la expresión de aldehído deshidrogenasas (ALDH). Esta superfamilia de enzimas es determinante para la desintoxicación de sustratos de aldehídos endógenos y exógenos al catalizar la oxidación de aldehídos a ácidos carboxílicos [90]. Las ALDH están altamente expresadas en CMC de diferentes tipos de cánceres, y sus niveles elevados de expresión se correlacionan con un deficiente pronóstico en pacientes con cáncer [91]. Además, están asociadas con la quimiorresistencia mediada por CMC, aunque sus mecanismos de acción no han sido bien dilucidados [92].

4. Resistencia a la apoptosis. Las CMC resisten la apoptosis mediada tanto por la vía de muerte intrínseca (dependiente de mitocondrias), como por la vía de los receptores de muerte celular extrínseca [93]. Por ejemplo, los CMC de glioma y leucemia humanos, expresan bajos niveles de Fas y ligando de Fas (Fas-L) (proteínas de membranas), lo que da como resultado resistencia a la muerte celular mediada por receptores extrínsecos [94]. Además, de la vía apoptótica extrínseca, la proteína pro-supervivencia Bcl-2 está in-

volucrada en la vía de muerte dependiente de mitocondrias, a través de sus interacciones inhibidoras con los proapoptóticos Bax y Bak. La proteína Bcl-2 (proto-oncogen), se encontró sobreexpresada en células madre de leucemia, glioma y glioblastoma [95]. La inactivación de las vías de muerte celular intrínsecas y extrínsecas, asegura una ventaja de supervivencia selectiva para las CMC.

5. Respuesta de reparación del ADN. Otra ventaja de supervivencia está relacionada con la capacidad de las CMC para activar oportunamente el sensor de daño del ADN [96]. Este proceso involucra vías de reparación del ADN que incluyen la reparación por escisión de nucleótidos (REN), reparación por escisión de bases (REB), reparación de errores de apareamiento (MMR), reparación directa y reparación de rotura de doble hebra (DSB). La evidencia de la resistencia de CMC a la radioterapia, fue proporcionada por un estudio que demostró la capacidad de las células madre de glioblastoma para activar eficientemente el *ATM* de la serina/treonina quinasa y el daño en el ADN de la proteína quinasa de control (Chk1) en respuesta a las radiaciones ionizantes [97].

6. Microambiente CMC. Los nichos de células madre representan áreas de tejido que proporcionan microambientes específicos, que mantienen y promueven la capacidad de las CMC para autorrenovarse y generar progenies diferenciadas [98]. El nicho de células madre es necesario para determinar el destino de estas células, ya que su comportamiento está influenciado por su asociación con otras células del nicho. Este concepto es aplicable a las CMC, donde la interacción con estos nichos es necesaria para el mantenimiento de las poblaciones de CMC. El microambiente es un complejo altamente heterogéneo compuesto por células tales como células estromales, células inmunes y células epiteliales, y una red de macromoléculas extracelulares que proporciona soporte a las células dentro de la MEC [99]. Las células que se encuentran en el nicho promueven el crecimiento, el mantenimiento y la diferenciación de las CMC. Las terapias contra el cáncer no han tenido éxito, ya que los fármacos eliminan la población masiva de células cancerosas, sin afectar a las poblaciones de CMC [100].

7. Fibroblastos asociados al cáncer (FAC). Expresan un apoyo mecánico para las CMC a través de la producción de colágeno fibrilar. Los FAC fibroblastos asociados al cancer (FAC), también secretan la citocina CXCL12 (ligando 12 de quimiocina del motivo CXC), factores de crecimiento como el HGF, el factor de crecimiento endotelial vascular (VEGF) y el factor de crecimiento derivado de plaquetas (PDGF), que contribuyen al aumento de la proliferación, invasión y metástasis de las CMC [101]. Los FAC participan en la heterogeneidad celular a través del TGFβ1, que promueve la TEM relacionada con CMC [102].

8. Células inmunes. Las células inmunes contribuyen al estado inflamatorio crónico del microambiente de las CMC, que potencia la proliferación, invasión y metástasis tumorales [103]. Los macrófagos asociados a tumores (TAM) y las células supresoras derivadas de mieloides (MDSC) secretan TGF-β, que contribuye a la TEM, la invasión y las metástasis [104]. Las células inmunes del microambiente contribuyen a la evasión tumoral a través de una variedad de mecanismos. Los TAM, a través del TGFβ reclutan células T reguladoras (Tregs) dentro del nicho y contribuyen a la inmunosupresión. Las MDSC, secretan factores de crecimiento como TGFβ, y las citocinas reclutan células T auxiliares para promover su actividad inmunosupresora [105].

9. Células madre mesenquimales (CMM). Son células estromales multipotentes que pueden diferenciarse en osteocitos, adipocitos y condrocitos. Estas células migran a sitios nflamatorios crónicos como es el caso del cáncer, donde contribuyen a la metástasis al secretar TGFβ que promueve la TEM [106]. También participan en la colonización de cáncer secundario en cánceres metastásicos de mama, próstata y pulmón

[107]. Las CMM promueven la proliferación del cáncer gástrico y la angiogénesis a través de la secreción de VEGF, proteína 2 inflamatoria de macrófagos (MIP-2), TGF-β1 y las citocinas proinflamatorias interleucinas IL-6 e IL-8 [108]. Las CMM pueden originar FAC, que contribuyen aún más a la heterogeneidad de las células del microambiente CMC y a su potencial metastásico [109,110].

10. Células endoteliales. La angiogénesis es clave para el microambiente de CMC, ya que proporciona nutrientes para el metabolismo, que son necesarios para su autorrenovación y capacidades invasivas y metastásicas. A través de los vasos tumorales, las células inmunitarias como las Treg, contribuyen a la supresión inmunitaria [111]. Las células endoteliales, junto con las células perivasculares, constituyen los componentes básicos de los vasos, y son estimuladas por factores angiogénicos como el VEGF dentro de un entorno hipóxico como el cáncer. Esto resulta en un aumento de la vasculatura del tumor y un mayor crecimiento y metástasis [112]. Además, las células endoteliales tumorales secretan citocinas como IL-3, granulocitos (globulos blancos)-CSF (factores estimuladores de colonias de células sanguíneas), granulocitos-macrófagos-CSF (GM-CSF), IL-1, IL-6, VEGF-A y bFGF (factor de crecimiento de fibroblastos), que promueven y conservan la autorrenovación y la progresión mediada por CMC [113].

11. Hipoxia. En el cáncer, la hipoxia ocurre como resultado de condiciones isquémicas [114]. Por lo tanto, la baja tensión de oxígeno es capaz de provocar cambios en el fenotipo celular y cooperar con otras vías para inducir la TEM. La TEM inducida por hipoxia, está estrechamente mediada por la vía de señalización de HIF (factor de transcripción), que contribuye al crecimiento tumoral agresivo y a la invasividad. Estudios experimentales han demostrado que el crecimiento en condiciones hipóxicas, puede reprogramar las células epiteliales a un fenotipo mesenquimatoso, debido a la activación de represores de la transcripción de cadherina-E, lo que conduce a la promoción del potencial invasivo [115]. Se han propuesto varios aspectos de los posibles mecanismos moleculares. En primer lugar, la activación de HIF-1 y 2 puede inducir TEM mediante la regulación positiva de factores de transcripción asociados a TEM o represores como Twist, Snail, Slug y SIP1/ZEB2 (factor de transcripción) en células cancerosas [116,117]. En segundo lugar, la hipoxia y la vía HIF activan las vías de señalización asociadas a TEM como TGF-β, Notch, NF-κB, Wnt/catetina- β y Hedgehog [118,119]. La hipoxia y la vía de HIF pueden inducir el fenotipo o las características de la TEM, mediante la regulación de las citocinas inflamatorias asociadas a la TEM, como el aumento de la expresión del factor de necrosis tumoral α inducido por hipoxia (TNF-α), la interleucina 6 (IL-6) y IL-1β, que promueve la inducción del fenotipo TEM [120,121]. Así mismo, la hipoxia y la vía de HIF pueden inducir la TEM mediante la regulación directa o indirecta de proteínas o enzimas que median las interacciones célula-matriz, y facilitan la motilidad y la invasión mediadas a través de la regulación de LOX / LOX2 (lisil oxidasa), Hey1, Hes1 (represores transcripcionales) y el activador del plasminógeno tipo uroquinasa (uPA) [122,123].

12. Estroma. El estroma desempeña un papel en la protección de las CMC contra la quimioterapia y otros tratamientos, proporcionando a las CMC, estímulos de señalización resistentes a través de receptores de superficie para activar otras líneas de defensa. Los miofibroblastos tumorales, secretan factores de crecimiento como el HGF y la periostina, para activar la señalización de Wnt en el cáncer colorrectal y el de mama [124,125]. Las células estromales secretan altos niveles de HGF, lo que hace que las células cancerosas humanas de muchos tipos co-cultivadas, adquieran resistencia a varios fármacos, en particular a los de RAF (proteína quinasa) [126]. Otros factores de crecimiento o citocinas, incluidos la IL-6, el FGF y la neuregulina 1, ayudan a formar el llamado "nicho quimiorresistente" de las CMC, activando

diversas vías de señalización de supervivencia [127]. Además, las integrinas de los receptores de MEC, están altamente asociadas con la supervivencia de la CMC y la resistencia a fármacos, lo que representa un papel crítico para el MEC [128]. El marcador de superficie de células madre CD44, inicialmente identificado como el receptor de localización de linfocitos, se expresa comúnmente en células madre embrionarias y adultas, así como en las CMC. Esto ocurre porque se une a su ligando hialuronano para regular muchos aspectos de la función de las células madre, incluida su autorrenovación. La señalización de CD44 también ayuda a mantener la integridad genómica de las células madre al protegerlas y reparar el ADN despuésdel daño oxidativo. Además, CD44 está implicado en la regeneración de CMC responsables de la quimiorresistencia [129].

13. Canales iónicos que promueven TEM. El microambiente hipóxico además de promover programas de supervivencia adaptativos en el cáncer, se asocia con la resistencia, la apoptosis, la angiogénesis y la migración, e induce la TEM en una variedad de tipos de células cancerosas [130]. El calcio (Ca^{2+}) es un mensajero intracelular que desempeña un papel crucial en el cáncer, incluída la proliferación [131]. La angiogénesis y la metástasis, también están involucradas en la inducción de TEM a través del EGF como una consecuencia de la entrada de Ca^{2+} a la célula [132]. El microambiente del tumor es rico en ATP, por lo tanto es probable que aumenten los niveles de Ca^{2+} libre intracelular, mediante la activación de los receptores purinérgicos [133] o indirectamente, a través de la activación de proteínas G y la generación de trifosfato de 1,4,5 inositol (IP_3) por el receptor P2Y [134].

El canal iónico del potencial receptor transitorio canónico-1 (TRPC1), es un componente clave de las respuestas a la hipoxia en las células de cáncer de mama. Esta regulación incluye el control de eventos específicos de TEM, y la activación de vías de señalización mediadas por hipoxia, como la activación del EGFR (receptor del factor de crecimiento epidermal), STAT3 (citoquina) y el marcador de autofagia LC3B, a través del factor inducible por hipoxia-1α (HIF1α). El receptor transitorio canonico-1 (TRPC1), regula los niveles de HIF1α en las líneas celulares de cáncer de mama MDA-MB-468 y HCC1569 deficientes en PTEN. Esta regulación surge de los efectos sobre la traducción constitutiva de HIF1α en condiciones normóxicas a través de una vía dependiente de Akt. En apoyo adicional al papel de TRPC1 en TEM, su expresión está estrechamente asociada con genes relacionados con TEM y metástasis en tumores de mama. La expresión de TRPC1 también es de pronóstico significativo para los cánceres de mama basales, en particular los clasificados como positivos para los ganglios linfáticos. Los roles definidos de TRPC1 se podrían investigar terapéuticamente para el control de rutas oncogénicas en células de cáncer de mama [135].

14. Hormonas

Insulina. La señalización del factor de crecimiento similar a la insulina (IGF), está constituida por una red dinámica de proteínas que incluye los ligandos (IGF-I e IGF-II) y sus receptores asociados, proteínas de unión a IGF (IGFBP) y proteasas de IGFBP [136]. La proteína IGF-1, ha estado más fuertemente implicada en la progresión del cáncer de mama debido a su efecto mitogénico y antiapoptótico sobre las células epiteliales mamarias [137]. El IGF-I puede actuar de forma endocrina, paracrina o autocrina. Por consiguiente, los niveles y la actividad de IGF-1 se han examinado de cerca en tejidos proliferativos, para estudiar su relación con los cambios en la morfología celular asociados con la progresión del cáncer. La insulina puede estimular la síntesis del factor de crecimiento similar a la insulina 1 (IGF-1), y ambos tienen potentes efectos mitogénicos sobre las células tumorales. La insulina y el IGF-1 pueden activar las vías PI3K/Akt/mTOR y Ras/Raf/MAPK, estimulando así el crecimiento tumoral [138]. Estos productos génicos dan como resultado la activación ex-

tracelular de MMP. Las MMP junto a la MEC pueden activar a TGF-β1. El TGF-β1 aumenta la producción de la matríz extracelular, como la fibronectina, como parte de su papel en la inducción de TEM**.** Por otra parte, la IGFBP-3 inducida por TGFβ puede actuar como un promotor tumoral en presencia de fibronectina, ya que podría mediar en la actividad pro-tumorigénica de TGFβ para inducir TEM [139]**.** En ratones inmunosuprimidos, las células epiteliales mamarias humanas sobreexpresaron IGF-IR, que se asoció con el inicio de TEM, mediante la regulación positiva de Snail y la regulación negativa de caderina-E [140]. En base a la sobreexpresión del IGF-IR, se asocia con un fenotipo agresivo en una variedad de tumores [141].

Leptina. Es pro-tumorigénica, actúa sobre las células epiteliales de mama promoviendo la proliferación a través de la señalización de estrógenos, la activación de MAPK y STAT3, y la inhibición de la apoptosis mediante la activación de AKT [142]. Además, es pro-angiogénica e induce la TEM en el cáncer de mama, además promueve la auto-renovación tanto de las CMC como del cáncer de mama [142]. La leptina participa en la señalización para la activación de un circuito proinflamatorio que promueve la progresión del cáncer. Se ha demostrado que la interferencia entre IL-1, leptina y Notch promueve la proliferación, la migración y la regulación positiva del factor de crecimiento endotelial vascular y del receptor 2 en el cáncer de mama [143]. Wang y col. en 2015, señalaron que la leptina promueve la TEM en las células del cáncer de mama a través de la regulación positiva de IL-8, mediante la activación de la vía de señalización PI3K/Akt [144]. La leptina también promueve la migración e invasión de las células del epitelio mamario mediante la activación de un complejo regulador formado por las cinasas FAK y Src, que favorecen la expresión de las proteínas relacionadas con la formación de estructuras proteolíticas, implicadas en la invasión y progresión del cáncer. Tizapa y col. en 2019, señalaron que la sobreexpresión y activación de la proteína Hic-5 durante la TEM, favorece la formación de invadopodios, promoviendo la degradación de los componentes de la MEC e induciendo la metástasis del cáncer [145].

Adiponectina. Tiene un papel importante en el metabolismo de la glucosa, los lípidos, la sensibilidad a la insulina, la angiogénesis, la inmunidad, la inflamación y el cáncer [146]. La adiponectina inhibe la proliferación de células de cáncer de mama con receptores estrógenos negativos, a través de la señalización de MAPK y AKT, promoviendo la apoptosis y regulando negativamente las propiedades metastásicas, al inhibir la señalización de mTOR mediante la activación del complejo enzimático (AMPK), mientras que a bajas concentraciones podría promover el crecimiento y la progresión del tumor en cáncer de mama con receptores de estrógenos positivos [147,148]. Funciona tambien como potencial supresor de tumores, inhibiendo la TEM, pero es frecuentemente silenciada en el cáncer de próstata por hipermetilación del promotor [149]. Existen estudios que han demostrado la asociación inversa entre la adiponectina y el riesgo de cáncer de próstata o cáncer de próstata de grado alto [149,150]. Así mismo, desempeña un papel en el bloqueo de la carcinogénesis, mediante la inhibición de la proliferación y la promoción de la apoptosis, e inhibe el VEGFα, evitando así, la neovascularización del cáncer [151]. La adiponectina provoca la detención del ciclo celular de las líneas de células del estroma y del epitelio prostático e induce la apoptosis aumentando la caspasa-3 y regulando negativamente el gen Bcl2 (linfoma de células B) [152]. En el cáncer de pulmón, la adiponectina puede ejercer un efecto antiproliferativo a través de la regulación por disminución de CREB (proteína involucrada en la tumorigénesis y significa: proteína de unión al elemento de respuesta a AMPc). Se demostró que las concentraciones fisiológicas disminuían significativamente la proliferación celular del adenocarcinoma de pulmón humano [153]. En un estudio de cáncer de

tiroides, Dossus y col. en 2018, detectaron bajos niveles de adiponectina circulante, que generalmente acompaña a la obesidad, y que se asocian con un mayor riesgo de cáncer de tiroides [154]. Porcile C y col. en 2014, demostraron que la adiponectina inhibía la proliferación celular de las líneas celulares de glioblastomas humanos, al inducir la detención del crecimiento dentro de la fase G1. Además, observaron que regulaba negativamente la acción del factor de crecimiento similar a IGF-1, aboliendo la proliferación inducida por IGF-1 de las líneas celulares de glioblastoma [155].

Estrógeno. El estrógeno E2 (estradiol) induce la TEM. Durante la progresión del tumor, el estrógeno puede fomentar la motilidad celular y la invasión del cáncer de mama ER positivo al promover la TEM [156]. La señalización ERα puede regular la caderina-E y TEM a través de Slug [157]. El ERβ (receptor de estrógeno β) inhibe TEM en el cáncer de próstata desestabilizando el factor de transcripción inducible por hipoxia e inhibiendo la localización nuclear de Snail, mediada por el factor de crecimiento endotelial vascular [158]. El Midkine (MK), factor de crecimiento que se une a la heparina, es capaz de ejercer actividades como la proliferación celular, la migración celular y la angiogénesis, también puede inducir TEM a través de la señalización Notch2/Jak2-Stat3(citoquinas) en queratinocitos humanos [159] e impulsar la TEM en las células de cáncer de páncreas, mediante la activación de la señalización Notch [160]. EL MK también se induce fuertemente durante la oncogénesis, la inflamación y la reparación de tejidos inducida por E2. En las líneas celulares de adenocarcinoma de pulmón LTEP-a2 and A549, E2, regula el incremento de la expresión de MK. Así mismo, E2 indujo el reclutamiento de ERβ como elemento de la respuesta a estrógenos (ERE) en el promotor MK. La transcripción de MK inducida por E2 fue mediada principalmente por ERβ, lo que sugiere que la MK inducida por E2 juega un papel importante en la progresión de la TEM. Resultados similares de los niveles de proteínas relacionados con E2, MK y TEM fueron obtenidos en tejidos clínicos del adenocarcinoma de pulmón [161].

Testosterona. En el cáncer de vejiga, la expresión del receptor de andrógenos (RA), es más elevada, además la expresión de RA aumenta con la etapa del tumor, especialmente en el tejido del cáncer metastásico, lo que señala que RA representa un factor crítico en el desarrollo del cáncer de vejiga [162]. Se demostró que TEM desempeña un papel vital en la metástasis del carcinoma urotelial, relacionado a través de VEGF, EGF y TGF-β [163]. El TGF-β puede regular RA para activar la TEM, proporcionando evidencias para que RA pueda considerarse como una terapia contra el cáncer urotelial. La acción de los andrógenos se ejerce a través del eje que implica la síntesis testicular de testosterona, su transporte a los tejidos diana y su conversión por la 5-reductasa en el metabolito activo 5-dihidrotestosterona (DHT). Los andrógenos ejercen sus efectos biológicos al unirse al RA e inducir su actividad transcripcional. Peng y col. en 2006, demostraron que la inactivación de la expresión de RA en las células T24 por micro-ARN inhibió la capacidad de migración de estas células. Esto puede estar asociado con una disminución de las concentraciones de MMP-9, que resultó del silenciamiento del gen RA. Las MMP promueven la progresión del cáncer y la metástasis al impulsar el crecimiento, la migración, la invasión y la angiogénesis [164]. Por otra parte, los andrógenos estimulan la expresión de MMP-9 en células de cáncer de próstata dependientes de andrógenos y la eliminación de RA suprimió las propiedades invasivas de estas células, debido a la disminución de la expresión de MMP-9 [165]. Jitao y col. en 2014, utilizando la línea celular de vejiga T24, determinaron que el silenciamiento de RA por micro-ARN podría suprimir la progresión del cáncer de vejiga de manera significativa tanto *in vitro* como *in vivo*. Realizaron estudios de silenciamiento para regular la expresión del RA y determinar la participación de RA en TEM, en pre-

sencia o ausencia de un RA-micro-RNA. Los resultados demostraron que RA indujo el patrón TEM en las células tumorales de vejiga y condujo a cambios significativos en la migración de células cancerosas de vejiga y en el potencial de invasión. La supresión de los niveles de expresión de RA, se correlacionó con la TEM mediada por andrógenos en las células T24, y en las cuales TGF-β puede desempeñar un papel importante, por lo que RA podría tener un gran potencial en la terapia del cáncer de vejiga [166]. Se ha identificado al RA como un represor de caderina-E y podría activar la TEM en células tumorales y la metástasis en el cáncer de mama [167,168]. Por otra parte, los andrógenos podrían inducir las características de TEM y la reorganización del citoesqueleto a través de la interacción con la señalización de TGF-β, lo cual podría contribuir al comportamiento metastásico del cáncer de próstata resistente a la castración [169]. Además, se demostró que existía una regulación recíproca entre RA y Slug en células de cáncer de próstata, lo que indica un papel importante de la señalización RA en TEM, y que Slug es un gen regulado por andrógenos en las células de cáncer de próstata [170]. En base a la señalización de RA en cáncer de vejiga, y el cual representa un factor crítico para TEM y metástasis, Jing y col. en 2014, en estudios tanto "*in vitro*" como "*in vivo*", demostraron que los andrógenos regulan positivamente la expresión de Slug e inducen la TEM en células de cáncer de vejiga RA-positivas, a través de la activación de la vía Wnt/β-catenina. Así mismo, además de Slug, se determinó la expresión de Snail, Twist y ZEB-1 con el tratamiento de DHT en células cancerosas de vejiga RA-positivas. Los resultados mostraron una regulación positiva significativa para Slug, pero no de los otros factores de transcripción. Esto es consistente con el hallazgo de que la expresión de RA en los tejidos del cáncer de vejiga, muestra una alta correlación con los niveles de expresión de Slug [171]. Slug es un miembro de la familia Snail de factores de transcripción de dedos de zinc, que ha sido identificado como un potencial oncogén en varios tipos de tumores, y que es capaz de reprimir la expresión de caderina-E y activar la TEM [172].

CONCLUSIÓN

Es fundamental que los inductores de TEM se mantengan en silencio para mantener la homeostasis y la integridad del epitelio. La aparición de TEM puede considerarse como la reactivación de programas del desarrollo similares que operan a nivel celular, aunque en este caso en el cáncer, genera consecuencias fatales. Es importante precisar cuáles son las señales micro ambientales inductoras de TEM, que permiten cambios en las células, que las hacen sensibles a tales señales, y determinar los mecanismos de señalización dentro de las células epiteliales que ordenan a los diversos programas de TEM. Obtenido este conocimiento, puede ser trasladado a la práctica clínica a través de la oncología de precisión que se basa en analizar el perfil genético, clínico y molecular del paciente, para posteriormente poder definir un tratamiento personalizado, con las mayores posibilidades de éxito, contribuyendo de esta forma a mejorar su calidad de vida y su supervivencia.

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Aspirin in primary cardiovascular prevention: the two faces of the coin and the importance of the Number Needed to Treat: a systematic review and meta-analysis.

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Keywords*:* aspirin; cardiovascular disease; primary prevention; bleeding risk; number needed to treat.

Abstract. Aspirin has been an essential treatment for the primary prevention of cardiovascular diseases (CVD). Several randomized controlled studies do not support the routine use of aspirin, mainly due to its association with bleeding risk. This systematic review aims to advocate aspirin prescription based on the Number Needed to Treat (NNT) and the Number Needed to Harm (NNH). This combination provides a good measure of the effort to avoid an unfavorable outcome, weighed against possible associated risks. A search of randomized studies on aspirin treatment was conducted in two separate periods. Four studies from 1988-1998 and six from 2001-2018 were included in the analysis (157,060 participants). The primary endpoint was a composite outcome of Non-fatal Myocardial Infarction (NFMI), Non-fatal Ischemic Stroke (NFIS), and CV mortality. Major bleeding was a safety endpoint. We calculated the Absolute Risk Reduction (ARR%), NNT, and NNH, alongside the Relative Risk (RR) and 95% CI of each primary endpoint. The results of all included studies (10) showed a net benefit with aspirin treatment for NFMI (NNT= 259) and the composite outcome (NNT=292) with a significant relative risk reduction of 20% ($p=0.003$; $I^2= 0\%$) and 10% ($p<0.001$; $I^2= 0\%$), respectively. There was a relevant 60% increase in the bleeding risk ($p<0.0001$, NNH=208; $I^2= 3\%$). The NNT and NNH may constitute measures of efficacy and risk in clinical shared decision-making. However, it is essential to consistently establish that patients' benefit-risk should be individualized and not represent a clinical guide for everyone.

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Aspirina en prevención cardiovascular primaria. Las dos caras de la moneda y la importancia del número necesario a tratar. Revisión sistemática y metanálisis.

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Palabras clave: aspirina; enfermedad cardiovascular; prevención primaria; riesgo hemorrágico; número necesario a tratar.

Resumen. La aspirina ha sido un tratamiento esencial para la prevención primaria de las enfermedades cardiovasculares (ECV). Varios estudios controlados aleatorizados no apoyan el uso rutinario de la aspirina principalmente debido a su asociación con el riesgo de sangrado. Esta revisión sistemática tiene como objetivo evaluar la prescripción de aspirina basada en el Número Necesario para Tratar (NNT) y el Número Necesario para Dañar (NNH). Esta combinación proporciona una buena medida del esfuerzo para evitar un resultado desfavorable, sopesado frente a los posibles riesgos asociados. Se realizó una búsqueda de estudios aleatorios sobre el tratamiento con aspirina en dos períodos separados. En el análisis se incluyeron cuatro estudios de 1988 a 1998 y seis de 2001 a 2018 (157 060 participantes). El criterio principal de valoración fue un resultado compuesto de infarto de miocardio no mortal (NFMI), accidente cerebrovascular isquémico no mortal (NFIS) y mortalidad cardiovascular. La hemorragia mayor fue el punto final de seguridad. Se calculó la reducción del riesgo absoluto (RAR), el NNT y el NNH, junto con el riesgo relativo (RR) y el IC del 95% de cada criterio principal de valoración. Los resultados de todos los estudios incluidos (10) mostraron un beneficio neto con el tratamiento con aspirina para NFMI (NNT= 383) y el resultado compuesto (NNT=445) con una reducción significativa del riesgo relativo del 20% (p=0,003; I^2= 0%) y 10% (p<0,001; I^2= 0%), respectivamente. Hubo un incremento relevante del 60% en el riesgo de sangrado (p<0,0001, NNH=208; I^2= 3%). El NNT y el NNH pueden constituir medidas de eficacia y riesgo en la toma de decisiones clínicas compartidas. Sin embargo, es importante establecer consistentemente que el riesgo-beneficio de los pacientes debe ser individualizado, y no una guía clínica para todos.

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INTRODUCTION

More than 30 years have passed since the Physician´s Health Study was published. This painstaking work demonstrated a 44% risk reduction of myocardial infarction (MI) with aspirin (RR: 0.56; 95 %CI, 0.45 to 0.70; p<0.0001) [1]. This effect was more pronounced in the group of individuals older than 50 years, while the presence of ulcer and transfusion demand as secondary events were not significant compared with the placebo group (RR: 1.22; 95%CI,0.98 to 1.53; p = 0.08 for ulcer). The conclusion of this work was a recommendation for the use of aspirin in the primary prevention of a first MI in healthy individuals. However, the US Food and Drug Administration (FDA) did not approve the professional labeling of aspirin for the prevention of MI because another

similar trial, The British Doctor´s Trial, did not show benefit from aspirin administration for cardiovascular prevention [2]. In 2003, the use of aspirin was updated in the primary prevention of cardiovascular disease [3]. In addition to the two mentioned trials, three more trials were also analyzed: The Thrombosis Prevention Trial [4], The Hypertension Optimal Treatment Study [5], and The Primary Prevention Project [6], including 55.580 randomized participants (11.466 women). The studies mentioned above revealed a 32% reduction in the risk of a first MI and a 15% reduction in the risk of all important vascular events following aspirin´s treatment, demonstrating strong evidence of the use of aspirin in the primary prevention of MI. At that time, the US Preventive Services Task Force (USPTF) [7] and the American Heart Association recommended aspirin for men and women whose 10-year risk of a first coronary event was 10% or greater [8] since the benefits of a reduction of cardiovascular (CV) events outweighed the risks in most of the patients presenting this sort of cardiovascular risk.

Additionally, there are gender-specific differences in platelet function and response to aspirin [9,10]. Women under 65 years old without known CVD have a minor response to aspirin therapy in the primary prevention of coronary artery disease [11]; the dose recommended was 81-100 mg every other day [12].

The net benefits for persons who have started taking aspirin continue accumulating over time without a bleeding event. The net benefits, however, generally become progressively smaller with advancing age because of an increased risk for bleeding, and modeling data suggest that it may be reasonable to consider stopping aspirin use at around age 75 [13] for primary prevention. Despite this, there is a gap in understanding the benefits and risks of giving aspirin to patients at moderate risk of CVD. Aspirin has been a primary preventive drug for cardiovascular and cerebrovascular diseases for years. What has happened recently to change the concept and the prescription for the use of aspirin in the primary prevention of CVD? Today, the use of aspirin for primary prevention has been a subject of debate. Based on well-conducted studies, organizations such as the European Society of Cardiology, European Association for Cardiovascular Prevention & Rehabilitation, and the USPSTF, have delivered an almost uniform verdict that substantially changed the aspirin prescription for primary CV prevention [13,14].

The Number Needed to Treat (NNT), calculated as the reciprocal of the absolute risk reduction percentage, is a concise, clinically useful parameter that provides quantitative information on the efficacy of therapeutic interventions. Moreover, NNT allows clinicians to understand how much effort is needed to prevent a given event. The NNT and its opposite, the Number Needed to Harm (NNH), can be helpful in medical decision-making, then the use of NNT or NNH could be the likelihood of obtaining a benefit or harm [15]. This systematic review aims to study the effect of aspirin in the primary prevention of CVD, under the scope of fundamental trials that have been an essential guide in the prescription or not of aspirin throughout decades till nowadays. Based on this premise, we believe that using the NNT and NNH could guide physicians in deciding whether aspirin could be prescribed to prevent primary CV events.

METHODOLOGY

Search Strategy

The present review exclusively focuses on aspirin as a preventive drug for primary CVD treatment. The search was divided into two periods to differentiate the times when the aspirin prescription was relevant (from 1988 to 1999, Table 1a) to the one where aspirin was questioned for primary prevention (from 2000 to 2018, Table 1b). The later period has been considered the modern era of cardiovascular primary prevention [16].

Table 1a.
Features of randomized controlled studies on the aspirin primary prevention in cardiovascular diseases (1989-1999).

Study	Number of individuals/ follow-up (years)	Intervention design	Primary events	Secondary events	Outcomes in primary events	Outcomes in secondary events:
The British Doctor´s Trial (1988)[2]	5139 (3429 vs 1710) 6	Aspirin 500 mg/d/ vs no aspirin	NFMI:	Not specified	NFMI: 1.03 (0.71-1.49), p=0,96. Stroke: 1.377 (0.72-2.60), p=0.40	Not specified
Physician´s health study (PHS)(1989)[1]	22071 (11037 vs 11034)/ 5	Aspirin 325 mg/beta-carotene every other day vs placebo	NFMI, non-fatal stroke, and death for CVD	Ulcer, hemorrhage of any cause, and transfusion	For non-fatal MI: 0.56 (CI:0.45-0.70), p<0,0001. For Stroke: 1.22 (0.93-1.60), p=0.15. For CV Mortality: 0.96 (0.60-1.54), p=0.87	Hemorrhage of any cause: 2979 (27%) in aspirin group vs 2248 (20,3%) RR=1.32 (1.25-1.40). p<0,0001
The Thrombosis Prevention Trial (TPT) (1998)[4]	5085 (2545 vs 2540)/ 6	Aspirin 75 mg/d vs placebo	Ischemic Heart Disease: Coronary death, fatal and NFMI	Major, intermediate and minor hemorrhage	For NFMI: 0.69 (0.53-0.89), p<0,0049. For fatal MI: 1.13 (0.78- 1.63), p= 0,57. For coronary death: 0.81 (0.66-0.99), p<0,048	Hemorrhage of any cause: 1.24 (1.12-1.37), p<0.0001. Major bleeding: 1.52 (1.01-2.28), p<0.055. Intermediate: 2,00 (0.61-6.65),p=0.38
The Hypertension Optimal Treatment (HOT) Study(1998)[5]	18790 (9399 vs 9391)/ 4	Aspirin 75 mg/d vs placebo	Major CV events: fatal and NFMI, stroke, and other CV deaths	Major fatal, non-fatal hemorrhage, and minor hemorrhage	Major CV events, 0·85 (0·73–0·99), p<0.03. Fatal and NFMI: 0·64 (0·49–0·85) p<0,002. Stroke: 0·98 (0·78–1·24), p=0.88 CV death: 0·95 (0·75–1·20), p= 0.65	Any type of hemorrhage: 1.74 (1.44-2.09), p<0,0001. Major hemorrhage: 1.84 (1.38-2.46), p<0,0001

NFMI: Non-fatal Myocardial Infarction; CV: Cardiovascular; CVD: Cardiovascular disease.

Table 1b
Features of randomized controlled studies on the aspirin primary prevention in cardiovascular diseases (2001-2018).

Study	Number of individuals/ follow up (years)	Intervention design	Primary events	Secondary events	Outcomes in primary events :	Outcomes in secondary events:
The Primary Prevention Project (PPP) (2001)[6]	4495 (2226 vs 2269)/ 3.6	Aspirin 100 mg/ d/vitamin E vs no aspirin	CV Death, NFMI, and non-fatal stroke.	Major fatal /non-fatal hemorrhage	CV deaths: 0·56 (0·31–1.00), p=0.06. All MI: 0·69 (0·38–1·23), p=0,27. Stroke: 0·67 (0·36–1·27), p=0,29	Any type of hemorrhage: 4.07 (1.67-9.96), p<0,0015 Major hemorrhage: 1.74 (1.32-2.30), p<0,0001
The Primary Prevention of Cardiovascular Disease in Women (WHS) (2005) [19]	39876 (19934 vs 19942) 10	Aspirin 100mg every other day vs placebo Patients; >45 year old	NFMI, non-fatal stroke, or death from cardiovascular causes	GI bleeding requiring transfusion	Major CV events: (RR, 0.91; (0.80 to 1.03); p=0.13. Stroke: RR;0.83; (0.69-0.99); p=0.04). Fatal or NFMI: RR: 1.02; (0.84-1.25); p=0.83, or death from cardiovascular causes RR 0.95; (0.74-1.22); p=0.68)	Major bleeding: RR, 1.40; (1.07- 1.83); p=0.02
The Japanese Primary Prevention Project (JPPP) (2014)[20]	14 464 (7220 vs 7244)/ 6.5	Aspirin 100mg daily vs no aspirin	Composite primary outcome was death from CV causes (MI, stroke, and other CV causes), non-fatal stroke (ischemic or hemorrhagic, including undefined cerebrovascular events), and NFMI	Secondary outcomes included in divide endpoints.	Composite outcome: HR:0.94 (0.77-1.15); p= 0.54. NFMI: HR:0.53 (0.31-0.91), p= 0.02. TIA:HR: 0.57 (0.32-0.99);p =0.04	Extracranial (major) hemorrhage: HR:1.85 (1.22-2.81); p = 0.004).

Table 1b.
CONTINUACIÓN

Study	Number of individuals/ follow up (years)	Intervention design	Primary events	Secondary events	Outcomes in primary events :	Outcomes in secondary events:
The ASPREE Trial (2018)[21]	19114 (9525 vs 9589)/ 4.7	Aspirin 100mg daily vs placebo	Endpoints included major hemorrhage and cardiovascular disease (fatal coronary heart disease, NFMI, fatal or non-fatal stroke, or hospitalization for heart failure).	Major Hemorrhage as secondary endpoint	For CVD: HR: 0.95; (0.83-1.08), p=NS. Fatal, NFMI: HR:0.93 (0.76-1.15). Stroke: HR: 0.89 (0.71-1.11)	Major Hemorrhage: HR: 1.38; (1.18-1.62); p<0.001
The ARRIVE Trial (2018)[22]	12546 (6270 vs 6276)/ 5 years	Aspirin vs placebo Eligible patients were aged 55 years (men) or 60 years (women) and older and had an average cardiovascular risk	The primary efficacy endpoint was a composite outcome of cardiovascular death, MI, unstable angina, stroke, or TIA.	Safety endpoints were hemorrhagic events and incidence of other adverse events	HR:0·96; (0·81–1·13), p=0·6038).; NFMI: HR 0·90, (0·67–1·20); p=0·4562	
The ASCEND Trial (2018)[23]	15,480 (7740 vs 7740)/ 7.4	Aspirin 100mg vs placebo in diabetic patients	The primary efficacy outcome (MI., stroke or TIA, or death from any vascular cause, excluding any confirmed intracranial hemorrhage)	The primary safety outcome was the first major bleeding event	Serious vascular events: 0.88; (0.79-0.97), p=0.01. NFMI: 0.98 (0.80–1.19). Stroke: 0.88 (0.73–1.06)	Major bleeding events:1.29 (1.09 -1.52); p=0.003

NFMI: Non-Fatal Myocardial Infarction; CV: Cardiovascular; TIA: Transient ischemic attack; HR: Hazard ratio; RR: Relative risk.

The bibliography search was conducted in PUBMED by MEDLINE and Google Scholar under the following MESH (Medical Subject Headings) terminology: *aspirin in primary prevention, aspirin in myocardial infarction, aspirin in stroke, aspirin in cardiovascular death or mortality, aspirin in bleeding or hemorrhage; those terms were connected thru a Boolean "and" with randomized controlled trials* (Fig. 1). Additionally, the term *number needed to treat* was used for the complementary bibliography. The primary endpoint to report was a composite of non-fatal myocardial infarction (NFMI), non-fatal ischemic stroke (NFIS), and cardiovascular mortality (CVM). The primary bleeding outcome was major bleeding, as stated by the studies. The exclusion criteria were: a) studies with less than 4000 participants, b) systematic reviews on aspirin treatment because there are good reviews about it [16-18], c) a combination of antiplatelet or anticoagulants treatments with aspirin (The Thrombosis Prevention Trial assessed warfarin and aspirin alone and in combination but data for participants who received warfarin were excluded from the analysis), d) duplicate publications and e) those works that do not contain the composite as the endpoint.

Finally, four studies from 1988-1998 [1,2,4,5] and six studies from 2001-2018 [6,19-23] were included in the analysis (Tables 1a and 1b). This article has been assessed according to the PRISMA 2020 statement and checklist [24]. The bias risk in each study was assumed according to a systematic review published previously [16], following the Cochrane risk of bias assessment, and the Jadad scale was used to evaluate the quality of the randomized controlled studies (< 3: high risk of bias, ≥ 3: low risk of bias) [25]. Ethical approval was not required for conducting this study.

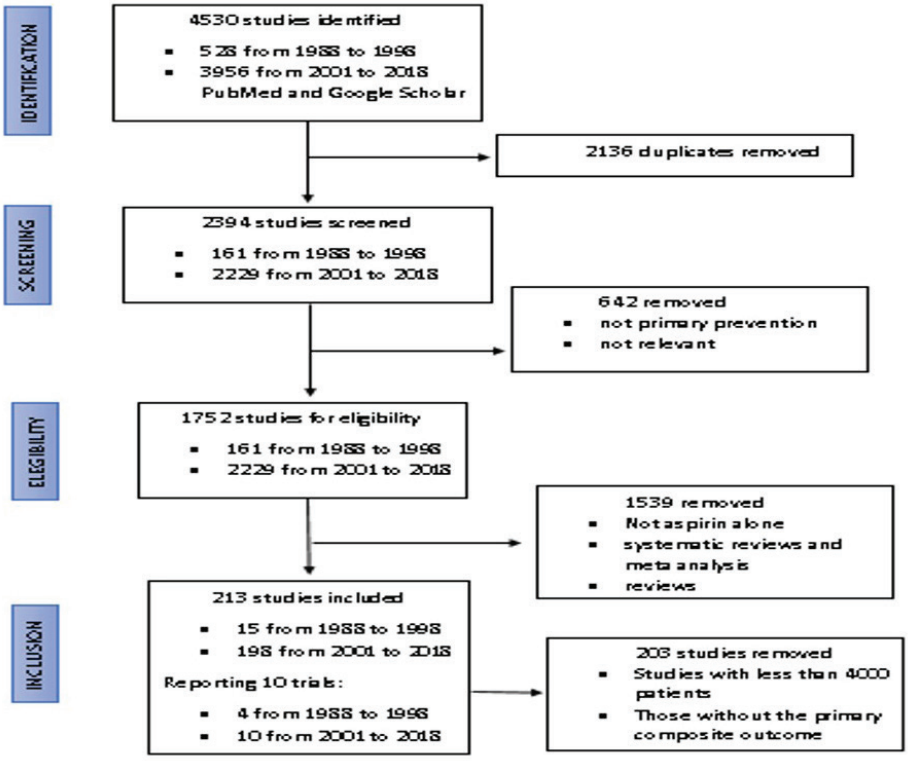

Fig. 1. Flow chart of literature search strategy according to the MESH terminology. Note: some data might be lost in the early stages of the search.

Statistical approach

NNT and NNH

NNT or NNH would be the number of patients to be treated to obtain one benefit or one harm in a predefined period [26]. The number needed to treat is simply the reciprocal of the absolute risk difference obtained from the percentage of events in the control group minus the percentage of events in the experimental group (also named as absolute difference). Depending on the treatment, when the difference in the two groups proportions is significant, the NNT is small, and vice versa. NNT is a concise, clinically helpful presentation of the effect of an intervention. The NNT and the NNH are calculated as the inverse of the absolute reduction (ARR) or absolute increment of the risk (ARI). A 95% CI for NNT can be constructed by simply inverting and exchanging the limits of a 95% CI from the ARR. When the result shows an ARR or ARI with 95% CI extended from negative to positive values, it means that the zero is included, and the NNT is infinity thus, we need to separate two intervals using via infinity (∞) and indicate that the treatment may be helpful or harmful [27].

As Altman has mentioned [27], the terms NNT and NNH may not be appropriate to denote benefit and harm. He proposes the abbreviations of NNTB (benefit) and NNTH (harm). However, we maintain the conventional abbreviations of NNH and NNT to refer to benefit or harm, respectively. We also constructed an arbitrary classification of the NNT or NNH effect as follow: Net benefit, Uncertain benefit (treatment could be harmful), Uncertain harm (treatment could be helpful), and net harm.

Because the included studies have different follow-up periods, we have to make assumptions about it and make a "time adjustment" because if we want to be able to compare these NNTs, it is necessary to adjust all of them to refer to the same tracking time. So it is necessary to uniform the time of these studies to obtain the same relative interpretation of the results regarding benefit and harm. For this purpose, we have to use the following formula:[28,29]

$$NNT_{(hypothetic)} \div Time_{(hypothetic)} = NNT_{(observed)} \div Time_{(observed)}$$

Rearranging the terms, we have;

$$NNT_{(hypothetic)} = NNT_{(observed)} \times [Time_{(hypothetic)} / Time_{(observed)}]$$

In the present study, we have adjusted five years as a hypothetical time for all the included studies to calculate NNT and NNH. We used a computer program for ARR, NNT, and NNH to obtain the data and their 95% CI (https://www.graphpad.com/quickcalcs/NNT1/). Additionally, as an effect measure, a Relative Risk and its 95% CI were estimated using the Comprehensive Meta-Analysis program (Biostat, Englewood, NJ) alongside the relative weight and random effect. As statistical parameters, the consistence for heterogeneity (I^2) was determined as low (<25%), moderate (25% to 75%,), and high (>75%) by testing the chi-square calculation of each meta-analysis (Cochrane Q) according to the Higgins formula[30]; and statistical significance was fixed as $p<0.05$.

The forest plot for meta-analysis of each primary effective endpoint and safety was constructed under NNT and NNH parameters, with all included studies performing a logarithmic scale with infinity value in the middle of the scale according to previous reference[30], the NNT 95%CI (benefit) values are shown to the left and NNH 95% CI (harm) values on the right with the overall estimate.

RESULTS

Table 2a shows the total results of the endpoints in the trials made in the last year of the 20th century (four trials with a total of 51,085 participants), with a net benefit for NFMI (NNT= 156) confirmed by a significant relative risk reduction of 31% [RR,95%CI: 0.69(0.61-0.79); p<0,0001; I^2= 10.5%].

Table 2a. Summarized results of the endpoints in the included studies, period 1988-1998 (n= 4).

ENDPOINTS	Aspirin events (%) n: 26410	No Aspirin events (%) n:24675	RR(95%CI), p value; I^2	ARR% (95%CI)	ARI% (95%CI)	NNT (95%CI)	NNH (95%CI)*	Observations
NFMI	385 (1.46)	518 (2.10)	0.69 (0.61-0.79) <0.0001; 10.5%	0.64 (0.41-0.87)		156 (115 - 244)		Net benefit of aspirin treatment
NFIS	338 (1.28)	300 (1.22)	1.02 (0.83-1.25) 0.87; 11.5%		0.06 (-0.13 to 0.26)		1667 (770 to ∞ to 390)	Uncertain harm (aspirin could be helpful)
CV Mortality	463 (1.75)	383 (1.55)	1.12 (0.99-1.30) 0.08 ; 0%		0.20 (-0.02 to 0.42)		500 (4986 to ∞ to 237)	Uncertain harm (aspirin could be helpful)
Composite Outcome	1186 (4.49)	1201 (4.87)	0.92 (0.85-0.99) 0.046; 0%	0.38 (0.01-0.74)		266 (135 - 10158)		Net benefit of aspirin treatment
Major bleeding	205 (0.78)	109 (0.44)	1.79 (1.42-2.26) <0.0001; 0%		0.33 (0.20 -0.47)		299 (213 - 500)	Net harm of aspirin treatment

The composite outcome shows an NNT of 266 and an 8% relative risk reduction [RR,95%CI: 0.92(0.85-0.99); p=0.046; I^2= 0%]. The major bleeding revealed a 79% increase in the relative risk [RR,95%CI; 1.79(1.42-2.26); p<0.0001; I^2= 0%, NNH=299;].

Table 2b points out the same elements of the previous table with 105,975 participants and six trials made in two decades of the 21st century. The result of the studies carried out showed a benefit with the treatment for NFIS (NNT= 553) with a 12% in relative risk reduction [RR,95%CI: 0.88 (0.80-0.98); p<0.01; I^2= 0%]. The composite outcome presented an NNT of 288 with a significant relative risk reduction of 9% [RR,95%CI: 0.91 (0.86-0.97); p<0.003; I^2= 0%], and for major bleeding, there was a 57% increase of the relative risk [RR,95%CI: 1.57 (1.30-1.91); p<0.0001; I^2= 0%, NNH= 175;].

The total of the results in the combined and separate primary endpoints of all studies are shown in Table 2c. From a total of 157,060 participants, there was a net benefit of 20% and 10% on the relative risk reduction for NFMI [RR= 0.80 (0.69 to 0.93); p=0.003; I^2= 0%; NNT= 259] and Composite outcome [RR= 0.90 (0.85 to 0.99); p<0.001; I^2= 0%; NNT= 292] respectively. Major bleeding presents a 60% increase in the relative risk [RR: 1.60 (1.38 to 1.85); p<0.0001; I^2= 3%; NNH= 208]. The rest of the endpoints showed an uncertain result because the aspirin treatment could be harmful or helpful compared with the control.

Forest plots of the meta-analysis of aspirin treatment in the total included studies are shown in terms of NNT (benefit) or NNH (harm) for each primary endpoint.

Table 2b. Summarized results of the endpoints in the included studies, period 2001-2018 (n= 6).

ENDPOINTS	Aspirin events (%) n: 52915	No Aspirin events (%) n: 53060	RR (95%CI) p value; I^2	ARR% (95%CI) r	ARI% (95%CI)	NNT (95%CI)	NNH (95%CI)	Observations
NFMI	669 (1.26)	718 (1.35)	0.93 (0.82-1.05) 0.21; 9.6%	0.09 (-0.05 to 0.23)		1112 (435 to ∞ to2086)		Uncertain benefit (aspirin could be harmful)
NFIS	721 (1.36)	819 (1.54)	0.88 (0.80-0.98) <0.01; 0%	0.18 (0.04 -0.33)		553 (303- 2500)		Net benefit of aspirin treatment
CV Mortality	508 (0.96)	551 (1.04)	0.92 (0.82-1.04) 0.20; 0%	0.08 (-0.04 to 0.20)		1250 (505 to ∞ to 2419)		Uncertain benefit (aspirin could be harmful)
Composite Outcome	1898 (3.59)	2088 (3.94)	0.91 (0.86- 0.97) 0.003; 0%	0.35 (0.12 - 0.58)		288 (174- 839)		Net benefit of aspirin treatment
Major bleeding	964 (1.82)	662 (1.25)	1.57 (1.30-1.91) <0.0001; 0%		0.57 (0.43 -0.72)		175 (139- 233)	Net harm of aspirin treatment

The overall estimate points out that concerning aspirin treatment revealed a net benefit for NFMI (Fig. 2a), an uncertain benefit for NFIS (Fig. 2b), and uncertain harm for CV mortality (Fig. 2c). As we expected, net harm was associated with major bleeding (Fig. 2d)

DISCUSSION

The light of the results of this systematic review, we argued that aspirin has an ambiguous place in the prescription for primary CV prevention. NFMI from studies of the 20th century and NFIS in this century showed a net benefit with the aspirin treatment. In some instances, the treatment could be harmful, given negative ARR 95% CI values. The composite outcome results also reported benefits in the two groups of studies. Globally there was a net benefit of aspirin treatment for NFMI and the composite outcome but also a significant bleeding risk. This study demonstrates that the absolute risk reduction for cardiovascular events and absolute risk increase for major bleeding associated with aspirin use were of similar magnitude. In terms of NNT and NNH, this represents notable data on the aspirin prescription despite some evidence that indicates the efficacy of aspirin could be uncertain in the NNT/NNH ratio since all of the studies and the combined results have shown a relevant bleeding risk. These findings have similarities with a recent meta-analysis [31]. A systematic review [32] revealed an ARR of 0.41% in the composite cardiovascular outcome with an NNT of 241 and an ARI of 0.47% for major bleeding risk, representing an NNH of 210. This confirmed a possible adverse effect of aspirin due to bleeding risk. Another systematic review [33] showed a reduction of 10% of MCE (major cardiovascular events) (0.90, 95% CI 0.85-0.96, p<0.001) with an

Table 2c
Summarized results of the endpoints in all included studies (n=10).

ENDPOINTS	Aspirin events (%) n: 79325 n:	No aspirin events% n:77735	RR (95%CI) p value; I^2	ARR% (95%CI)	ARI% (95%CI)	NNT (95%CI)	NNH (95%CI)	Observations
NFMI	954 (1.20)	1236 (1.59)	0.80 (0.69-0.93) 0.003; 0%	0.39 (0.27-0.50)		259 (199-369)		Net benefit of aspirin treatment
NFIS	1059 (1.34)	1119 (1.44)	0.93 (0.83-1.01) 0.09; 0%	0.10 (-0.01 to 0.22)		958 (454 to ∞ to 8910)		Uncertain benefit (aspirin could be harmful)
CV. Mortality	971 (1.22)	934 (1.20)	0.96 (0.88-1.05) 0.39; 0%	0.02 (-0.09 to 0.13)			4433 (1167 to ∞ to 765)	Uncertain harm (aspirin could be helpful)
Composite Outcome	3084 (3.89)	3289 (4.23)	0.90 (0.85-0.99) <0.001; 0%	0.34 (0.15-0.54)		292 (186-676)		Net benefit of aspirin treatment
Major bleeding	1169 (1.47)	771(1.00)	1.60 (1.38-1.85) <0.0001;3%		0.48 (0.37-0.59)	208 (269-170)		Net harm of aspirin treatment

NFMI: Non-fatal Myocardial Infarction, NFIS: Non-fatal Ischemic Stroke, CV: Cardiovascular, RR: Relative Risk, ARR: Absolute Risk Reduction, ARI: Absolute Risk Increase, NNT: Number Needed to Treat, NNH: Number Needed to Harm, CI: Confidence Interval. Note: In the present study, each individual study was adjusted to 5 years a hypothetical time to calculate NNT and NNH: NNT(hypothetic) = NNT(observed) X [Time(hypothetic) / Time (observed)].

When the result shows an ARR with 95%CI positive values means a net benefit (NNT); when the result shows an ARI with 95%CI means net harm (NNH). When the result shows an ARR or ARI with 95%CI extended from negative to positive values means that the zero is included thus we need to separate two intervals using via infinity (∞) and indicates that the treatment may be helpful or harmful [27].

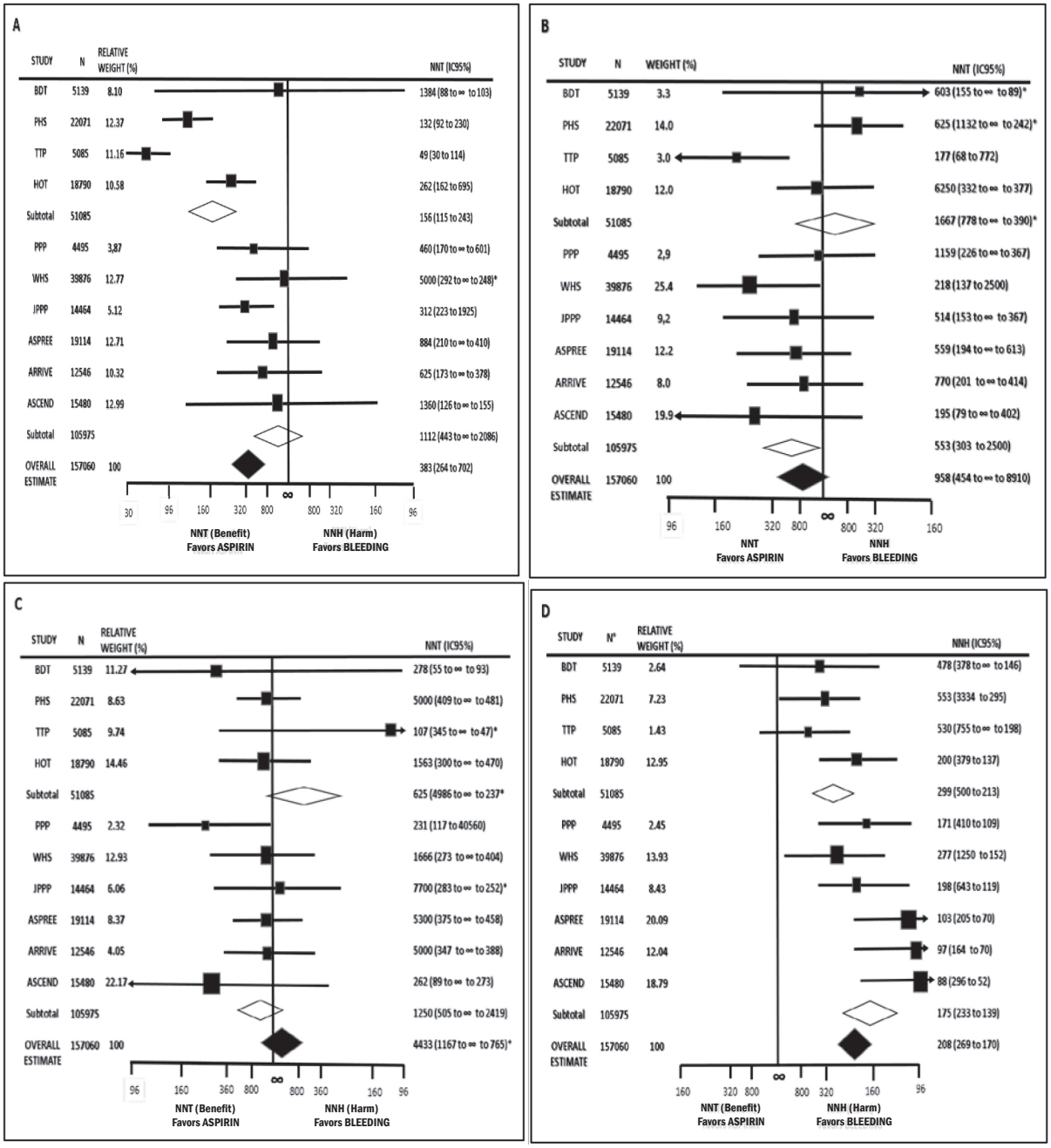

Fig. 2. Forest plot of meta-analysis of the included studies calculating from ARR95%CI as effect measure the NNT or NNH with their respective 95%CI. Non-fatal myocardial infarction (A), Non-fatal ischemic stroke (B), Cardiovascular mortality (C), and Major bleeding (D) as endpoints. *Denotes Absolute Risk Increase (ARI). When the ARR95%CI includes zero we need to separate two intervals using via infinity (∞) in the middle of the scale [27].

ARR of 0.39% (95%CI: 0.18-0.61), which correspond to NNT of 253 (95%CI: 163-568) to prevent one single MCE, and the NNH (major bleeding) was 306. These results are similar to our work, indicating potential harm in aspirin use due to increased bleeding risk. Abdelaziz *et al*. [34] showed in an illustration the NNT for MI (357), ischemic stroke (500), TIA (370), and MACE (263) with the NNH for major bleeding (222), hemorrhagic stroke (1000), and GI bleeding (385), based on pooled data from 15 randomized controlled trials. Therefore, it was deduced that bleeding risk is present when calculating the NNTs over NNH with major bleeding. These findings suggest that the decision to use aspirin for primary prevention should be tailored to the individual patient based on the estimated CV risk. Another approach to evaluate aspirin treatment in the primary prevention of CV events is to compare benefits (prevention of MI and stroke) and harms (major GI bleeds and hemorrhagic stroke) using relative weights in the assessment of systematic reviews of the NNT and NNH as the number of person-years with treatment need to prevent one adverse event [35]. This approach demonstrated a net benefit for aspirin; nevertheless, in the sensitivity analysis, aspirin was harmful due to greater relative weight for GI bleeds.

We tried to give a better scope of aspirin as a primary preventive treatment for CV events, but unfortunately, the controversy persists. To treat or not to treat with aspirin is the question, and this situation is under debate. To better understand this complex scenario, we follow the timing of the declarations of the USPSTF about the statements on aspirin prevention in CVD [7,36-38].

In this contemporary period, aspirin passed from being a good prescription for primary prevention of CVD at any age to a restriction for its use only in 40-59 years old individuals, with a strong recommendation against its use in older people. The last decision came from another six studies, whose results regarding the therapy with aspirin in primary prevention for CVD were unfavorable for its recommendation (Table 1b). Additionally, in an analysis of 17 RCT (164.862 participants) [39], aspirin did not show any significant reduction in all-cause mortality compared with placebo (RR:0.97;95%CI:0.93-1.01; p=0.13). However, when ≥ 65 years old patients were excluded, it significantly reduced all-cause mortality (RR 0.94; 95% CI 0.90–0.99; p = 0.01) in the aspirin group. These results concord with the age arguments recently expressed by the USPSTF. The European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice colleagues and other systematic reviews share the same opinion since its guidelines do not recommend aspirin for the primary prevention of CVD at all [14,40]. However, those agreements can lead to misinterpretation or confusion for patients in whom aspirin therapy may be essential: such as those on primary or secondary prevention with an established CV risk of more than 10% [41].

The only explanation for this change is attributed to the bleeding risk that increases with age. However, there is a debate about this new scenario, and doubts must be cleared. For example, should they stop taking aspirin for people who have been using aspirin for years and do not have evidence of bleeding? If yes, what could we probably expect? So we could expect an increase in CV events in this particular group unless there is an indication of another alternative surrogate for aspirin prescription.

A comment arises regarding the different conduct over time on aspirin prescription, during the last decades of the 20th century and then the change of opinion in the two decades of the 21st century. The first studies on aspirin and prevention of cardiovascular risk focused mainly on the significant relative reduction of the risk of myocardial infarction (44% PHS, 31% TPT, and 36% HOT, only the BDT showed a non-significant 3%). Perhaps this finding eluded the atten-

tion to the side effects of bleeding caused by the administration of aspirin, which was barely mentioned in those papers, but was not given its due importance, despite the relevant relative increase in the risk of major bleeding (71% PHS, 52% TPT, and 84% HOT). On the contrary, the trials that were performed in the two decades of the 21st century addressed with great interest the risk of bleeding with aspirin prescription in the prevention of CVD. For this reason, these papers highlighted the relevance of major hemorrhage as a contraindication to aspirin intake. They concluded that serious but no fatal bleeding [42] is frequent with aspirin administration compared with the benefit achieved in CV prevention. As a representative example, the myocardial infarction in terms of RRR (Relative Risk Reduction for efficacy) vs. major bleeding as RRI (Relative Risk Increase for safety) is shown as follows: PPP (31% vs. 74%), WHS (2% vs. 40%), JPPP (47% vs. 85%), ASPREE (7% vs. 38%), ASCEND (2% vs. 29%) and the ARRIVE (10% vs 110%), all of them favoring the bleeding risk. The different points of view above about aspirin treatment changed the opinion of doctors and their patients regarding the routine use of aspirin as a primary preventive drug in CV events.

Another point of view of this conflictive situation is that the prescription of aspirin is unnecessary in some instances because it is an over-a-counter (OTC) drug that can be freely purchased, and patients have been buying the drug without considering the potential bleeding risk [41].

Although there are relevant evidence and guidelines as instruments for shared decision-making to help clinicians in the use of aspirin in primary prevention [43-46] and the search for a benefit versus risk prediction tool, we propose the NNT and NNH as valuable measures in the balancing benefit-risk with aspirin in the therapy of the effective primary prevention of CV events. Knowing or estimating NNT and NNH for an individual patient's risk could be a guide for the overall or net value of a prophylactic intervention [47]. This combination provides a good measure of the effort in avoiding an unfavorable outcome, weighed against possible associated risks. The calculation of these measures is straightforward, and also its interpretation so that physicians could make an individual clinical decision based on the results of the interventions for CV primary prevention guided by the calculation of how many patients can be treated to avoid one adverse event, counterbalancing with the collateral side effects of the aspirin prescription. However, although the calculation of the NNT is simple, we need to consider the treatment time to ensure its correct interpretation [48]. An essential limitation of NNT and NNH is that these metrics are limited to dichotomous (rather than continuous) outcomes [16].

Limitations

The present study has several limitations: first, the dose of aspirin was different in the studies, oscillating between 75mg and 500mg with six trials using 100mg daily. Second, there were some difficulties in classifying endpoints (MI and Stroke are sometimes not defined as non-fatal, and several studies did not specify major bleeding as a safety endpoint). On the other hand, one crucial point that is missing is the evolution of the definition of non-fatal MI: in the "modern era", the use of troponin captures minor MIs which were previously missed by ECG and/or CPK only, and on the other hand, we did not include total mortality as an outcome, and this could probably be a significant cause of bias. Third, in the studies made in the 20th century, particularly bleeding events were poorly reported despite the apparent evidence. Fourth, the studies were not analyzed, separating participants from high and low cardiovascular risk. Fifth, The NNT and NNH were calculated in hypothetical results that were expressed as five years of tracking time as a standard

unit for all studies and their calculations, thus the results of this review need to be interpreted with prudence. Sixth, the forest plots for meta-analysis for NNT and NNH were constructed with a scale including infinity (∞) and quoting to separate in two confidence intervals when there were negative values.

CONCLUSION

Recent studies have demonstrated that aspirin should not be recommended in the primary prevention of CVD, although it has a place in the secondary prevention of CVD [17,45,49]. The use of aspirin in primary cardiovascular disease was associated with a lower risk of cardiovascular events and an increased risk of major bleeding. NNT as a measure of effect and NNH to determine harm could be helpful in clinical share decision-making. However, as the "two faces of the coin" (not determined by a coin flip), it is essential to establish consistently that the benefit-risk for patients should be individualized and not be a clinical practice guide for everyone.

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Author's contribution

GV was responsible for the study rationale, manuscript drafting, and statistical analysis. JWM performed the literature search and made the final review of the manuscript. All authors were involved in the conception and the design of the study and interpretation of the data, revised the manuscript critically for important intellectual content, and finally approved the manuscript submitted.

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Clinical efficacy and safety of folic acid and vitamin B_{12} for the adjuvant treatment of schizophrenia: a systematic review and meta-analysis.

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Key words: folic acid; meta-analysis; schizophrenia; vitamin B_{12}.

Abstract. Given the different effects of folate and vitamin B_{12} on the adjuvant treatment of schizophrenia (SCH), their efficacy and safety as adjuvant therapies for SCH were systematically evaluated by evidence-based medicine. Publication retrieval was performed using authoritative databases such as the Cochrane Library, PubMed, and Web of Science to screen randomized controlled trials (RCTs). After the quality evaluation and data extraction of included studies, eligible RCTs were systematically reviewed using Review Manager 5.2 software. In total, 14 RCTs were included. The results of the meta-analysis revealed that as the adjuvant therapy for SCH, vitamin B_{12} differed significantly from folate in terms of anxiety relief rate [odds ratio (OR)=1.28, 95% confidence interval (CI) (1.02, 1.61), p=0.03, I^2 =0%, Z=2.13]. However, there were no significant differences in the incidence rate of mania [OR=1.13, 95% CI (0.78,1.65), p=0.65, I^2=36%, Z=0.65], total efficacy [OR=1.06, 95% CI (0.72, 1.56), p=0.77, I^2=0%, Z=0.30] and incidence rate of adverse reactions [OR=1.15, 95% CI (0.88, 1.49), p=0.31, I^2=0%, Z=1.03]. Although folate and vitamin B_{12} exhibit no significant differences in the adjuvant treatment of SCH, vitamin B_{12} exerts markedly fewer side effects than folate drugs, and it is of determinant significance for the clinical adjuvant medication of SCH.

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Eficacia clínica y seguridad del ácido fólico y la vitamina B_{12} como tratamiento adyuvante de la esquizofrenia: una revisión sistemática y metanálisis.

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Palabras clave: ácido fólico; metanálisis; esquizofrenia; vitamina B_{12}.

Resumen. En vista de los diferentes efectos del folato y la vitamina B_{12} en el tratamiento adyuvante de la esquizofrenia (SCH), su eficacia y seguridad como terapia adyuvante para SCH fueron evaluadas sistemáticamente mediante la medicina basada en la evidencia. La recuperación de publicaciones se realizó en base a bases de datos autorizadas como Cochrane Library, PubMed y Web of Science para la selección de ensayos controlados aleatorios (ECA). Después de la evaluación de la calidad y la extracción de datos de los estudios incluidos, los ECA elegibles se revisaron sistemáticamente mediante el software Review Manager 5.2. En total, se incluyeron 14 ECA. Los resultados del metanálisis revelaron que, como terapia adyuvante para la SCH, la vitamina B_{12} difería significativamente del folato en términos de tasa de alivio de la ansiedad [odds ratio (OR) = 1,28, intervalo de confianza (IC) del 95% (1,02, 1,61), p=0,03, I^2=0%, Z=2,13], pero no hubo diferencias significativas en la tasa de incidencia de manía [OR=1,13, IC 95% (0,78,1,65), p=0,65, I^2=36%, Z= 0,65], eficacia total [OR=1,06, IC 95% (0,72, 1,56), p=0,77, I^2=0%, Z=0,30] y tasa de incidencia de reacciones adversas [OR=1,15, IC 95% (0,88, 1,49), p=0,31, I^2=0%, Z=1,03]. Aunque el folato y la vitamina B_{12} no presentan diferencias significativas en el tratamiento adyuvante de la SCH, la vitamina B_{12} ejerce notablemente menos efectos secundarios que los fármacos de folato y tiene una importancia orientativa para la medicación adyuvante clínica de la SCH.

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INTRODUCTION

Schizophrenia (SCH) is a chronic mental disorder that involves social, cognitive, and emotional problems and has a lifetime prevalence of 1% in the population. As a chronic disease, SCH is usually accompanied by physical illness, malnutrition, and reduced self-care practices, all of which can cause vitamin deficiency [1]. Nutritional deficiencies often coexist with SCH, which is attributed to the patients' tendency to have higher calorie diets with saturated fat instead of fiber foods such as fruits and vegetables. Previous studies have revealed deficiencies of nutrients, such as folate and vitamin B_{12} in SCH patients resulting from less time on outdoor activities [2,3]. Vitamin B_{12} and folate levels decline in patients with SCH [4]. Alcoholism and drug addictions are viewed as chronic diseases featured by recurrence, in common with SCH. However, the prevalence data of illicit drug addiction worldwide are still lacking. Like SCH, alcoholism, and drug addictions may cause adverse effects on cognitive function and

induce malnutrition [5]. In addition, excessive drinking is a common phenomenon in developed countries, which affects nutrient intake and metabolism, leading to malnutrition, and also causes damage to multiple organs. Although folate and vitamin B_{12} deficiencies are frequently present in chronic alcohol drinkers, the impact of long-term drinking on vitamin B_{12} and folate levels has not yet reached a consensus. Thus, the data about correlations of vitamin D and other vitamin deficiencies with the risk of alcoholism are also insufficient to support the final conclusion [6]. Recently, vitamin B_{12} has been demonstrated to be vital for hemostasis and multiple physiological functions, as well as the pathogenesis of diseases such as cancer, autoimmune disorder, senile dementia, cognitive impairment, and SCH [7]. It was also reported in another study that vitamin B_{12} acts as an essential player in SCH and cognitive function and has immunomodulatory, anti-inflammatory, and antioxidant effects [8]. Homocysteine is thought to be a pro-atherogenic molecule that has toxic effects on endothelium, but its associations with diagnosis and prognostic evaluation of SCH have not been systematically analyzed and summarized [9]. Consistent with vitamin B_{12}, folate is a vitamin B used to modulate cell division. Folate deficiency is closely implicated in megaloblastic anemia, cardiovascular disease, osteoporosis, bone dysplasia, depression, and SCH, and folate insufficiency during pregnancy may cause fetal neural tube developmental defects. SCH involves neurodegenerative processes, although the impact extent of vitamin deficiency on these processes remains unclear[10], and the potential deterioration of such processes resulting from folate and vitamin B_{12} deficiencies cannot be ignored. For this reason, efforts should be made to identify patients at risk of specific vitamin deficiencies and then provide prompt and appropriate interventions.

Consequently motivated, the clinical efficacy and safety of folate and vitamin B12 as adjuvant therapy in SCH were systematically reviewed and meta-analyzed to guide the clinical medication of SCH.

MATERIALS AND METHODS

Retrieval methods

Publication retrieval was performed using databases such as the Cochrane Library, PubMed, Web of Science, and EMBASE, as well as related websites for registration of clinical trial institutions with "schizophrenia", "cognitive impairment", "SCH", "folate", and "vitamin B_{12}" as subject words and trademark names of relevant drugs as free words. In addition, relevant studies published in English were retrieved to avoid bias due to language restrictions (Fig. 1).

Inclusion criteria

The inclusion criteria of studies were as follows: i) studies using randomized controlled trials (RCTs), ii) those with research subjects meeting the diagnostic criteria for SCH following the Minnesota Multiphasic Personality Inventory or schizophrenia scale and also the ICD-11 diagnosis of schizophrenia [11], iii) those researching comparisons of efficacy and safety between folate and vitamin B_{12} as adjuvant therapies for SCH, iv) those mainly evaluating indexes including anxiety relief rate, incidence rate of mania, total efficacy and incidence rate of adverse reactions, v) those whose research subjects had no history of drug abuse, and vi) those including research subjects who underwent 6-12 weeks of treatment and were aged ≥8 years old and lack of folate or vitamin B_{12}.

Exclusion criteria

The exclusion criteria involved: i) studies adopting non-RCTs, ii) repeated studies, iii) studies with incomplete data, or iv) studies published for the second time.

Quality evaluation of studies

Evaluation was performed as required: i) whether patients were ranked randomly

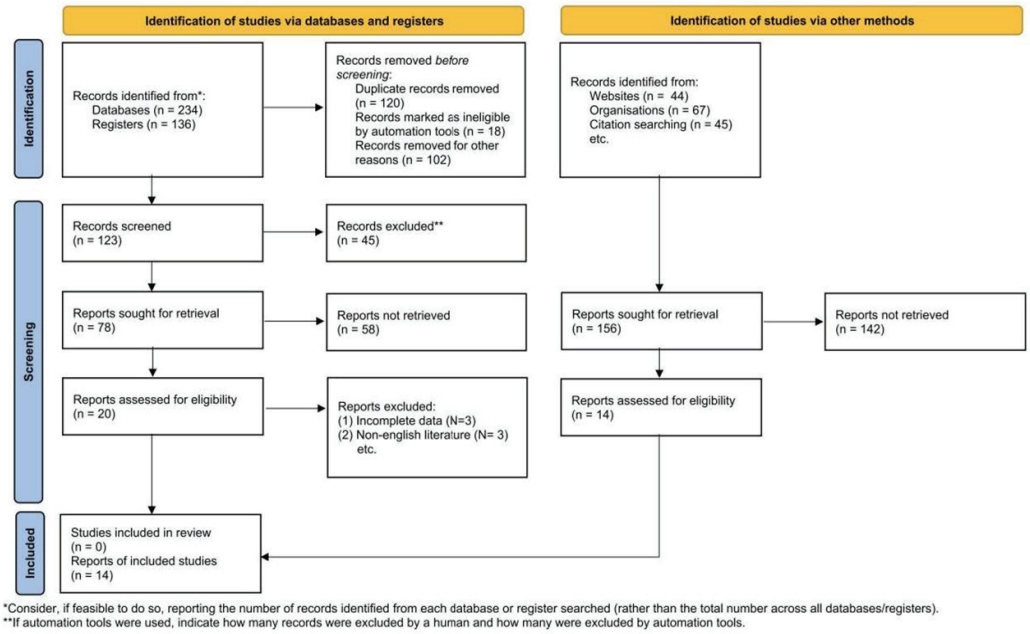

Fig. 1. Flow chart of literature retrieval.

("yes" =2, "unclear" =1, "no" =0), ii) random hiding ("yes" =2, "unclear" =1, "no" =0), iii) blind trial ("yes" =2, "clear" =1, "no" =0), and iv) withdrawal or not withdrawal ("yes" =1, "no" =0). Data involving author information and country, Jadad score, type, patient's age and gender, the dose of study drugs, number of cycles, effective treatment, total efficacy score after treatment, and adverse reactions were extracted. Then two commentators were responsible for comparing these data, where inconsistencies were discussed, and missing information was supplemented as far as possible.

Bias analysis of studies

Two investigators independently conducted data extraction and cross-checking to ensure the data's accuracy. Quality evaluation was conducted for RCTs with the Cochrane Handbook 5.0.2 as a reference. As for the included studies, the presence or absence of publication bias was evaluated using a funnel chart (performed through the Egger's test), which illustrated that all studies were within the triangle area, without obvious publication bias (Figs. 2 and 3).

Statistical analysis

The Review Manager 5.2 software [Cochrane Information Management System (IMS)] provided by Cochrane Collaboration was utilized for statistical analysis using the hazard ratio of binary variables. The meta-analysis analyzed the efficacy and incidence rate of adverse reactions using relative risk (RR) and 95% confidence interval (CI). Besides, the chi-square test (the significance level was set at $p<0.05$) and *t*-test expressed by *Z* and P values for the hypothesis test were applied, and $p<0.05$ was considered statistically significant. The hypothesis test results were displayed in the forest plot, and the χ^2 test was employed to analyze heterogeneity, divided into low, medium, and high heterogeneity and represented by I^2=25%, 50%, and 75%, respectively. The inverted funnel chart was used and displayed no obvious publication bias.

RESULTS

Basic information of included patients

A total of 123 studies were obtained by the preliminary retrieval based on databases such as PubMed, Cochrane Library, Web of

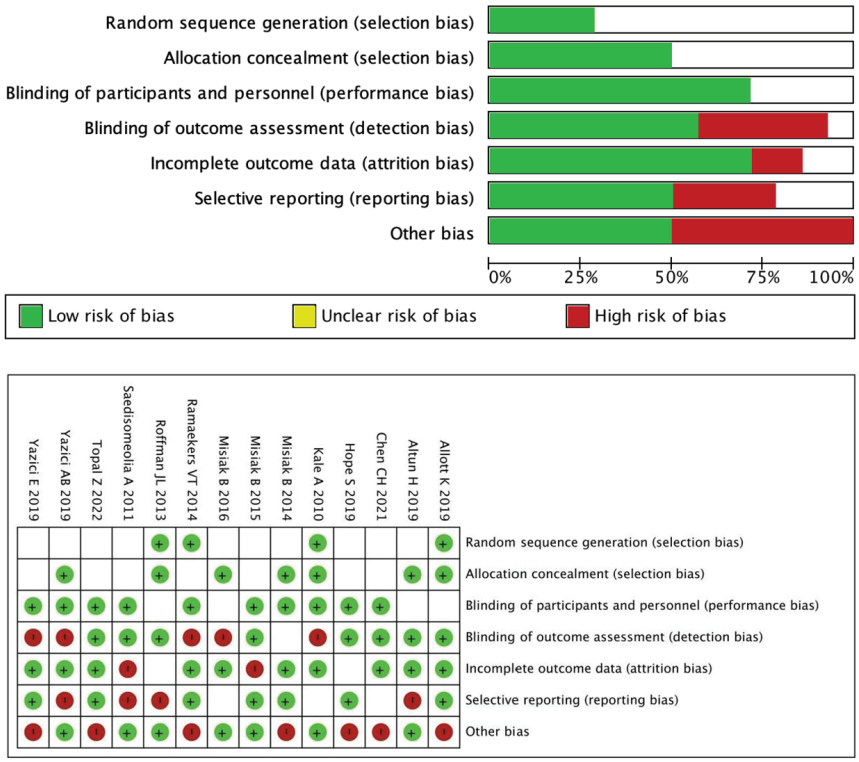

Fig. 2. Quality evaluation chart of literatures.

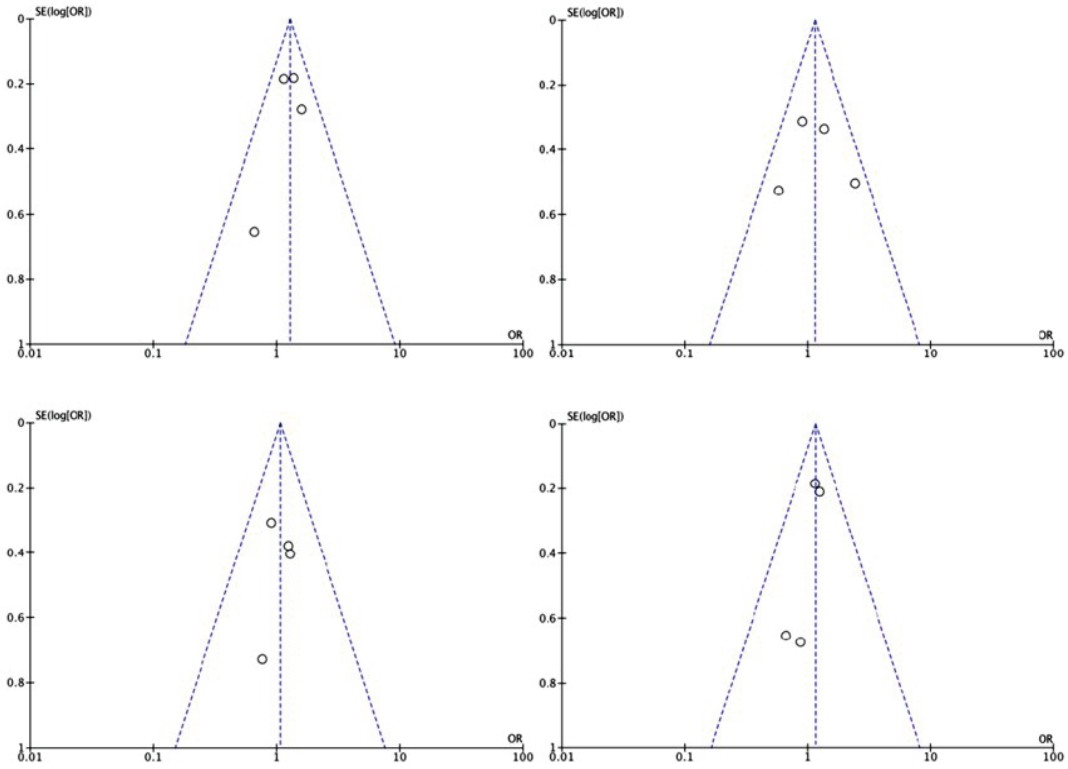

Fig. 3. Funnel chart for analysis of literature publication bias.

Science, and EMBASE. Furthermore, relevant references were also retrieved to avoid omission. Fourteen studies adopting RCTs were included (Table 1) [12-25].

Anxiety relief rate in SCH patients undergoing adjuvant therapy with folate and vitamin B_{12}

A heterogeneity test found that a low level of heterogeneity existed in 14 studies adopting RCTs for detecting anxiety relief rates in SCH patients undergoing adjuvant therapy with folate and vitamin B_{12}, so fixed models were utilized for meta-analysis. The experimental group was given folate in combination with vitamin B_{12}, and the control group was only given folate. The two groups had significantly different remission rates of anxiety [odds ratio (OR)=1.28, 95% CI (1.02, 1.61), p=0.03, I^2 =0%, Z=2.13] (Fig. 4).

Incidence rate of mania in SCH patients undergoing adjuvant therapy with folate and vitamin B_{12}

A heterogeneity test was conducted and revealed that 14 studies adopting RCTs for investigating the incidence rate of mania in SCH patients undergoing adjuvant therapy with folate and vitamin B_{12} exhibited a low level of heterogeneity, which could be subject to meta-analysis with fixed models. The results manifested that there was no significant difference in the incidence rate of mania in SCH patients undergoing adjuvant therapy with folate and vitamin B_{12} between the experimental group and the control group [OR=1.13, 95% CI (0.78, 1.65), p=0.65, I^2 =36%, Z=0.65] (Fig. 5).

Total efficacy of folate and vitamin B_{12} in the adjuvant treatment of SCH

A heterogeneity test was performed and manifested that 14 studies adopting RCTs for examining the total efficacy of adjuvant therapy with folate and vitamin B_{12} for SCH showed a low level of heterogeneity, which were subject to meta-analysis with fixed models. The results demonstrated that the total efficacy of adjuvant therapy with folate and vitamin B_{12} for SCH showed no significant difference between the experimental group and the control group [OR=1.06, 95% CI (0.72, 1.56), p=0.77, I^2 =0%, Z=0.30] (Fig. 6).

Incidence rate of adverse reactions of folate and vitamin B_{12} in the adjuvant treatment of SCH

Through a heterogeneity test, it was uncovered that 14 studies adopting RCTs for detecting the incidence rate of adverse reactions of folate and vitamin B_{12} in the adjuvant treatment of SCH had a low level of heterogeneity, so fixed models were used for meta-analysis. The results confirmed that the incidence rate of adverse reactions of adjuvant therapy with folate and vitamin B_{12} for SCH exhibited no significant difference between the experimental group and the control group [OR=1.15, 95% CI (0.88,1.49), p=0.31, I^2 =0%, Z=1.03] (Fig. 7).

DISCUSSION

Folate and vitamin B_{12} have immunomodulatory, anti-inflammatory, and antioxidant properties. It has been reported that the incidence of vitamin D deficiency is raised in patients newly diagnosed with SCH[26]. Another study demonstrated that vitamin B_{12} is not only associated with malnutrition but also correlated with the occurrence of disease and increased incidence rate of autoimmune thyroid diseases. Consequently, the loss of the neuroprotective effect of vitamin B_{12} may be implicated in the pathogenesis of SCH [27]. Like SCH, substance use disorder (SUD) is a chronic disease usually related to malnutrition. In a study about associations of folate and vitamin B_{12} levels with the severity of symptoms in patients with SCH, those with low levels of vitamin B_{12} may be at particular risk of poor prognosis. A previous study also revealed lower folate levels in SCH patients than in healthy controls [28]. Howev-

Table 1
Basic information of patients in 14 literatures adopting RCTs.

Study item	Age	Gender (Male)	Observation index of outcome	Experimental group (N)	Control group (N)	NOS score	Study type
Yazici *et al.* 2019	41.44±12.28	57.14%	Anxiety relief rate, incidence rate of mania, etc.	119/189	109/189	8	RCT
Yazici *et al.* 2019	40.63±13.50	100%	Anxiety relief rate, incidence rate of mania, etc.	24/30	23/28	7	RCT
Altun *et al.* 2018	9.33±1.80	76.67%	Anxiety relief rate, incidence rate of mania, etc.	23/30	25/30	8	RCT
Hope *et al.* 2020	30.0±9.0	55.78%	Anxiety relief rate, incidence rate of mania, etc.	420/ 483	401/483	8	RCT
Allott *et al.* 2019	20.2±3.0	65.40%	Anxiety relief rate, incidence rate of mania, etc.	88/120	76/120	8	RCT
Topal *et al.* 2022	8.5±3.1	73.90%	Anxiety relief rate, incidence rate of mania, etc.	178/203	180/203	7	RCT
Ramaekers *et al.* 2014	19.5±2.56	78.20%	Anxiety relief rate, incidence rate of mania, etc.	12/18	13/18	9	RCT
Chen *et al.* 2021	44.3±10.7	49.60%	Anxiety relief rate, incidence rate of mania, etc.	125/232	117/232	9	RCT
Misiak *et al.* 2014	26.0±5.3	58.97%	Anxiety relief rate, incidence rate of mania, etc.	27/39	31/39	7	RCT
Kale *et al.* 2010	33.57±8.35	56.32%	Anxiety relief rate, incidence rate of mania, etc.	23/31	26/48	8	RCT
Roffman *et al.* 2013	45.3±1.1	71.0%	Anxiety relief rate, incidence rate of mania, etc.	31/56	28/56	8	RCT
Saedisomeolia *et al.* 2011	37.25±16.0	66.44%	Anxiety relief rate, incidence rate of mania, etc.	44/60	41/60	8	RCT
Misiak *et al.* 2016	28.51±8.6	68.44%	Anxiety relief rate, incidence rate of mania, etc.	117/135	121/146	9	RCT
Misiak *et al.* 2015	25.12±4.48	43.22%	Anxiety relief rate, incidence rate of mania, etc.	34/83	36/83	7	RCT

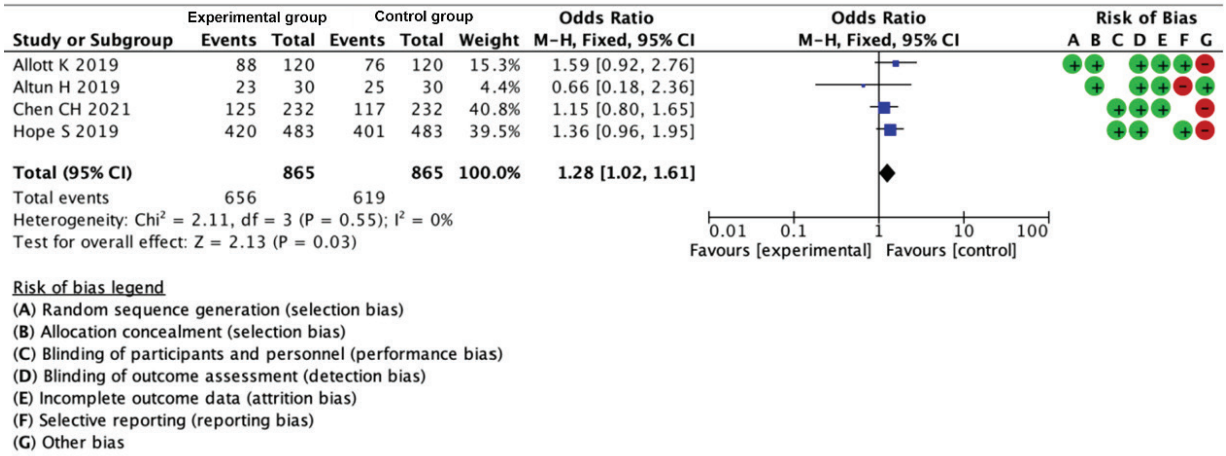

Fig. 4. Meta-analysis of anxiety relief rate in SCH patients undergoing adjuvant therapy between the two groups.

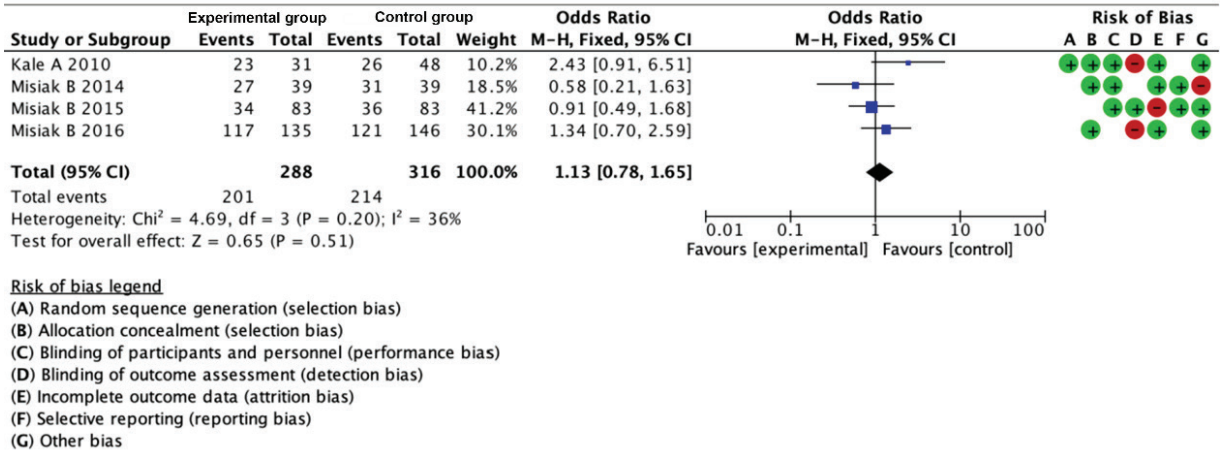

Fig. 5. Meta-analysis of incidence rate of mania in SCH patients undergoing adjuvant therapy between the two groups.

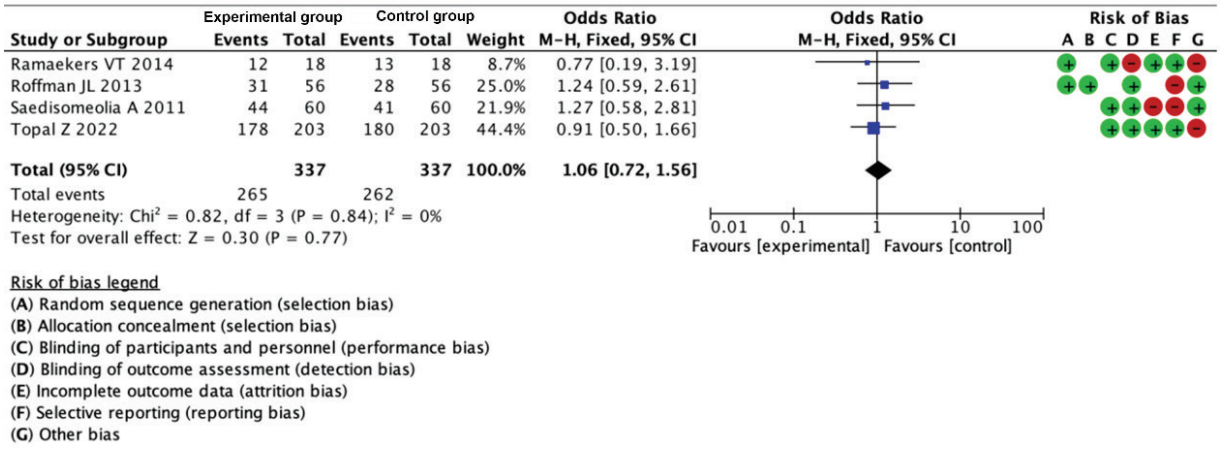

Fig. 6. Meta-analysis of total efficacy of adjuvant therapy for SCH between the two groups.

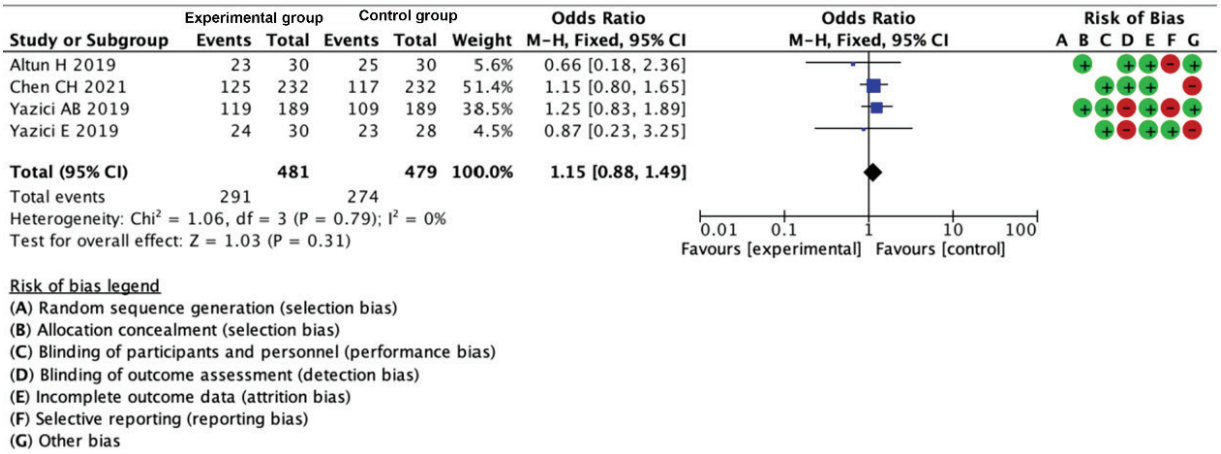

Fig. 7. Meta-analysis of incidence rate of adverse reactions in SCH patients undergoing adjuvant therapy between the two groups.

er, these findings have not been reported in other studies. Consistent with previous studies, folate, and vitamin B_{12} are relatively deficient in older adults, verifying associations with gender and age [29-31].

It has long been considered that abnormal one-carbon metabolism is one of the mechanisms for the neuropathology and psychopathology of SCH [32]. The changes in levels of one-carbon metabolic components (folate and vitamin B_{12}), homocysteine, and docosahexaenoic acid (DHA) are primarily found in patients receiving drug administration. For instance, daily administration of 2 mg folate plus 1 mg vitamin B_{12} for 12 weeks can significantly reduce the serum homocysteine level ($p<0.0001$) [33]. In a relevant study, the impact of change levels of one-carbon metabolic components (folate and vitamin B_{12}) on the severity of SCH was reported, and the subsequent alterations of homocysteine and DHA in phospholipids were also notably correlated with the pathogenesis of SCH [34]. In a study conducted by Satoskar *et al.* [35], the associations of folate and vitamin B_{12} deficiencies with the pathogenesis and prognosis of SCH patients were investigated, and the mechanism of one-carbon metabolism was also further explored. In the study, the clinical efficacy and safety of agents were analyzed between first-episode psychosis (FEP) patients (n=31) and healthy controls (HC, n=48), and folate and vitamin B_{12} were matched with confounding factors such as race, diet, and lifestyle, to reduce variability. Compared with HC, the DHA level in patients with FEP noticeably declined. The unique cohort used in the study provided an extensive mechanism for changing one-carbon metabolism (disturbed folate-vitamin B_{12}-DHA balance). Besides, the increased level of homocysteine contributes to the mechanism research on the neuropathology of SCH, and the data mentioned above may be of great significance for the psychopathology of SCH [36-39].

The limitations of this meta-analysis include: i) Potential publication bias existed because of too few studies and small sample size. ii) There were few studies included and no subgroup analysis for comparison of efficacy. iii) Only therapeutic effects at the end of treatment were evaluated, but long-term effects were not assessed. iii) We only included adult SCH patients over the age of 18, whose results would be inapplicable to adolescents.

Vitamin B_{12} and folate levels are notably lower in patients with SCH [40,41]. Herein, further analysis on the clinical efficacy and

safety of adjuvant therapy with folate and vitamin B_{12} for SCH revealed that vitamin B_{12} differed significantly from folate in terms of anxiety relief rate ($p<0.05$). However, there were no significant differences in the incidence rate of mania, total efficacy, and incidence rate of adverse reactions ($p>0.05$). Although vitamin deficiency commonly occurs in patients with SCH, vitamin B_{12} has notably fewer side effects than folate drugs, which is consistent with the findings of Roffman *et al*. [42]. Hence, this meta-analysis is of great guiding significance for the adjuvant clinical medication of SCH.

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Conflict of interest

The authors declare no conflict of interest.

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Authors' contribution

Study design: KN, XZ; Data collection: YW, YFW; Data analysis: YW, YFW; Writing: KN, XZ. KN and XZ contributed equally to this study.

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