Centro de Investigación en Alimentación y Desarrollo, A.C.

CARACTERIZACIÓN MORFOMÉTRICA Y CONTENIDO DE MIRNA-30, -145 Y -155 EN VESÍCULAS EXTRACELULARES COMO POTENCIALES BIOMARCADORES DE ESTADO NUTRICIONAL EN PACIENTES CON CÁNCER DE MAMA: ESTUDIO EXPERIMENTAL

Por:

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CONTENIDO

RESUMEN

El cáncer de mama (CM) es una de las neoplasias más frecuentes en el mundo. En mujeres con CM, el aumento de peso enmascara la pérdida de masa muscular, y se asocia a mayor recurrencia tumoral y mayor morbimortalidad. Estas alteraciones nutricionales pueden repercutir en la expresión de miRNAs (miRNA-155, miRNA-145, miRNA-30) relacionados con complicaciones asociadas a la desnutrición, progresión y crecimiento tumoral. Una nutrición adecuada previene complicaciones de salud y mala nutrición. Sin embargo, no existen biomarcadores específicos que detecten los cambios tempranos en composición corporal de las pacientes con CM. Las vesículas extracelulares (VE) son estructuras que actúan en la comunicación celular y que pueden participar en alteraciones de salud. Por tanto, las características de VE y su contenido (miRNA), podrían funcionar como biomarcadores relacionados con el estado nutricional de mujeres con CM. Por lo anterior, el objetivo es caracterizar la población de vesículas extracelulares en plasma y su expresión de miRNA-155, miRNA-145, miRNA-30 en pacientes con CM antes y después de una intervención dietética. Los datos clínicopatológicos (HER2neu, receptor de estrógeno y Ki67), datos antropométricos y de composición corporal y muestras de plasma fueron recuperadas de un proyecto previo. Las VE se aislaron del plasma de 16 mujeres con diagnóstico reciente de cáncer de mama. El tamaño de las VE y su potencial zeta se analizaron mediante análisis de dispersión dinámica de luz (DLS) y el contenido de miRNA-155, miRNA-145 y miRNA-30 se determinó mediante qPCR. No hubo diferencias significativas en el tamaño de VE y su expresión de miRNA-155, miRNA-145 y miRNA-30 tras 6 meses de intervención nutricional. El potencial zeta se asoció con: HER2neu (β=2.1; p=0.00), ki67 (β=-1.39; p=0.007), estrógeno positivo (β=1.57; p=0.01), peso (β=-0.09; p= 0,00), y grasa visceral (β=0,004; p=0,00). miRNA-30 se asoció con LDL (β=-0,012; p=0,01) y HDL (β=-0,02; p=0,05). miRNA-155 se asoció con grasa visceral (β=-0,0007; p=0,05) y ki67 (β=-0,47; p=0,04). No encontramos asociaciones entre la expresión de miRNA-145 y alguna de las variables. Estos resultados revelan una asociación significativa entre la expresión de miRNA-30, miR-NA-155 y el potencial Zeta de las VE con biomarcadores de riesgo metabólico y pronóstico de la enfermedad en mujeres con cáncer de mama; en particular, el potencial Zeta de las vesículas extracelulares puede ser un nuevo biomarcador sensible a los cambios en el estado nutricional y la progresión del cáncer de mama.

Palabras clave: exosomas, microvesículas, biomarcadores, estado nutricio.

ABSTRACT

Breast cancer (BC) is one of the most frequent neoplasms in the world. In women with BC, weight gain masks the loss of muscle mass, and is associated with greater tumor recurrence and higher morbidity and mortality. These nutritional alterations can alter the expression of miRNAs (miRNA-155, miRNA-145, miRNA-30) related to complications associated with malnutrition, progression, and tumor growth. Proper nutrition prevents health complications and poor nutrition. However, there are no specific biomarkers that detect early changes in body composition in patients with BC. Extracellular vesicles (EVs) are structures that act in cellular communication and that can participate in health alterations. Therefore, the characteristics of EV and its content (miRNA) could function as biomarkers related to the nutritional status of women with BC. Our objective is to characterize the population of extracellular vesicles in plasma and the expression of miRNA-155, miRNA-145, miRNA-30 in their content in patients with BC before and after a nutritional intervention. Clinicopathological data (HER2neu, estrogen receptor, and Ki67), anthropometric and body composition data, and plasma samples were retrieved from a previous project. EVs were isolated from the plasma of 16 women newly diagnosed with breast cancer. The size of the EVs and their zeta potential were analyzed by dynamic light scattering (DLS) analysis and their miRNA-155, miRNA-145 and miRNA-30 content was analyzed by qPCR. There were no significant differences in the size and expression of miRNA-155, miRNA-145 and miRNA-30 after 6 months of nutritional intervention. Z potential was associated with: HER2neu (β=2.1; p=0.00), ki67 (β=-1.39; p=0.007), estrogen positive (β=1.57; p=0.01), weight (β=-0.09; p = 0.00), and visceral fat (β=0.004; p=0.00). miRNA-30 was associated with LDL (β=-0.012; p=0.01) and HDL (β=-0.02; p=0.05). miRNA-155 was associated with visceral fat (β=-0.0007; p=0.05) and ki67 (β=-0.47; p=0.04). We found no associations between the expression of miRNA-145 and any of the variables. Our results reveal a significant association between the expression of miRNA-30, miRNA-155 and the Zeta potential of EVs with biomarkers of metabolic risk and disease prognosis in women with breast cancer; in particular, the Zeta potential of EVs may be a new biomarker sensitive to changes in nutritional status and breast cancer progression.

Keywords: exosomes, microvesicles, biomarker, nutritional status.

1. SINOPSIS

1.1. Justificación

En mujeres el cáncer de mama es la neoplasia más frecuente. Los cambios en composición corporal que ocurre durante el desarrollo de la enfermedad, así como en respuesta al tratamiento antineoplásico, provoca principalmente la ganancia de grasa y pérdida de músculo de las mujeres con cáncer de mama. Ante estos cambios, la intervención nutricional oportuna puede mejorar el estado nutricional de las mujeres con cáncer de mama. La búsqueda de biomarcadores que detecten cambios tempranos en la composición corporal pudiera ayudar en el desarrollo de estrategias terapéuticas, así como de prevención dirigidas a mujeres con cáncer de mama. Las vesículas extracelulares, sus características y contenido de ácidos nucleicos han sido estudiados por su sensibilidad ante cambios del ambiente celular en presencia de enfermedad. Por lo que, el estudio de vesículas extracelulares y su contenido de miRNA antes y después de una intervención nutricional en mujeres con cáncer de mama, pudieran ser estudiados como potenciales biomarcadores de cambios en el estado nutrimental.

1.2 . Antecedentes

El cáncer de mama es la neoplasia más común a nivel mundial, afectando a 2.3 millones de personas por año. En la mayoría de los países, el cáncer de mama es la principal neoplasia en mujeres (Cancer, 2021). A la fecha, la Sociedad Americana de Cáncer en Estados Unidos, estima que 1 de cada 8 mujeres padeció esta enfermedad (BREASTCANCER.ORG, 2023); mientras que, en México, en al año 2021 el cáncer de mama fue la primera causa de muerte en mujeres mayores de 30 años (*COMUNICADO DE PRENSA NÚM. 77/23 2 DE FEBRERO DE 2023 PÁGINA 1/6 COMUNICACIÓN SOCIAL ESTADÍSTICAS A PROPÓSITO DEL DÍA MUNDIAL CONTRA EL CÁNCER (4 DE FEBRERO) DATOS NACIONALES*, n.d.).

El estado de nutrición y composición corporal de las mujeres, previo al diagnóstico de cáncer de

mama, influye en los cambios de composición corporal que se presentarán a lo largo de la enfermedad y que pueden ser efecto secundario de los tratamientos antineoplásicos (Combs et al., 2013a; Raiten et al., 2011).

Un óptimo estado nutricional y de composición corporal, son factores clave para hacer frente a diferentes afecciones y tratamientos durante el cáncer de mama (Custódio et al., 2016). Los principales cambios en composición corporal que influyen en el pronóstico de la enfermedad en mujeres con cáncer de mama son el aumento de la masa grasa, la pérdida de masa muscular y/o la densidad mineral ósea (Agurs-Collins et al., 2019; Aleixo et al., 2019, 2020). Los desequilibrios en la composición corporal pueden aumentar el riesgo de toxicidad por fármacos antineoplásicos, complicaciones en intervenciones quirúrgicas, progresión de la enfermedad y recurrencia metastásica (Ethun et al., 2017; Guigni et al., 2018).

En nuestro grupo de trabajo ya se ha reportado, anteriormente, las mejorías al estado nutricio de mujeres recientemente diagnosticadas con cáncer de mama, tras llevar una intervención nutricional de 6 meses (Limon-Miro et al., 2021), para ese estudio se diseñó una intervención nutricional individualizada utilizando el método de menú de equivalente de comida de macronutrientes dinámicos (Limon-Miro et al., 2019). Los resultados mostraron una pérdida significativa de grasa visceral, peso corporal y ningún cambio en la masa del músculo esquelético apendicular, la densidad mineral ósea y la masa libre de grasa, después de 6 meses de intervención nutricional (Limon-Miro et al., 2021). Los cambios en la composición corporal discutidos anteriormente se detectaron después de los primeros seis meses de tratamiento antineoplásico, utilizando herramientas y técnicas de evaluación nutricional convencionales aplicados en la práctica clínica. Sin embargo, hace falta realizar más investigación sobre técnicas y biomarcadores más sensibles, ahora a nivel molecular, para comprender mejor los cambios que ocurren en las primeras etapas del desarrollo de la enfermedad, así como en respuesta a una intervención nutricional. Contar con biomarcadores a nivel celular que complementen los biomarcadores tradicionales para el control metabólico y progresión del cáncer nos brindarían una poderosa herramienta para discernir el comportamiento asociado a las alteraciones en composición corporal que padecen las pacientes con cáncer de mama.

Un biomarcador se define como una “característica que se mide como un indicador de procesos biológicos normales, procesos patogénicos o respuestas biológicas a una exposición o intervención, incluidas las intervenciones terapéuticas”(FDA-NIH Biomarker Working Group, 2016). Aunque los biomarcadores utilizados en nutrición y cáncer pueden detectar cambios en la composición

corporal, la progresión de la enfermedad, el efecto de un medicamento o tratamiento antineoplásico, etc., solo son sensibles en etapas posteriores de la progresión de la enfermedad y hay poca o ninguna especificidad para los cambios en el estado nutricional (Combs et al., 2013b; Fougère et al., 2015).

A su vez, se han expuesto las discrepancias entre los biomarcadores utilizados en la práctica clínica y la investigación, lo que limita la comprensión de las enfermedades crónicas y la nutrición (Califf, 2018)**.** La mayoría de los biomarcadores utilizados en la investigación nutricional (p. ej., albúmina, creatinina, glucosa, etc.) están relacionados con el desarrollo y la progresión de enfermedades, pero en general, hay una falta de biomarcadores sensibles a alteraciones nutricionales tempranas o diseñados para responder a intervenciones nutricionales, lo que ha impulsado la búsqueda de nuevos biomarcadores (Raiten et al., 2011); entre las opciones de investigación destacan las vesículas extracelulares (Delgadillo-Velázquez et al., 2022; Mendivil-Alvarado et al., 2022)**.**

Las VE son micropartículas liberadas por todas las células al espacio extracelular, su liberación y contenido responden a las condiciones del microambiente celular (Oliveira Rodríguez et al., 2017; Théry et al., 2018). Utilizando la clasificación de la Sociedad Internacional de Vesículas Extracelulares (ISEV), las VE se dividen según su tamaño (nm) en vesículas extracelulares pequeñas y medianas (Théry et al., 2018; Witwer et al., 2021). Las VE se pueden identificar mediante marcadores específicos, entre ellos CD63, CD81, ALIX y TSG101 (Théry et al., 2018). Sin embargo, el enriquecimiento de proteínas y marcadores ya sea en el contenido o en proteínas citoplasmáticas y/o transmembrana de las VE, es muy variado. Actualmente, se está investigando qué determina el contenido de las VE, pero se cree que depende de la naturaleza de la célula de donde provienen, así como de las condiciones del microambiente celular o la presencia de enfermedades como el cáncer.

Las vesículas extracelulares pequeñas, también conocidos como exosomas, tienen un tamaño que va desde 50nm hasta 150nm, mientras que las VE medianas abarcan a las vesículas de menos de 1000nm, también conocidas como microvesículas. Los primeros estudios de VE pequeñas son derivados de células hematopoyéticas y células dendríticas (Harding, C., Heuser, J. & Stahl, 1984; Théry et al., 1999; Zitvogel et al., 1998). Por su parte, las VE medianas originalmente eran consideradas producto de desecho celular (Wolf, 1967). La biogénesis de las VE aún sigue en estudio, pero se sugiere que las VE pequeñas provienen de la maduración de cuerpos multivesiculares en el interior de la célula, mismos que al fusionarse con la membrana plasmática expulsan las VE al medio extracelular (Johnstone et al., 1987). Las VE medianas surgen de la

evaginación de la membrana plasmática, involucrando la remodelación de esta para expulsar al medio extracelular la VE mediana. Involucrando con ello remodelación de actina, calcio y proteínas transmembranales (Théry et al., 2018).

Este proceso de biogénesis hace que las VE contengan una variedad de proteínas muy variadas, mismas que pueden incluir proteínas involucradas en el proceso de biogénesis o tráfico vesicular, tales como las tetraspaninas. Aun cuando la caracterización específica de las VE representa un reto actual, se sabe que su composición y características serán influenciadas por el estrés celular. Algunos de los cambios en las características de las VE en respuesta al estrés celular es su modificación de tamaño, potencial Zeta y/o contenido. Esta sensibilidad de las VE es la que las destaca como posibles biomarcadores en condiciones patológicas que van desde enfermedades neurodegenerativas y crónico-degenerativas hasta cáncer de mama (Dimassi et al., 2018; Eitan et al., 2017; Holvoet et al., 2016; Hu et al., 2019).

Entre las características de las VE encontramos el potencial Zeta, una propiedad biofísica que explica las cargas en la superficie de una partícula, lo que resulta en diferencias de potencial electrostático (Cuadros-Moreno et al., 2014; Foord et al., 1970). El potencial Zeta otorga a las VE la capacidad de interactuar con otros tejidos distales e incluso con otras vesículas; sin embargo, en nuestra opinión se ha descrito poco sobre los cambios en el potencial Zeta durante enfermedades como el cáncer o bajo terapias de apoyo como una intervención nutricional. Por lo que aún se desconoce si el uso y validación del potencial Zeta puede ser considerado como un biomarcador del estado nutricional y/o seguimiento de intervenciones nutricionales (Martinez et al., 2011; Martínez & Andriantsitohaina, 2017).

Finalmente y aunado a lo anteriormente expuesto, el contenido de ácidos nucleicos en VE ha sido estudiado, entre ellos el contenido de miRNA. Los miRNA son segmentos cortos de ARN (22 bases) que regulan la expresión génica a través del emparejamiento de bases con secuencias complementarias de los ARNm diana (Lewin, 2002). La mayoría de los miRNA detectables en saliva y suero se concentran en vesículas extracelulares pequeñas (Gallo et al., 2012). Lo que sugiere que los miRNA se transportan de un tejido a otro, utilizando VE como vehículo protector de las transgresiones del entorno extracelular.

Específicamente en el cáncer de mama, miRNA-145, miRNA-30 y miRNA-155 han sido identificados como responsables de promover la angiogénesis, la progresión y la invasión tumoral (Han et al., 2018; Huntzinger & Izaurralde, 2011; Jiang et al., 2019; Katsuda et al., 2014; Zou et al., 2012); además, su expresión se ha asociado con resistencia al tratamiento antineoplásico y

recurrencia tumoral (Sayyed et al., 2021). Sin embargo, la información es limitada sobre la presencia y transporte de estos miRNA en VE y sobre cómo están involucrados en el proceso, progresión y desarrollo de la enfermedad; así como si pudieran ser considerados biomarcadores potenciales para el seguimiento de una intervención nutricional. Es por ello que el objetivo de este trabajo fue caracterizar morfométricamente la población de vesículas extracelulares y su contenido de miRNA-30, miRNA-145 y miRNA-155, en pacientes con cáncer de mama antes y después de una intervención dietaria.

1.3. Hipótesis

La heterogeneidad morfométrica y expresión de miRNA-30, miRNA-145 y miRNA-155 en vesículas extracelulares plasmáticas de pacientes con cáncer de mama será diferente tras una intervención dietaria.

1.4.Objetivo General

Caracterizar morfométricamente la población de vesículas extracelulares plasmáticas y su contenido de miRNA-30, miRNA-145 y miRNA-155, en pacientes con cáncer de mama antes y después de una intervención dietaria.

1.5. Objetivos Específicos

- Aislar y caracterizar morfométricamente las vesículas extracelulares en plasma de mujeres con cáncer de mama al inicio y final de los primeros seis meses de tratamiento antineoplásico y nutricional.
- Explorar expresión de miRNA-30, miRNA-155, miRNA-145, contenidos en las vesículas

extracelulares circulantes en plasma en mujeres con cáncer de mama al inicio y final de los primeros seis meses de tratamiento antineoplásico y nutricional.

- Comparar las características morfométricas y la expresión de miRNAs (miRNA-30, miRNA-145 y miRNA-155) de las vesículas extracelulares en mujeres con cáncer de mama al inicio y final de los primeros seis meses de tratamiento antineoplásico y nutricional.
- Comparar los resultados de la caracterización morfométrica de VE con la composición corporal de mujeres con cáncer de mama al inicio y final de los primeros seis meses de tratamiento antineoplásico y nutricional.
- Comparar los resultados de la caracterización de vesículas extracelulares y su contenido de miRNAs con biomarcadores tradicionales de riesgo cardiovascular (Colesterol total, HDL, LDL, triglicéridos) y resistencia a la insulina (glucosa e insulina) mujeres con cáncer de mama al inicio y final de los primeros seis meses de tratamiento antineoplásico y nutricional.

1.6.Sección Integradora del Trabajo

El capítulo 1 integra la información actual sobre vesículas extracelulares, definición, clasificación, y su relación con el estado nutricional. Se conocen los cambios al estado nutricional que se producen por alguna enfermedad, también se ha descrito y sugerido la participación de las vesículas extracelulares en diferentes enfermedades. Sin embargo, poco se sabe sobre los cambios que pueden presentar las VE ante diferentes estados de mala nutrición. El capítulo 1, resumen algunos de los reportes de VE y estado nutricional en obesidad, desnutrición, cambios en componentes corporales como grasa y masa muscular; así como la respuesta de VE ante cambios específicos de macronutrientes en la dieta. Además, en este capítulo se describe cómo las vesículas extracelulares actúan en la comunicación celular y cómo sus características pueden ser alteradas por condiciones fisiopatológicas, convirtiéndolos en potenciales biomarcadores del estado nutricional en humanos.

Los cambios al estado nutricional de las mujeres con cáncer de mama difieren en comparación a otras neoplasias. Mientras que en diferentes tipos de cáncer destaca la pérdida de masa grasa y

masa muscular; las mujeres con cáncer de mama presentan una ganancia de masa grasa, misma que enmascara la pérdida de masa muscular, ocasionando obesidad sarcopénica. Si bien las VE se han investigado como potenciales biomarcadores de estado nutricional, poco se ha descrito sobre sus cambios ante una intervención nutricional durante una enfermedad. En el capítulo 2, se resumen los resultados del análisis de características de VE en (tamaño, potencial Zeta y contenido de miRNA-30, miRNA-145 y miRNA-155) en mujeres recién diagnosticadas con cáncer de mama que recibieron una intervención nutricional durante 6 meses. Así mismo, se reporta la asociación de estos cambios con la composición corporal de las mujeres y sus resultados en biomarcadores de riesgo metabólico. Nuestros resultados revelaron asociaciones positivas y negativas con características clinicopatológicas, composición corporal y biomarcadores de riesgo metabólico. Por lo que se revela el potencial uso de las características de vesículas extracelulares como biomarcadores de riesgo metabólico y pronóstico de la enfermedad en mujeres con cáncer de mama.

2. MALNUTRITION AND BIOMARKERS: A JOURNEY THROUGH EXTRACELLULAR VESICLES

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Review

Malnutrition and Biomarkers: A Journey through Extracellular Vesicles

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Abstract: Extracellular vesicles (EVs) have been identified as active components in cellular communication, which are easily altered both morphologically and chemically by the cellular environment and metabolic state of the body. Due to this sensitivity to the conditions of the cellular microenvironment, EVs have been found to be associated with disease conditions, including those associated with obesity and undernutrition. The sensitivity that EVs show to changes in the cellular microenvironment could be a reflection of early cellular alterations related to conditions of malnutrition, which could eventually be used in the routine monitoring and control of diseases or complications associated with it. However, little is known about the influence of malnutrition alone; that is, without the influence of additional diseases on the heterogeneity and specific content of EVs. To date, studies in "apparently healthy" obese patients show that there are changes in the size, quantity, and content of EVs, as well as correlations with some metabolic parameters (glucose, insulin, and serum lipids) in comparison with non-obese individuals. In light of these changes, a direct participation of EVs in the development of metabolic and cardiovascular complications in obese subjects is thought to exist. However, the mechanisms through which this process might occur are not yet fully understood. The evidence on EVs in conditions of undernutrition is limited, but it suggests that EVs play a role in the maintenance of homeostasis and muscle repair. A better understanding of how EVs participate in or promote cellular signaling in malnutrition conditions could help in the development of new strategies to treat them and their comorbidities.

Keywords: exosomes; microvesicles; obesity; undernutrition; microparticles

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1. Introduction

The term malnutrition encompasses disorders associated with deficit or excess in the consumption of nutrients, which manifests in conditions such as undernutrition and excess weight (obesity), known as the double burden of malnutrition. The figures for malnutrition worldwide are alarming, and show that it affects children and adults alike. According to the World Health Organization, in 2014, there were approximately 462 million underweight adults. It has also been reported that global obesity prevalence has risen approximately 2 percentage points per decade since 1975 [1]. Worldwide, in 2016, 678 million adults were reported to have obesity [2], and it is proposed that this amount will increase to 1.12 billion obese individuals by 2030 [3].

Obesity is defined by the World Health Organization as excess body fat, using a body mass index (BMI) greater than or equal to 30 kg/m^2 as a reference. The double burden of malnutrition causes health issues such as muscle wasting; the propensity to develop

cardiovascular, chronic-degenerative diseases; and an increase in the incidence of infections, among other maladies, which affect the health status and quality of life of the sufferers.

Currently, anthropometric tools and indicators, biochemical parameters, and biomarkers are used to assess the nutritional status of the population, aiming at the timely detection of malnutrition in some of its forms [4]. Among these tools, the use of biomarkers of nutritional status stands out. Biomarkers are measurable parameters or molecules, that can be used to assess the stability or, alternatively, the degree of abnormality of a particular biological process, making it possible to detect or monitor the deterioration of health and, in some cases, nutritional alterations. However, the predictive power of traditional biomarkers used in the field of nutrition (e.g., plasma metabolites and/or body parameters) does not adequately reflect the nutritional status of the individual [5]. They are generally late in showing results of clinical value, because their quantification varies depending on the presence of disease, disease stage, pathological condition, or metabolic alteration [6]. Thus, the possibility of early detection of malnutrition conditions or complications associated with them is limited, which in turn increases the morbidity and mortality of sufferers. Given this, it is essential to go beyond specific metabolites and to explore new mechanisms of signaling and/or cellular communication [7].

In the last decade, the study of extracellular vesicles (EVs) has gained considerable interest due to the active role they play in cellular communication. It is known that EVs are particles released by cells into the extracellular space, and that both their release and content respond to the conditions surrounding the cellular microenvironment [8]. This response seems to be a highly calibrated one, such that it promises predictability of response in the event of alterations within the microenvironment surrounding the cells. This makes the study of EVs not only fascinating, but also relevant in that, precisely because of this predictability, it allows for the possibility for detectability and measurability, thereby helping to close the gap between the deterioration of health and time to detection. Henceforth, when we speak of the sensitivity of EVs, we are referring to this seemingly highly calibrated response, which is manifested with variations in the size, quantity, and content of EVs. Indeed, due to the response of EVs to situations of cellular stress, their use as potential biomarkers in some pathological conditions has been suggested, such as in neurodegenerative, cardiovascular, and chronic-degenerative diseases and some types of cancer [9–12].

Although the literature does show that there is an association between EVs and diseases or health complications associated with obesity and undernutrition, little is known about the influence of malnutrition alone (that is, without the influence of additional illnesses) on the heterogeneity and specific content of EVs. A better understanding of how EVs participate in or promote cellular signaling in situations of malnutrition could help in the development of new strategies to treat them and their comorbidities. With this in mind, the aim of this review is to examine the current research on the effect of malnutrition on EVs and the likely role of EVs in the development of comorbidities associated with undernutrition and obesity.

This review is divided into five sections. The first section deals with the basic general knowledge about EVs and their classification. The subsequent four sections examine the current evidence on the sensitivity of EVs to specific conditions of nutritional status: excess weight, adipose tissue, diet, weight loss, and undernutrition.

2. Definition and Biogenesis of Extracellular Vesicles

The study of EVs is broad and branches out into many areas such as renal diseases, cancer, and autoimmune and cardiac diseases. For a better understanding of this rapidly evolving and developing field of study, it is worth reviewing the theoretical bases about its biogenesis and classification that have so far been proposed, bearing in mind that little is known about the influence of nutritional status on the sensitivity of EVs.

It is well known that cells use different signals and vehicles to transmit information to other cells [13,14]. They expel cytoplasmic and membrane material through vesicles

that, when found in the extracellular medium, are called EVs. The process of the production of EVs is carried out by most cells in the body and is a phenomenon that has been maintained throughout evolution, both in eukaryotic and prokaryotic organisms [15]. The International Society for Extracellular Vesicles (ISEV) proposed, as a generic concept, the term "extracellular vesicles" for all those particles delimited by a lipid bilayer, which are naturally released from the cell to the extracellular medium, with the characteristic that they cannot replicate nor have a nucleus [14].

At first, EVs were thought of as waste carriers only. It was not until 1987 that the first hypotheses about their existence as active components in cellular communication were raised [16]. Since then, research on EVs has increased, providing information about their components, possible biogenesis mechanism, and even their classification. More recently, a debate as to the most accurate classification of EVs subpopulations took place. As part of this debate, considerations regarding their content and morphometric characteristics were at issue, as questions were raised as to whether the mechanism of biogenesis and content of EVs were dependent on the type of cell that produced them. Faced with this problem, the ISEV proposed a series of guidelines regarding nomenclature, as well as the minimum requirements to define populations of EVs [14].

The classification of EVs is based on their biogenesis and size, broadly separates them into two groups, exosomes (50–150 nm) and microvesicles (<1000 nm), also known as small and medium EVs, respectively. However, the ISEV has encouraged caution when using the terms exosomes and microvesicles, as they could be confused with terms that were historically used to refer to something else, and might be contradictory and inaccurate when referring to concepts about EVs. Such is the case for small EVs, also known as exosomes, which should not be confused with the exosomal complex [14].

2.1. Exosomes

Exosomes represent a group of small vesicles with sizes ranging from 50 to 150 nm [14]. They can come from any type of cell, although initial investigations have suggested that they came from hematopoietic and dendritic cells [17–19].

Exosomes are initially generated within the lumen of endosomes as intraluminal vesicles (ILVs), and during their maturation they undergo a process to become late endosomes, also known as multivesicular bodies (MVBs). MVBs fuse their contents with the plasma membrane of the cell and are then expelled into the extracellular medium, by the cell, as EVs [16]. Some of the mechanisms through which this process takes place are still under study. However, within the main mechanisms involved, the endosomal transport sorting complex (ESCRT—endosomal sorting complexes required for transport) and its subcomplexes (ESCRT-0, -I, -II, and -III) are known to play a role in the sorting and conformation of multivesicular endosomes (an earlier form of MVBs), as well as in the secretion and excretion of exosomes [20–22]. Thus, the ESCRT complex, together with its four subcomplexes, represent an important step in the formation of exosomes. It has been shown in dendritic cells that the depletion in the formation of exosomes directly affects their production [22]. This mechanism does not seem to be the only one, however. The lipid microdomains in the plasma membrane, the activity of sphingomyelinase (nSMase) [23], the presence of flotiline [24], and the affinity of proteins associated with tetraspanins [25] have been shown to also be involved in the genesis of ILVs.

The presence and enrichment of proteins in the exosomes is varied and can include proteins involved in the process of biogenesis and/or vesicular traffic. These include the family of tetraspanins (CD81, CD63, CD82, and CD9), a group of transmembrane proteins that form complexes with each other, as well as with different transmembrane and cytosolic proteins [25–28]; associated proteins such as integrins and immunoglobulins; cytoskeleton proteins (tubulin and actin); ESCRT complex-related proteins (ALIX and TSG-101) [29,30]; and heat shock chaperone proteins (HSP70 and HSP90), which are found in most exosomes [31].

Exosome biogenesis is a complex process whose understanding is made more difficult by the fact that the regulation of each of the mechanisms hitherto described remains unknown; furthermore, the possibility that they could coexist in a given cell type also cannot be ruled out completely. Likewise, the targeting of these small vesicles is not fully understood, but to date, it is known that this could depend on their content, type of originating cell, mechanism of biogenesis, and/or the pathological situation of the cell [21]. Much work remains to be done to unravel the mechanisms of biogenesis and that of the targeting of exosomes.

2.2. Microvesicles

Microvesicles (MVs), also known as microparticles or ectosomes, are vesicles that measure from 100 to 1000nm [14]. They were originally considered tiny particles from platelets called "platelet dust/debris", found in the plasma and serum [32]. Today, it is known that they come from different types of cells and are vesicles generated by direct sprouting of the plasma membrane, whose process involves the reorganization of actin and the subsequent detachment of the vesicle towards the cell exterior [14]. Although the biogenesis of MVs, as it relates to their release into the extracellular medium, is yet to be fully described, it is known that different mechanisms are required to integrate the rearrangement of lipids and membrane proteins to complete this process, including calcium-dependent and independent mechanisms [33].

The content of MVs can vary. However, within their components, lipids and proteins involved in their biogenesis can be found. An example of these is the group of RHO proteins (GTPases) and RHO (rock)-associated protein kinases. As for lipids, the most enriched in the MVs are different types of lipids/phospholipids, among them lysophosphatidylcholine, sphingolipids, ceramides, and cholesterol. The components of MVs, as well as their mechanisms of genesis and traffic, are still areas under study.

In general, the composition and specific markers of MVs and exosomes are different and depend on the biogenesis of each subpopulation and the type of cell from which they come. The use of membrane markers is one of the most used techniques to classify both MVs and exosomes. However, there are proteins or markers that are shared by both groups, which makes exact identification more difficult. In an attempt to standardize the classification and composition of specific markers, the ISEV has suggested minimum information for studies of EVs, considering the use of operational terms for EV subtypes to be referenced, including their physical characteristics (size and/or density) and chemical composition; a list of EV specific markers is also suggested [14]. Despite ISEV efforts, the characterization of these subpopulations remains a challenge.

3. Extracellular Vesicles and Excess Weight

Obesity, an excessive accumulation of body fat (BMI $\geq$ 30 kg/m^2), adversely affects body function and favors the development of comorbidities, such as cardiovascular and metabolic diseases [34,35]. The early identification of these conditions is key to timely treatment. However, there are people with obesity who do not develop metabolic disorders and show apparent health, known as "metabolically healthy obese" (MHO) [36]. The situation of the MHO, however, does not preclude future deterioration of health. In fact, it is the MHO who present a higher cardiovascular risk, as alterations in communication at the cellular level continue to occur, even without the apparent changes traditional metabolic biomarkers [37–41]. Due to the sensitivity of EVs to situations of metabolic stress, recent studies conducted in animals and just a few in humans on the characteristics of EVs and excess weight have shown significant and interesting advances (Table 1). Even so, research on the use of EVs as potential biomarkers in situations of obesity remains limited.

Table 1. Studies of extracellular vesicles in obesity.

Author, Year (Refs.)	Source of Isolation	EVs Size /Method of Isolation	EVs Classification	Specific Cell Marker	EVs Characteristics	Main Finding
Goichot, 2006 [42]	Plasma	NR [A]	MP	Annexin V	Increase in EVs concentration (ug/mL)	Negative association with BMI
Esposito, 2006 [43]	Plasma	NR [A]	MP	CD31, CD42	Increase in the number of EVs	Association with waist-hip ratio; C-reactive protein; HOMA-IR
Murakami, 2007 [44]	Plasma	NR [A]	MP	CD41	Increase in the number of EVs	Association with BMI; waist circumference; subcutaneous body fat
Stepanian, 2013 [45]	Plasma	NR [A]	MP	CD41, CD31, Annexin V	Increase in the number of EVs	The characteristics of EVs are independent of the metabolic syndrome
Kranendonk, 2014 [46]	Explant subcutaneous and omental adipose tissue	NR [B]	EVs	CD9 Adiponectin	Association between the amount of EVs and WC and liver enzymes	Adipose tissue EVs can stimulate or inhibit insulin signaling at the liver level, depending on their adipokine content
Campello, 2015 [47]	Plasma	NR [A]	MP	Annexin V, CD62, CD61, CD45	Increase in the number of EVs	Association with BMI, waist, fibrinogen, IL6, and FVIII; overproduction of EVs could induce the generation of thrombin
Koeck, 2015 [48]	Subcutaneous and visceral adipose tissue	50–100 nm [C]	EXO	CD63	Increase in EVs concentration (ug/mL)	Higher BMI decreases the concentration of EVs
Togliatto, 2016 [41]	Visceral adipocyte stem cells primary culture	<1000 nm [D]	EVs	CD63, CD81	No apparent change in size or quantity	Obesity impacts on the proangiogenic potential of EVs
Eguchi, 2016 [49]	Adipose tissue	NR[D]	EXO & ET	Perilipin A	Increase in EVs quantity	Association with biomarkers: glucose, insulin, and HOMA-IR; presence of perilipin A in adipocyte EVs
Mleczko, 2018 [50]	Plasma and adipocytes culture	100–150 [D]	EXO	CD81, MHCI TSG101	No apparent change in size or quantity	EVs of obese subjects decrease insulin-stimulated 2-deoxyglucose caption in adipocytes

Table 1. *Cont.*

Author, Year (Refs.)	Source of Isolation	EVs Size /Method of Isolation	EVs Classification	Specific Cell Marker	EVs Characteristics	Main Finding
Mendivil, 2019 [51]	Plasma	<100 nm [C]	EXO	ALIX	Increase in size of EVs	Association with BMI, TG, and % body fat
Santamarina, 2019 [52]	Plasma	<116 nm [D]	EVs	NR	Smaller EVs size	Glucose, HOMA-IR, BMI, TG, HDL, and HA1c
Reza, 2020 [53]	Plasma	161 nm [D]	EXO	CD63	No changes between groups were find	Participation in the insulin signaling pathway; increase in the intracellular content of TG and decrease the secretion of FGF21 in hepatocytes

BMI: body mass index; EVs: extracellular vesicles; EXO: exosomes; ET: ectosomes; method of isolation: [A] none reported, [B] sucrose gradient and ultracentrifugation, [C] synthetic polymer precipitation, [D] ultracentrifugation; MP: microparticles; MV: microvesicles; TG: triglycerides; WC: waist circumference; HOMA-IR: insulin resistance index; HDL: high density lipoprotein; HA1c: hemoglobin A1c.

Most of the studies on EVs have been conducted in murine models without metabolic complications; only a few have been carried out in obese subjects. In both, changes in the general characteristics of EVs, size, number, and content, e.g., nucleic acids (mRNA, miRNA, etc.), have been reported. Once these changes occur, they might be the subject of further changes, depending on the degree of obesity. Furthermore, some of these characteristics and the content of EVs have been positively correlated with indicators such as BMI and biomarkers such as glucose, insulin, and serum lipids [45,49,54–57], among others. This correlation has led some authors to suggest that the characteristics of EVs (level and size) are affected by the inflammatory microenvironment caused by obesity.

Studies in obese adults have found that the characteristics of EVs could depend on the level of development of obesity, but not on its associated metabolic complications [45]. Goichot, for example, reported that the increase in plasmatic EVs concentration in obese subjects could explain their higher risk of thrombotic complications [42]. Based on this, it has been suggested that endothelial and platelet-derived EVs could be involved in the pathogenesis of endothelial dysfunction in obesity. Additionally, the increase in the number and concentration of EVs has been correlated with the increase in the insulin resistance index (HOMA-IR) [43,49,52] and associated components in the insulin signaling pathway [53], as well as with high levels of triglycerides in blood and excess body fat [44,51]. Taken together, this research suggests that EVs could be involved in the development of metabolic complications, but more evidence is needed to reveal the cellular pathway by which these changes occur, which translate into metabolic alterations.

Despite the fact that most of the evidence to date suggests that EVs increase in number and size in many body fluids in obese people, there is one study that suggests otherwise. Santamaria et al. reported that miRNA cargo of plasma EVs are associated with obesity, as well as smaller sizes of EVs in obese women than in those with normal weight [52]. However, the number of small EVs isolated from obese and lean participants was found to be equivalent in obese and normal weight women [52]. It is not clear what explains these results, but Santamaria suggested that the significant differences in glucose parameters and increased fatness in obese women were responsible for the plasma EVs changes. In agreement with Santamaria et al., Durcin suggested that the production and expulsion of EVs occurs following exposure to different biological stimuli related to the chronic low-grade inflammation state associated with obesity [54].

So, the relationship that seems to exist between the characteristics of EVs and metabolic biomarkers suggests that EVs are also clear indicators of the development of metabolic disorders or diseases. This is promising for the study of EVs as biomarkers in excess weight morbidities. However, more research is needed in order to describe the specific role that EVs play in these pathological processes, as well as their subsequent validation as potential biomarkers in humans.

4. Extracellular Vesicles and Adipose Tissue

The study of EVs as potential biomarkers of alterations to nutritional status includes EVs from specific cells or tissues, such as endothelial, platelet, and adipose tissue [58,59].

It appears that alterations in the adipose tissue of MHO subjects have an influence on the characteristics of extracellular platelet and endothelial vesicles, which, in turn, seem to have an effect on the development of cardiovascular and metabolic diseases [45,47]. The reason for this, as has been explained, is that the adipose tissue of adults MHO secretes cytokines alter endothelial function and this, in turn, promotes the activation of the transcription factor NF-KB [60] and pro-inflammatory pathways. This mechanism has also been shown in murine models with hypertension [61–64]. Furthermore, it has been shown that the activation of NF-KB can also be stimulated by the EVs of macrophages [65] and adipocytes [50], which induce abnormalities in the glucose−insulin balance. The insulin-dependent decrease in glucose assimilation has been reported to be due to the inhibition, at least in part, of Akt phosphorylation [46,48], which in turn interferes with the translocation of GLUT-4 in adipocytes. Given this, it has been proposed that the decrease in insulin-stimulated glucose absorption is mediated by the activation of NF-KB induced by EVs [50]. However, this is just a small part of the role that EVs play in the main metabolic pathways involved in the development of comorbidities of obesity, such as insulin resistance.

Studies using adipose tissue explants (from obese adults) have shown changes in the expression of key proteins in signaling pathways, such as TFG-B, which is involved in the development of fibrosis in various processes of chronic inflammation, especially in the liver [48]. Koeck et al. reported that EVs from adipose tissue could play an important role in the pathogenesis and development of nonalcoholic fatty liver, commonly present in obesity [48]. Moreover, Eguchi et al. suggested that EVs from adipose tissue induce the recruitment and migration of macrophages, associated with obesity [57]. Furthermore, different EVs subpopulations (large and small), which differ in their lipid and protein content and that could be responsible for the inflammatory and metabolic alterations typical of obesity, have been found [54,66–68]. The mechanisms by which the content of EVs triggers any particular metabolic alteration, however, are not fully understood.

5. Extracellular Vesicles, Diet and Weight Loss

It is known that EVs are sensitive to many cellular stress situations, including sensitivity to nutrient deficiencies. Crewe et al. propose that EVs contain and transport proteins and lipids capable of modulating cellular signaling pathways between endothelial cells and adipocytes [69]. This transport event, which is made necessary in situations of fasting, refeeding, and obesity, is likely to be physiologically regulated so that EVs may participate in the tissue response to changes in the concentration of nutrients in the organism [69]. Thus, EVs could also be involved in the communication between adipose tissue and other cells. Gao et al. proposed that this involvement occurs as a means of communication between adipocytes and neurons [70]. This communication could modulate signaling pathways in the hypothalamus, which regulate appetite and weight gain.

A high-fat diet has been shown to cause morphometric changes (size) in EVs [56,71]. In addition, a high fat diet has been shown to cause changes in the expression of specific miRNAs contained in the EVs of hepatocytes, which, in turn, modulate the expression of various genes in other organs, such as the pancreas, causing hyperplasia in its islets [72]. Qi Fu et al. suggested that these changes caused by hepatocyte-derived EVs may be a compensatory measure of the B cells of the pancreatic islets under conditions of obesity

and insulin resistance [72]. The sensitivity of EVs to insulin has also been shown by Eichner et al., who reported that, in humans, after receiving a glucose load, their levels of circulating EVs in the plasma decrease, and that this reduction could be associated with arterial stiffness, physical exercise, and insulin sensitivity [73]. Taking this research as a whole, it suggests that EVs are sensitive to specific modification of lipids and glucose in the diet. It is conceivable that, in humans without metabolic complications, EVs are also sensitive to the modifications of macronutrients, but research is needed to prove this.

Diet modification in obese subjects is a therapeutic tool for weight loss and improvements in metabolic biomarkers [74,75]. The evidence suggests that changes in the levels of EVs in the event of weight loss depend on the amount of weight lost and the degree of excess weight [62]. In adults with a BMI >35 kg/m2, weight loss of >25% does not show significant differences in the amount of EVs before and after weight loss [76]. In contrast, when compared to a control group, adults with an average BMI of 26 kg/m^2 that presented weight losses of 5% had a significant decrease in the level of plasma EVs [44,75]. In addition, comparisons made between an excess vs. a normal weight group have shown a distinct EVs composition in the case of excess weight; in particular, differences in the profiles of proteins and nucleic acids (mainly miRNAS) involved in the development of cardiovascular diseases and diabetes [77–79].

The composition of EVs is known to be affected by changes in body weight, diet, and after bariatric surgery. Thrush reviewed the little information available on the changes in the characteristics of EVs that occur in successful- and unsuccessfully-treated subjects [80]. He reported that the EVs of obese patients that successfully respond to dietary treatment and weight loss stimulate oxidative metabolism in muscle cells to a greater extent than the EVs of obese patients resistant to treatment and weight loss [80]. This could help explain the variability that exists in response to dietary treatment and weight loss in obese subjects, but more evidence is needed to evaluate potential therapeutic goals to promote an appropriate dietary strategy for unsuccessfully treated patients.

Research has shown that the concentration and composition of EVs could change after bariatric surgery [47,81,82]. The physiological changes that occur after bariatric surgery also seems to bring about the modification of specific markers contained in EVs, which are implicated in the development of some alterations in adipose tissue, such as the free fatty acid transporter protein (FABP4) [82]. Given that FABP4 is mainly expressed in adipocytes, its changes in EVs after this surgical procedure could reflect the changes that occur in the adipose tissue, such as a reduction of adipocytes and of the total fat mass [82].

Although it is known that EVs are particularly sensitive to the early development of metabolic and cardiovascular disorders, the physiological role that they play in the pathogenesis of these disorders needs to be better understood so that they can eventually be used as biomarkers of these conditions, even before the first symptoms appear. Furthermore, more research is needed to validate their use in a clinical setting.

6. Extracellular Vesicles in Undernutrition

To date, the evidence pertaining to EVs in situations of undernutrition is limited and most of it has been developed in murine models or in vitro studies. For this reason, the available evidence on EVs and muscle depletion will be considered as a means to understand their possible involvement in undernutrition.

The skeletal muscle is one of the largest organ systems in the human body, representing ~40% of the body weight of an average adult. It plays a major role in maintaining homeostasis [4,83]. For instance, muscle mass plays an important role in the storage of glucose and amino acids, which are used by the body in stress or fasting situations, providing the backbone for the liver and gluconeogenesis process. Likewise, it plays an important role in the development of insulin resistance and other metabolic diseases [84]. However, there are situations that can affect the muscle mass composition and size, such as physical activity, chronic inflammation, sarcopenia, and malnutrition [83,85,86].

The communication between the muscle and other tissues such as adipose tissue, liver, and pancreas, is carried out through the release of myokines, "cytokines or peptides which are secreted by skeletal muscle cells and subsequently released into the circulation to exert endocrine or paracrine effects in other cells, tissues or organs" [87]. Researchers studying the transport mechanisms used by myokines to reach the bloodstream have suggested that they are transported by EVs [88]. This makes sense, as it is known that the muscle, like any other tissue in the body, releases EVs in the course of pathological conditions such as cancer [89], HIV [90], heart attacks [91], and kidney disease [92], or due to specific stimulus such as exercise [93]. Some authors have even suggested that the beneficial health effects that occur as a result of exercising are due to the content of myokines and miRNAs, among others, which are produced by muscle cells and transported via EVs [94,95]. Others have suggested that most of the circulating EVs during exercise are released by the muscle tissue, as it is the organ with the highest secretory activity [93,96,97]. The mechanisms for this process have not yet been fully understood. However, we could assume that the secretory activity of the muscle is not only reflected in the response to a specific physical activity, but also in response to another type of stimuli or condition that directly affects the proper functioning of muscle mass.

Based on the evidence that the muscle is responsible for the release of EVs during exercise, we could assume that this same process is replicated in other circumstances where the muscle is affected—among them, muscle loss in situations of undernutrition. Muscle mass is lost in undernourished adults in response to a deficient consumption of protein and to inflammatory response caused by pathological processes, which could manifest chronically or acutely [98]. The degree of inflammation is a key factor in the development and severity of undernutrition, including the development of extreme undernutrition (cachexia) [99]. Cachexia typically occurs in response to affections such as cancer, infectious diseases, or some autoimmune disorders [100]. Consequently, it leads to a greater propensity to develop infections, to a diminished response to pharmacological treatments, and to higher mortality [101].

The evidence on EVs and muscle wasting shows that these play a role in the development of cancer cachexia [102,103]. It has been proposed that the communication between cancer cells and other organs and tissues can cause an endocrine effect on muscle tissue. A mechanism has also been proposed in which EVs participate in muscle wasting, whereby the EVs content of HSP70/90 heat shock proteins activate, at the membrane level, the signaling pathway by TLR-4, which, in turn, triggers the degradation of regulatory and myofibrillar proteins [103]. It has also been hypothesized that the miRNA content in EVs promote myoblast death in murine cancer models via the TLR7 pathway [102]. Furthermore, various miRNAs in EVs, which are probably involved in the development of cachexia, have been identified and shown to participate in altering the signaling pathways that induce muscular apoptosis or dystrophy of this tissue [104,105], although the circulating levels of EVs with miRNAs are mainly attributed to the proliferation and communication of cancer cells and not specifically to muscle wasting. The identification of changes in EVs (content and composition) in neoplastic cachexia could potentially be a marker of muscle loss and wasting. Given this sensitivity of the EVs to this condition, which results in muscle loss and wasting, it could be hypothesized that undernutrition, free of cancer, could also bring about changes in EVs, which in turn could cause the muscle waste that typically accompanies undernutrition. To date, little is known about the changes in the size, characteristics, and content of EVs in undernutrition status when free of any disease.

Given the evidence that muscle is one of the most active tissues in releasing EVs into the blood stream during exercise and that inflammatory processes (e.g., cancer) affect the content of EVs, one could assume that the typical malnutrition abnormalities occur in response to the transport of proteins and of different nucleic acids transported by EVs. Thus, EVs could be responsible for or initiators of the typical muscle depletion observed in conditions of undernutrition. However, to our knowledge, there is no study that has tested this theory. Although much remains unknown about the physiological

mechanisms that EVs follow in situations of undernutrition, if our theory is correct, the content and characteristics of EVs could serve as an early risk marker of muscle depletion in situations without associated comorbidities; for example, this could apply to patients with anorexia nervosa, as well as those undergoing muscle mass loss due to natural physiological changes associated with ageing, or due to lack of protein consumption for those with food insecurity. If EVs could be used as biomarkers of early risk of undernutrition, this would also contribute to the development of new therapeutic strategies for the prompt treatment of muscle depletion, which is a feature of this nutritional status. This is important, as current biomarkers only detect the condition in advanced stages.

7. Conclusions

The sensitivity of EVs to the cellular microenvironment could reflect early cellular alterations related to conditions of malnutrition (undernutrition and obesity). Despite the limited research to date on EVs in the area of nutrition, research in this area is increasing and could herald the discovery of mechanisms involved in the development of malnutrition and its pathological complications. This may lead to a better understanding of how EVs participate in or promote cellular signaling in malnutrition situations, which could help in the development of new strategies to treat them and their comorbidities. Thus, EVs could come to be excellent future biomarkers of early conditions associated with malnutrition and help to close the gap between the deterioration of health and time to detection. Of course, the use of EVs as biomarkers would not substitute the use of current ones. Rather, their use seeks a greater understanding of the physiological changes that occur prior to the development of health complications associated with malnutrition. This could also lead to them being used as a routine diagnostic tool in the future.

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3. EXTRACELLULAR VESICLES AND THEIR ZETA POTENTIAL AS FUTURE MARKERS ASSOCIATED TO NUTRITION AND MOLECULAR BIOMARKERS IN BREAST CANCER.

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Extracellular Vesicles and Their Zeta Potential as Future Markers Associated with Nutrition and Molecular Biomarkers in Breast Cancer

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Abstract: A nutritional intervention promotes the loss of body and visceral fat while maintaining muscle mass in breast cancer patients. Extracellular vesicles (EVs) and their characteristics can be potential biomarkers of disease. Here, we explore the changes in the Zeta potential of EVs; the content of miRNA-30, miRNA-145, and miRNA-155; and their association with body composition and biomarkers of metabolic risk in breast cancer patients, before and 6 months after a nutritional intervention. Clinicopathological data (HER2neu, estrogen receptor, and Ki67), anthropometric and body composition data, and plasma samples were available from a previous study. Plasma EVs were isolated and characterized in 16 patients. The expression of miRNA-30, miRNA-145, and miRNA-155 was analyzed. The Zeta potential was associated with HER2neu ($\beta = 2.1$; $p = 0.00$), Ki67 ($\beta = -1.39$; $p = 0.007$), estrogen positive ($\beta = 1.57$; $p = 0.01$), weight ($\beta = -0.09$; $p = 0.00$), and visceral fat ($\beta = 0.004$; $p = 0.00$). miRNA-30 was associated with LDL ($\beta = -0.012$; $p = 0.01$) and HDL ($\beta = -0.02$; $p = 0.05$). miRNA-155 was associated with visceral fat ($\beta = -0.0007$; $p = 0.05$) and Ki67 ($\beta = -0.47$; $p = 0.04$). Our results reveal significant associations between the expression of miRNA-30 and miRNA-155 and the Zeta potential of the EVs with biomarkers of metabolic risk and disease prognosis in women with breast cancer; particularly, the Zeta potential of EVs can be a new biomarker sensitive to changes in the nutritional status and breast cancer progression.

Keywords: nutritional status; extracellular communication; exosomes; microvesicles

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1. Introduction

The lifestyle and food intake prior to the diagnosis of breast cancer influence the patients' body composition; additionally, changes in body composition over the course of the disease can be a secondary effect of antineoplastic treatments [1,2].

The nutritional status and body composition are critical to cope with different conditions and treatments, including breast cancer [3]. Increased fat mass, loss of muscle mass, and/or bone mineral density can influence the disease prognosis in women diagnosed with breast cancer undergoing antineoplastic treatment [4–6]. Imbalances in body composition may increase the risk of toxicity from antineoplastic drugs, complications in surgical interventions, disease progression, and metastatic recurrence [7,8].

A pre-test post-test study conducted by our research group showed positive changes in the body composition of women recently diagnosed with breast cancer ($n = 22$) [9], where an individualized nutritional intervention was designed using the dynamic macronutrients meal-equivalent menu method [10]. In the above-mentioned study, the dietary plans were designed according to the clinical practice guidelines of the World Cancer Research Fund American Institute for Cancer Research [11] and the review of current evidence [12]. Resting energy expenditure was calculated with an equation designed in the Mexican population [13]. In patients with a BMI >25 kg/m^2, a caloric restriction of 500–1000 kcal per day was made. The macronutrient distribution of the total caloric value was as follows: protein 1.2–1.5 g of protein per kilogram of weight, fat <30%, and carbohydrates 50%. Moreover, anthropometric and body composition analyses were performed on the patients at the beginning and 6 months after the nutritional intervention and antineoplastic treatment (weight, height, waist circumference, dual X-ray densitometry analysis [DXA], and electrical bioimpedance).

Results showed a significant loss of visceral fat, body weight, and no change in appendicular skeletal muscle mass, bone mineral density, and fat-free mass, after 6 months of nutritional intervention [9]. However, the changes in body composition discussed above can be detected after the first 6 months of antineoplastic treatment, when using conventional nutritional assessment tools and techniques. Further research into more sensitive techniques is needed at the molecular level, to better understand the molecular changes that occur in the early stages of disease development as well as in response to a nutritional intervention, instead of relying exclusively on traditional biomarkers for metabolic control and cancer progression. A biomarker is defined as a "characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions" [14]. Although biomarkers used in nutrition and cancer can detect changes in body composition, disease progression, the effect of a medication or antineoplastic treatment, etc., they are only sensitive at later stages of disease progression, and there is little to no specificity for nutritional changes [1,15].

The discrepancies between biomarkers used in clinical practice and research have been exposed, limiting the understanding of chronic diseases and nutrition [16]. Most biomarkers used in nutrition research (e.g., albumin, creatinine, glucose, etc.) are related to the development and progression of diseases. Still, there is a lack of biomarkers to assess early nutritional alterations or the response to nutritional interventions. This situation has driven the search for new biomarkers [2], among which extracellular vesicles (EVs) stand out.

In the last decade, EVs have been studied due to their active role in cell communication (see Mendivil et al., 2022 [17]). EVs are microparticles released by all cells into the extracellular space; their release and content respond to the conditions of the cellular microenvironment [18,19]. Using the classification of the International Society of Extracellular Vesicles, EVs are divided according to their size (nm) into small EVs and large EVs [19,20]. EVs are identified by specific markers that include but are not limited to CD63, CD81, ALIX, and TSG101 [19]. Additionally, the enrichment of proteins and markers in either the content or cytoplasmic and/or transmembrane proteins of EVs is highly varied. What determines the EVs content is still under investigation, but it is believed to depend on the nature of the cell where the EVs come from, as well as the conditions of the cellular microenvironment or the presence of diseases such as cancer.

As described above, EVs are sensitive to cellular stress, making EVs and changes in their characteristics (i.e., size, content, or Zeta potential) potential biomarkers in pathological conditions, ranging from neurodegenerative and chronic-degenerative diseases to breast cancer [21–24]. Among EVs characteristics, the Zeta potential is a biophysical property that explains the charges on the surface of a particle, resulting in electrostatic potential differences [25,26]. The Zeta potential gives EVs the ability to interact with other distal tissues and even with other EVs; however, little has been described about the changes in the Zeta potential for EVs throughout the course of diseases or support therapies (e.g., nu-

tritional intervention). It is still unknown whether the use and validation of Zeta potential can be considered as a biomarker of the nutritional status and/or follow-up of nutritional interventions [27,28].

The nucleic acid content of EVs (i.e., miRNAs) has also been studied. miRNAs are short segments of RNA (~22 bases) that regulate gene expression through base pairing with complementary sequences of the target mRNAs [29]. Most of the detectable miRNAs in saliva and serum are concentrated in small EVs [30]. Therefore, miRNAs are transported from one tissue to another using EVs as a vehicle that provides protection from the transgressions of the extracellular environment. Specifically in breast cancer, miRNA-145, miRNA-30, and miRNA-155 have been identified as responsible for promoting angiogenesis, progression, and tumor invasion [31–35]; additionally, their expression has been associated with resistance to antineoplastic treatment and tumor recurrence [36]. Even when miRNA-145, miRNA-30, and miRNA-155 have been identified in breast cancer patients, where they play an active role in the breast cancer process, the information is limited regarding how these miRNAs that are being transported in EVs are involved in the progression of the disease or the development of comorbidities and metastases, as well as if they can be potential biomarkers for follow-up after a nutritional intervention.

Herein, we aim to explore the changes in the Zeta potential of EVs, as well as their miRNA-30, miRNA-145, and miRNA-155 content in plasma EVs of women recently diagnosed with breast cancer before and after 6 months of an individualized nutrition intervention and during active antineoplastic treatment.

2. Results

Herein, we show the analysis of EVs characteristics (the size, Zeta potential, and their content) in a subsample of women diagnosed with breast cancer, who participated in a quasi-experimental study in which they received a nutritional intervention during the first 6 months of antineoplastic treatment (see [9]). In this subsample, even when the number of volunteers decreased compared to the work by Limon-Miro et al. [9], the positive changes in body composition, such as decrease in total fat, visceral fat, and weight, were maintained.

Table 1 shows anthropometric and body composition characteristics in the sub-sample (n = 16) of women at baseline and after 6 months of a food-based nutritional intervention during active antineoplastic treatment.

Table 1. Anthropometric and body composition characteristics of 16 women diagnosed with breast cancer at baseline and after 6 months food-based nutritional intervention during active antineoplastic treatment [1].

Variables	Nutritional Intervention		p-Value
	Baseline	6 Months	
Weight (kg)	72.5 (21.5)	69.6 (19.5)	0.002
Body mass index (kg/m^2)	26.4 (8.1)	26.2 (7.3)	0.002
Fat mass (kg)	30 (16.9)	26.3 (16.2)	0.002
Fat mass (%)	41.6 (7.95)	39.3 (7.4)	0.03
Fat mass index (kg/m^2)	11.1 (5.3)	10.1 (5.7)	0.002
Visceral fat (g)	697 (530.7)	590 (582.2)	0.01
Fat-free mass (kg)	40.3 (8.9)	41.1 (9.73)	0.08
Appendicular mass index (kg/m^2)	5.1 (1.6)	5.7 (1.48)	0.06

[1] Data are presented as median (IQR) interquartile range.

Additionally, we analyzed the plasma concentration of biochemical biomarkers. At baseline, their median concentrations were glucose (114 mg/dL), triglycerides (109 mg/dL), triglyceride-glucose index (TyG index [8.6]), total cholesterol (206 mg/dL), HDL (52 mg/dL), VLDL (21 mg/dL), and LDL cholesterol (133 mg/dL). These values were not statistically different at the end of the intervention ($p > 0.05$).

In this study, we isolated small- and large-sized EVs at baseline and 6 months after a food-based nutritional intervention. The median size of isolated EVs at baseline was 141 nm and 174 nm after 6 months ($p > 0.05$). Results show that the median Zeta potential was −8.8 mV at baseline and −8.0 mV at 6 months after the intervention ($p > 0.05$). When the morphology and size of the EVs were evaluated using transmission electron microscopy (TEM), spheroid shape vesicles with structures delimited by membranes were observed, consistent with the morphology of EVs. These EVs were dispersed and did not form aggregates; the approximate EV size was 200 nm (Figure 1A). Moreover, the expression of specific EV markers was confirmed (Figure 1B).

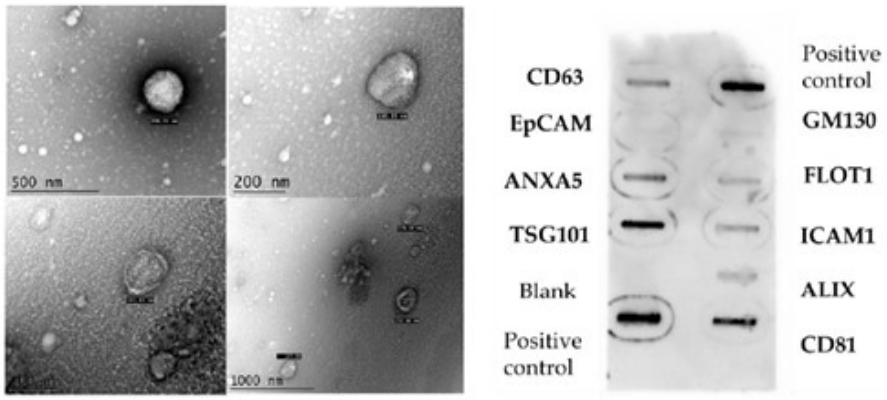

Figure 1. Morphology of the EVs through TEM and the expression of specific EVs markers. (**A**) The morphology and size of EVs using TEM; (**B**) Identification of specific EVs´ markers using the Exo-Check Exosome antibody Array™.

In this study, we also analyzed the EVs content for the expression of miRNA-145, miRNA-155, and miRNA-30 at baseline and after 6 months. The results showed a relative expression of 1.0 and 1.14 for miRNA-145; 0.72 and 0.84 for miRNA-155; and 1.0 and 0.93 for miRNA-30, before and after the nutritional intervention, respectively. However, the nutritional intervention had no impact on the normalized relative expression of the analyzed miRNAs ($p > 0.05$).

Results for the regression analyses for mixed effects models are summarized in Tables 2 and 3. The outcome variables were the EVs diameter (nm), Zeta potential (mV), and their expression of miRNA-30, miRNA-145, and miRNA-155. As explanatory variables, we included anthropometric variables (weight and height), indexes (BMI, fat mass index, lean mass index, and triglyceride-glucose index [TyG index]), body composition (fat mass and visceral fat), physical activity (min/d), biochemical biomarkers (glucose, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides), tumor molecular biomarkers (HER2neu, Ki67, and estrogen positive), and grade of breast cancer tumor (II, III). The EV size and miRNA-145 expression were not associated with any of the explanatory variables in the mixed effects models analysis ($p > 0.05$). The regression analyses for mixed effects models for the outcome variables miRNA-30, miRNA-155, and Zeta potential are summarized in Table 2.

Table 2. Regression analyses using mixed effects models using miRNA-30, miRNA-155, and the Zeta potential as outcome variables in 16 women diagnosed with breast cancer.

Outcomes	miRNA-30				miRNA-155				Zeta Potential			
Explanatory Variables	**Regression Coefficient**	***p*-Value**	**95%CI**		**Regression Coefficient**	***p*-Value**	**95%CI**		**Regression Coefficient**	***p*-Value**	**95%CI**	
TyG index [1]	−0.375	0.344	−1.151	0.401	0.009	0.97	−0.493	0.512	0.231	0.706	−0.9696	−1.432
Glucose (mg/dL)	−0.006	0.394	−0.020	0.008	−0.0001	0.977	−0.009	0.008	−0.017	0.092 *	−0.038	0.002
VLDL (mg/dL)	−0.030	0.267 *	−0.084	0.023	−0.003	0.835	−0.036	0.029	0.057	0.125 *	−0.015	0.13
LDL (mg/dL)	−0.012	0.003 *	−0.020	−0.004	−0.004	0.204 **	−0.010	0.002	−0.008	0.239 **	−0.023	0.005
HDL (mg/dL)	0.024	0.121 *	−0.006	0.054	0.015	0.118 *	−0.003	0.033	−0.015	0.564	−0.066	0.036
Triglycerides (mg/dL)	−0.001	0.597	−0.009	0.005	0.0008	0.708	−0.003	0.005	0.007	0.124 *	−0.002	0.017
Total cholesterol (mg/dL)	−0.009	0.021 *	−0.016	−0.001	−0.002	0.461	−0.007	0.003	−0.005	0.374	−0.018	0.007
Lean mass index (kg/m^2)	−0.094	0.576	−0.428	0.238	−0.103	0.326	−0.310	0.103	0.013	0.957	−0.492	0.520
Visceral fat (g)	−0.0006	0.289 *	−0.001	0.0005	−0.0007	0.087 *	−0.001	0.0001	0.001	0.21 **	−0.0007	0.003
Fat mass index (kg/m^2)	−0.028	0.698	−0.171	0.114	−0.081	0.062 *	−0.167	0.004	0.235	0.029 *	0.024	0.446
Physical activity (minutes)	−0.0001	0.898	−0.002	0.002	0.0001	0.84	−0.001	0.001	−0.002	0.192 *	−0.005	0.001
Weight (kg)	−0.015	0.298 *	−0.043	0.013	−0.017	0.05 *	−0.035	0.00003	0.026	0.291 **	−0.022	0.075
Ki67	0.408	0.334	−0.419	1.235	0.462	0.086 *	−0.065	0.989	−0.937	0.199 *	−2.368	0.493
ER-positive	−0.004	0.993	−0.966	0.958	−0.378	0.242 **	−1.013	0.255	1.643	0.032 *	0.138	3.147
HER 2/NEU (+)	0.852	0.048 *	0.006	1.698	0.066	0.834	−0.553	0.686	1.374	0.063 *	−0.075	2.825
Luminal A	−0.515	0.575	−2.315	1.284	−0.292	0.63	−1.486	0.900	−1.206	0.456	−4.383	1.969
Luminal B	−0.808	0.384	−2.626	1.010	−0.707	0.25 **	−1.912	0.498	−0.128	0.937	−3.338	3.080
BC Grade II	0.102	0.876	−1.191	1.397	−0.077	0.862	−0.950	0.795	−0.947	0.423	−3.266	1.370
BC Grade III	0.124	0.886	−1.570	1.819	0.412	0.479	−0.730	1.555	−1.082	0.485	−4.118	1.953

[1], [fasting triglycerides (mg/dL) × fasting plasma glucose (mg/dL)/2] [39]. ER-positive, estrogen positive; HER2/NEU(+), human epidermal growth factor 2; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; 95%CI, confidence intervals; MC, molecular classification of the tumor. BC, breast cancer grade. * When $p \leq 0.2$ in the univariate analysis, the variables were considered for the stepwise multivariate analysis. ** Variables selected by suggestion of the conceptual framework and they were of interest to the research group.

Table 3. Mixed model effects analysis for miRNA-30, miRNA-155 expression, and the Zeta potential in 16 women diagnosed with breast cancer.

Outcomes	Explanatory Variables	Regression Coefficient	*p*-Value	95%CI		AIC
miRNA-155	Visceral fat (g)	−0.0007	0.050	−0.001	-7.04×10^{-7}	87.731
	Ki67	0.478	0.049	0.002	0.953	
Zeta potential	ER-positive	1.572	0.010	0.370	2.775	132.326
	HER2/NEU(+)	2.159	0.000	1.111	3.206	
	Ki67	−1.391	0.007	−2.401	−0.381	
	Weight (kg)	−0.094	0.005	−0.160	−0.028	
	Visceral fat (g)	0.004	0.000	0.002	0.0075	
miRNA-30	HDL (mg/dL)	0.026	0.053	−0.0003	0.053	116.137
	LDL (mg/dL)	−0.012	0.001	−0.020	−0.004	

AIC, Akaike Information Criteria; 95%CI: confidence intervals; ER-positive, estrogen positive; HER2/NEU(+), human epidermal growth factor 2; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Prior to the multivariate analysis, we performed a univariate analysis of the Zeta potential, selecting the following explanatory variables: HER2, Ki67, physical activity, weight, fat mass index, visceral fat, triglycerides, VLDL cholesterol, LDL cholesterol, and glucose (Table 2). To test our hypothesis on the influence of body composition on the characteristics of EVs, and in view of our previous results [37], we decided to include the variables weight, LDL cholesterol, and visceral fat in the multivariate analysis, despite their *p*-value limiting to 0.2. For the multivariate model, we excluded the fat mass index variable due to collinearity with two previously selected variables: weight and visceral fat (Table 2). In the end, the best variables that explained the Zeta potential of EVs were ER positive, HER2, Ki67, weight, and visceral fat (Table 3). The Zeta potential was negatively associated with Ki67 and weight, and positively associated with visceral fat, HER2, and ER positive (see Table 3).

On the other hand, for the univariate analysis of miRNA-155, the following variables were selected as explanatory variables: tumor molecular classification, ER positive, Ki67, weight, fat mass index, visceral fat, and HDL and LDL cholesterol (Table 2). We excluded the fat mass index variable, to avoid collinearity with weight and visceral fat. On the multivariate model, we decided to include the variables of molecular classification of the tumor, ER positive, and LDL cholesterol due to their previously reported association with the expression of miRNA-155 [38] and fat mass in patients with breast cancer; additionally, we included variables whose *p*-value was close to 0.2 (Table 2). The multivariate model showed that visceral fat (negative) and Ki67 (positive) were significant, and when combined, they formed the best association model (Table 3). Although the significance value for visceral fat is borderline at 0.05, it indicates a trend in its association with miRNA-155.

The selection of explanatory variables for the miRNA-30 were HER2, weight, visceral fat, total cholesterol, VLDL, LDL, and HDL (Table 2). On the multivariate analysis, we decided to exclude total cholesterol and include instead its components HDL, LDL, and VLDL, to be more specific with potential associations. For the multivariate analysis, we selected HDL and LDL cholesterol. We found a positive association between miRNA-30 expression and HDL cholesterol, and a negative association between LDL cholesterol (Table 3). Although HDL cholesterol has a level of marginal statistical significance ($p = 0.053$), we included it considering that the subsample of patients in our study is limited; thus, associations should be interpreted with caution until they are verified in a larger sample (Table 3).

3. Discussion

In this study, we isolated extracellular vesicles from the plasma of breast cancer patients and found EVs size to be classified as small and large. Changes in body composition of the women included in the sub-sample of the study are similar to that previously reported by our research group for the study population [9]. Despite the changes in body

composition, there were no significant changes in traditional biomarkers such as glucose, insulin resistance, and serum lipids, at the beginning and end of the intervention.

In search of potential biomarkers that are sensitive to early nutritional changes, we found the characteristics of the EVs such as content, size, and their membrane Zeta potential, which to our knowledge has been poorly discussed in the literature.

3.1. Different Populations of Extracellular Vesicles

Extracellular vesicles have been proposed as cellular communication vehicles and as potential biomarkers for early disease diagnosis, treatment response, and prognostic monitoring [40–42]. Even though the use and validation of EVs are still being studied, the change in their characteristics (size, Zeta potential, or content) can provide information about the response that occurs at the cellular level to external aggressions, specific conditions such as therapeutic interventions [43–46], and potentially nutritional support therapies. Herein, we obtained small and large populations of EVs, but no significant difference in size was observed before and after the intervention, despite evidence suggesting that changes in EVs characteristics are associated with the nutritional status of the study subjects [47–54]. Most literature to date reports the use of the EVs size to classify them and do not use their size as a potential biomarker and its association with patient's characteristics.

The changes observed in the characteristics of EVs in this work add to the limited evidence that exists to date on changes in the size and content of EVs in breast cancer, where it is suggested that EVs population size in cancers are mostly (but not limited to) small EVs [55]. Available literature is contrasting; on one hand, small EVs have been reported the most, and on the other, EVs larger than 1 µm [55] have been associated with progression of cancer and other processes related to the disease. EVs larger than 1 µm are suggested as large oncosomes, which belong to large EVs [19]; oncosomes have been characterized in the last decade as solely coming from tumor cells, so their content and membrane components have been related to tumor development and cancer progression [55–57]. However, in our results, we found EVs with sizes smaller than 1 µm; small EVs are also reported as potential carriers of key integrins in the development of metastases, such as the integrin ITGB3, which can favor communication between breast cancer cells [58]. In any case, the relation of EVs size should continue to be studied as an essential variable to better understand the process of progression and evaluation in cancers.

3.2. Associations between the Zeta Potential and Patient Characteristics

The EVs membrane is a highly interactive and dynamic surface area that is responsible for facilitating EVs interactions with the extracellular environment [59]. It has been suggested that the interactions of small EVs act as modulators of signaling in biological processes, such as metastasis, cancer regulation, and tissue regeneration [60]. Some of the mechanisms proposed to understand the interaction of EVs with other tissues are as follows: (1) fusion of the plasma membrane of EVs with the target cell, releasing its content inside; (2) vehicles of proteins and cellular components; upon arrival to the target cell, they can be fragmented by the action of proteases and act as a ligand for target cell receptors; and (3) endocytosis and pinocytosis [59,61]. Our results showed that the Zeta potential of EVs had several significant associations with clinical characteristics of the patients, as well as weight and visceral fat.

Based on our results, we hypothesize that the association of the Zeta potential and the transmembrane protein complex HER2 could be explained by a certain charge and membrane intensity that HER2 can exert over the EVs, contributing to its affinity for specific tissues. However, the evidence is limited [62], and more studies incorporating the Zeta potential within the characterization of EVs are needed. The presence of HER2 in the plasma EVs of patients with breast cancer has been reported [63]; additionally, the study conducted in 2016 by Sina et al. [64] showed that in small EVs from women diagnosed with breast cancer, 14 to 35% were enriched with HER2. Fei Tian et al. [65] suggest that the presence of proteins in the EVs membrane of tumor cells from different cell lines will confer

a particular intensity percentage when using light scattering analysis; these properties will allow EVs to be differentiated among themselves, while potentially allowing their use in diagnostic, prognostic, and early detection of biomarkers, or in response to a specific pharmacological treatment [65,66]. Studies have shown that the presence of HER2 can be a promising biomarker in the identification of EVs whose origin is breast or prostate cancer [67]. Additionally, the heterogeneity in the components of EVs membrane from cancer, which can include HER2, has been associated with the overall survival of cancer patients [66]. To date, only a limited number of studies have tested the above results as predictive biomarkers of survival in cancer patients [68]; however, it is interesting to see how the understanding of the increasingly fine connections of EVs are being traced in their participation in cellular and molecular processes.

Our results showed that for the explanatory model of the Zeta potential, the variables included were visceral fat and weight. In studies of EVs and body composition, the EVs content was associated with the total and visceral fat mass [37,51,69,70]. Specifically, miRNAs linked to the development of comorbidities associated with excess weight have been identified in EVs from visceral adipose tissue; these microRNAs are linked to macrophage infiltration in adipose tissue, insulin resistance, and endothelial damage, among others [47,71]. Likewise, the size and composition of the EVs membrane have been positively associated with the amount of total body fat in overweight individuals [37,51,72,73]. However, to date, there is no evidence that links the amount of visceral fat with the Zeta potential of EVs in cancer. The Zeta potential of the EVs is determined by the amount and distribution of phospholipids, proteins, and carbohydrates of the membrane [74]. The positive association between visceral fat and the Zeta potential observed in our model could be associated with the loss of visceral fat that the women experienced in response to the intervention, in turn altering the composition of the EV membrane; unfortunately, there is no additional evidence to support our theory.

The Zeta potential determined in isolated EVs of women diagnosed with breast cancer was negative; this finding is similar to previous reports [40]. It is proposed that the negative charge can be a function of the content of carbohydrates, lipids, and proteins, as well as the side chains of its different ligands and receptors such as aspartate, lysine, arginine, and histidine, among others [40,75]. Additionally, EVs charge may be associated with transmembrane proteins such as tetraspanins, MHC class I (major histocompatibility complex), integrins, and GPI-anchored molecules (CD55 and CD59) that have been found in small EVs [20], as well as high levels of cholesterol, ceramides, and sphingomyelin [76,77]. In large EVs, its main components are phosphatidylserine, flotillin-1, and B1 integrins [78,79]. The components discussed above could coexist in the same group of EVs and confer a specific membrane charge.

The membrane composition and the Zeta potential of EVs will allow them to be part of different cell signaling pathways such as apoptosis, tumor growth, and proliferation, among others [48,80,81]. Some researchers have suggested that the protein content of EVs will depend on the cell of origin; for example, in breast cancer, Akagi et al. [82] suggest that depending on the cell line responsible for the disease, their load will be different and therefore communication in the cellular environment. However, it has also been proposed that the protein content of EVs can undergo post-transduction modifications such as glycosylation [46,77], which will expand their participation in different cellular signaling. Even the presence of glycans and glycoproteins have been studied as unique components of EVs originating from tumor cells, which would help to identify EVs in breast cancer and in subjects free of cancer [83]. In breast cancer, around 77 glycopeptides have been identified in plasma EVs from women with the disease, as well as changes in glycopeptides related to the type of cancer (metastatic and non-metastatic) [84], and its prognosis [85–87]. This suggests specific components of EVs as potential biomarkers for early diagnosis and response to treatment. However, validation studies are needed to corroborate the application of these as biomarkers. The identification of these components in EVs and their morphometric characteristics (Zeta potential and size) provide valuable information

on molecular targets, which can be used in the development of targeted pharmacological therapies and nutritional interventions that help maintain the nutritional status of patients with breast cancer.

Although we found associations between the Zeta potential and biomarkers of cell proliferation and response to treatment such as Ki67 and estrogen receptor, to date, the evidence is scarce to try and understand to a better extent the involvement of EVs and the changes in their Zeta potential during processes such as cancer. Our hypothesis suggests that EVs change their Zeta potential, depending on the physiological context, such as metastasis, development, or progression of cancer. The change in the surface charge of the EVs membrane can potentially function as the first step in a signaling cascade that would include all the mechanisms by which the EVs can produce an effect, from protein transport or content release, to acting as a ligand of the same target cell. Even though promising information is known to date about the participation of EVs in different cellular processes in cancer, we are far from understanding the process of EV participation in detail. However, the study of EVs represents a research challenge; it is in constant development and our results add to the evidence and narrow the existing knowledge gap.

3.3. Relevance of miRNA Expression-Containing Plasma EVs in Breast Cancer Patients

The extracellular vesicles can carry and transport regulatory molecules such as oncogenic proteins, coding and non-coding RNAs, DNA, and lipids between neighboring cells and to distant sites. The presence of miRNAs has been identified in extracellular vesicles from patients with bone marrow, bladder, lung, liver, and breast cancers [88–90]. The contents of EVs have been proposed as potential biomarkers of the above diseases, especially as biomarkers of early risk and prognosis in different types of cancer [91,92]. Some of the most studied components as biomarkers of early risk in cancer are miRNAs, including miRNA-145, miRNA-155, and miRNA-30.

3.3.1. Expression of miRNA-145 in EVs

The expression of miRNA-145 has been associated with tumor suppressor activity in breast cancer, inhibiting proliferation, migration, and invasion [93,94]; however, the suppressive activity and the mechanisms associated with the overexpression of miRNA-145 is still being studied. It has been suggested that tumor suppression by miRNA-145 is associated with the inhibition or stimulation of tumor angiogenesis; potential mechanisms of action are associated with the regulation of the insulin receptor substrate 1 (IRS1) gene, which suppresses N-RAS and VEGF-A (RAS mediates vascular endothelial growth factor), inhibiting tumor angiogenesis [32,95]. On the other hand, it has been suggested that tumor angiogenesis is promoted by reducing the expression of miRNA-145 in EVs from breast cancer cells [96]. The role of miRNA-145 has been highlighted in the development of metastasis and tumor proliferation in breast cancer cells by upregulating the expression of Transforming Growth Factor-beta Receptor 2 (TGFBR2) [97]. In addition, there is evidence that miRNA-145 could regulate tumor suppressor ZMYND10, because it can inhibit the miRNA-145 signaling pathway, thereby inhibiting the tumorigenicity of breast cancer [98].

On the other hand, the expression of miRNA-145 has been associated with key metabolic parameters in obesity and glucose metabolism, such as visceral fat area, HbA1c, plasma glucose, and circulating levels of leptin, adiponectin, and interleukin- 6 [99], as well as the risk of development cardiovascular disease (CVD).

Women with breast cancer have a higher risk of CVD and associated mortality, although the risk may vary depending on the history of antineoplastic treatment [100]. The risk of developing CVD is further increased in postmenopausal women [101]. Although our study subjects were free of cardiovascular diseases, some of them did have excess body weight [9], which can in turn increase the risk of developing CVD in adults with and without cancer [102]. In women with breast cancer, cardiovascular risk includes a series of factors ranging from eating habits, alcohol consumption, smoking, physical activity, and antineoplastic treatment. However, in breast cancer, the additional burden comes from the

antineoplastic treatment, since it can cause hypertension, arrhythmias, valvular disease, and pericarditis, among other issues [103]. The novelty of our results adds to the limited evidence on the presence of miRNA-145 in EVs of breast cancer patients, and its use as a sensitive biomarker associated with CVD.

Mohasen et al. [94] reported the role of small EVs secreted from adipose tissue-derived mesenchymal stem cells (MSCs) on the transfection of miRNA-145 into breast cancer cells; they aimed to weaken their expansion and metastasis. Their results show that miRNA-145 can be considered as a potential therapeutic strategy in breast cancer. The presence of miRNA-145 in EVs has been documented in small EVs in several diseases including cancer, such as ovarian cancer [104], where miRNA-145 has even been suggested as the most promising biomarker for the preoperative diagnosis of ovarian cancer [105]. miRNA-145 has also been reported in urinary EVs from prostate cancer patients [106], in vascular smooth muscle cells [107], colorectal cancer [108], hepatocarcinoma [109], cerebral injury [110], as well as its active role in the recovery of skeletal muscle mass in mice with chemotherapy [111], osteoarthritis [112], and pancreatic cancer [113].

3.3.2. Expression of miRNA-155 in EVs Was Associated with Ki67 and Visceral Fat

The presence of miRNA-155 in EVs from breast cancer has previously been reported [30,114,115]. Here, we showed the presence of miRNA-155 in EVs, but no significant difference was found between the initial and final expression; the limited sample size may have influenced the lack of power. The overexpression of miRNA-155 in metastatic breast cancer exosomes suggests its participation in the progression of breast cancer by suppressing the function of tumor suppressors such as PTEN and DUST14 [116,117]; the expression of miRNA-155 found in our study subjects may suggest that patients had a lower progression of breast cancer owing to downregulation, thereby promoting tumor growth and metastasis [118]. The underexpression of PTEN promotes the inhibition of Akt, a pathway that has already been reported to be deregulated in breast cancer [117,118]. Additionally, the expression of miRNA-155 has been previously reported and associated with resistance to antineoplastic treatment [36,119,120], tumor recurrence, and progression [119,121,122]. Likewise, the expression of miRNA-155 has been reported in EVs in diseases such as kidney cancer [123], lung injury [33], brain injury [124], and epilepsy [125], among others.

A low expression of miRNA-155 has been reported in patients under breast cancer treatment [126]. Stevic et al., reported a series of miRNAs contained in the EVs, associated with characteristics and clinicopathological parameters of breast cancer subtypes; coincidently, our results concur with their work, where they show an association in the expression of miRNA-155 with Ki67.

In breast cancer, the progression of the disease and the secondary effects of antineoplastic treatment promote the loss of muscle mass, reflected later in the development of cachexia [127,128]. The nutritional status of our study subjects improved post-intervention, but this might not have influenced the expression of miRNA-155. Evidence shows that the overexpression of miRNA-155 has been associated with a worsening of the nutritional status in patients with breast cancer [119]. It has been proposed that the development of alterations in the nutritional status of women with breast cancer is a function related to EVs, especially their content. In the case of miRNA-155, it has been suggested that EVs from breast cancer are emerging mediators of neoplastic cachexia [121], since they have an important role in the catabolism of adipocytes and muscle cells by targeting PPARγ (peroxisome proliferator-activated receptor gamma). In adipocytes, EVs from cancer cells contain miRNA-155, which promotes beige/brown differentiation and remodels metabolism in resident adipocytes by downregulating PPARγ expression but does not significantly affect biological conversion to C2C12 (myoblast cell line) [121]. Therefore, the transfer of miRNA-155 in EVs acts as an oncogenic signal that reprograms systemic energy metabolism and leads to cachexia associated with breast cancer [121,129].

In our results, we found the trend between visceral fat and expression of miRNA-155 of particular interest, because visceral fat was one of the body composition parameters

that had a significant loss post-intervention. Thus, we hypothesize that this loss of visceral fat has an implication beyond anthropometric changes and extends to molecular changes such as miRNA-155 expression. This evidence provides the foundation for future studies to investigate miRNA-155 and its association with body composition in a larger cohort of patients. Although the expression of miRNA-155 in EVs in cancer has been related to the downregulation of PPARγ expression, it could also be associated with visceral fat [127]. PPARγ is essential for the differentiation and proliferation of adipocytes, and it has also a beneficial effect on inflammatory processes of the vascular wall [130,131]. In this sense, it has been reported that EVs from smooth muscle produce endothelial injury and promote atherosclerosis in endothelial cells [132,133]. Additionally, miRNA-155 has been associated with crosstalk between fibroblasts and macrophages, as well as in cardiac repair after an acute myocardial infarction [134]. Considering the current information on miRNA-155, we hypothesize that the relationship between the expression of miRNA-155 and visceral fat in EVs could be related to the development of cardiovascular diseases due to excess weight. However, research is lacking for validating our hypotheses.

Our results add to the current evidence and provide essential information on the expression of miRNAs contained in EVs in women with breast cancer undergoing anti-neoplastic treatment and a nutritional intervention. Even though the current evidence continues to expand the participation of this miRNA in the development of processes, the exact mechanisms and the role of the EVs in the transport, expression, or overexpression of this miRNA are still under study.

Although the analysis of miRNAs in EVs is promising as a diagnostic and prognostic biomarker of disease, it is not intended to replace the use of current biomarkers used in clinical practice. Rather, the expression of different miRNAs contained in the EVs reveal important information about the transport that these carry under conditions such as cancer. This information contributes to the understanding of the EVs in the body under different scenarios; in this case, that of patients with breast cancer who received a nutritional intervention.

3.3.3. miRNA-30 Expression Was Associated with HDL and LDL Cholesterol

The expression of miRNA-30 has been related to different processes such as muscle depletion in situations of metabolic stress, muscular dystrophy [135,136], cardiovascular diseases [137], insulin resistance, diabetes [138], and breast cancer [35]. In our work, we found a negative association between LDL cholesterol and the expression of miRNA-30 and a positive association with HDL cholesterol. The association with metabolic biomarkers and miRNA-30 is still under study but the evidence from murine models suggests that this miRNA contained in EVs is a potential diagnostic biomarker for diabetes [138,139]. Likewise, in diabetes, miRNA-30 is attributed to active participation in the oxidation of fatty acids and endothelial dysfunction, suggesting it as a possible biomarker of coronary microvascular dysfunction [137,140], since the overexpression of miRNA-30 synergizes with exposure to fatty acids, thereby regulating eNOS (endothelial nitric oxide synthase) underexpression, a key regulator of microvascular function at the cardiac level [137].

In our results, the patients showed the expression of miRNA-30 in isolated EVs. A low expression of miRNA-30 in EVs has been associated with increased expression of genes involved in fibrosis and inflammation in ischemic remodeling, in murine and human models [141]. Herein, the expression of miRNA-30 was negatively associated with the level of LDL cholesterol and positively associated with HDL cholesterol, which may suggest an increased risk for the development of cardiovascular diseases in our study subjects. In murine models, miRNA-30, contained in EVs, has been proposed as a biomarker for the early diagnosis of cardiovascular disease, as well as a prognostic biomarker for tumor recurrence, improved survival, and metastasis in patients with breast cancer [142–146]. Additionally, miRNA-30 has been studied as a mediator in cancer invasion and migration by targeting KLF11 (krüppel-like factor 11), and activating the STAT3 pathway (Signal transducer and activator of transcription 3) [35]; these effects also play a role in different

types of tumors, controlling critical signaling pathways and relevant oncogenes [147]. Some oncogenes have been associated with the expression of miRNA-30 and other miRNAs, as well as other breast cancer biomarkers such as HER2 [148].

The association of miRNA-30 with biomarkers of cardiovascular risk indicates a possible participation in the metabolism of LDL and HDL cholesterol; however, more studies are needed to help us understand the exact mechanisms by which this recently found effect occurs.

Although our sample size was a limitation, the models and associations were statistically significant. However, the associations we found should be taken with caution, since the presence of non-vesicular components will include but are not limited to lipoproteins owing to the isolation method we used. However, the associations we detected in our results support the continuation of the study of miRNA in EVs and their associations in future studies. In subsequent studies, our findings could be verified and/or expanded, but with a larger number of samples and considering our experimental limitations.

4. Materials and Methods

4.1. Subjects

Our work group developed and implemented a food-based nutrition intervention (6 months) in 22 recently diagnosed breast cancer patients undergoing antineoplastic treatment (for more information see [9,10]); here we worked with cryopreserved (−80 °C) plasma samples collected at baseline and after the intervention from a sub-sample ($n = 16$) of this study's participants. The overall project was reviewed and approved by the ethics committee of the Center for Research in Food and Development (CIAD) (Code CE/05/2015). In accordance with the International Organizations of Medical Sciences, in bioethical guidelines 11 and 12 [149], we contacted the patients in the subsample who own the stored plasma and requested their authorization to carry out complementary plasma analyses: EVs size, Zeta potential, and content (miRNA-145, miRNA-155 and miRNA-30 content), as well as biochemical markers (i.e., glucose, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, and insulin resistance).

Briefly, we will describe the previous study and variables used in the current study. The dietary intervention was based on the design of an individualized meal plan for each patient diagnosed with breast cancer [10]. Baseline and final assessments included body composition (visceral fat, total body fat, and fat-free mass) using dual X-ray absorptiometry (whole body mode; Discovery WI QDR SERIES. Hologic, Waltham, MI, USA) as well as physical activity and tumor molecular biomarkers (HER2neu, Ki67, and estrogen positive).

4.2. Isolation and Analysis of Extracellular Vesicles

Cryopreserved plasma samples were thawed at 4 °C and subsequently homogenized and centrifuged at 10,000× *g* for 30 min and then 3000× *g* for 15 min; subsequently, we performed the isolation and analysis of extracellular vesicle size, Zeta potential, and content (miRNA-145, miRNA-155, and miRNA-30). The isolation and analysis of EVs, as well as biochemical markers, were performed at baseline and 6 months after the intervention.

4.2.1. Isolation of Plasma Extracellular Vesicles

EVs were isolated using ExoQuick™ (System Biosciences, Cat. No. Exoq20a-1, Palo Alto, CA, USA) and the manufacturer's protocol with additional centrifugation. We added 63 µL of ExoQuick™ per 250 µL of plasma and left to precipitate overnight at 4 °C. After this time, the tubes were centrifuged at 1500× *g* for 30 min at 4 °C, discarding the supernatant and recovering the pellet. This was centrifuged again at 1500× *g* for 5 min at 4 °C, where the residues of the supernatant were carefully removed to maintain the EVs pellet.

The EVs pellet was processed and resuspended in 250 µL sterile 0.05X PBS for further analyses or storage. After isolation with ExoQuick™, we used just the purification columns of the commercial kit ExoQuick® ULTRA 125 (System Biosciences, Cat. No. EQULTRA-

20A-1). Subsequently, we performed a protein quantification assay with the Micro BCA™ Protein Assay Kit (Thermo Scientific™ Product No. 23235, Waltham, MA, USA).

4.2.2. Size and Zeta Potential Analyses

The size distribution and Zeta potential of EVs were analyzed using dynamic light scattering analysis (DLS) on a Möbiuζ (Wyatt Technology Corp., Santa Barbara, CA, USA) at 37 °C. From the resuspended EV pellet, we added 1500 µL of sterile 0.05X PBS, and then, it was loaded into disposable polystyrene cells for DLS. The size and Zeta potential were obtained using the software DYNAMICS 7.3.1.15 (Wyatt Technology Corp., Santa Barbara, CA, USA).

4.2.3. Analysis of miRNA in Extracellular Vesicles

From the EVs pellet resuspended in PBS, total RNA extraction was performed using the miRNAeasy serum/plasma kit (Cat. No./ID: 217184, QIAGEN. Hilden, Germany). Subsequently, the miRNeasy Serum/Plasma Spike-In Control (Cat. No. # 219610, QIAGEN) was added. For cDNA synthesis, the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Ref 4366596, Waltham, MA, USA) was used, as well as the specific primers for miRNA-145 (TaqMan™ MicroRNA Assay-Applied Biosystems, ID 002278) and miRNA-155 (TaqMan™ MicroRNA Assay-Applied Biosystems, ID 002623); for miRNA-30, the primer design was based on previous literature by Chen et al. [150] and Kramer et al. [151] and synthesized using Integrated DNA Technologies (see Supplementary Material: Table S1). Reverse transcription was performed in a T100 thermocycler (Bio-Rad. Serial # 621BR18873), with the following conditions: 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min, at 4 °C.

The expression levels of miRNAs were quantified using the StepOne™ Real-Time PCR (Applied Biosystems) and duplex PCRs were performed; we used a synthetic miRNA from Caenorhabditis elegans (cel-miR-39-3 p. TaqMan™ MicroRNA Assay-Applied Biosystems, ID 000200) as a reference control to normalize the data. The normalized expression of mature miRNAs was calculated for all samples, using miRNA-39 as the reference control gene. The Ct was defined as the PCR cycle in which the reporter dye fluorescence signal crosses an amplification threshold. Ct data were collected in the exponential phase of the PCR amplification [152]. The normalized relative expression was obtained with the ΔCt value of each target miRNA (145, 155 or 30); then, the relative quantity (RQ) with $2^{-\Delta Ct}$ was calculated for the target miRNA and reference (miRNA-39). Finally, the result was normalized via logarithmic transformation [153]. Log-transformed normalized expression was used for different statistical analyses.

4.2.4. Negative Staining Electron Microscopy Analysis

The morphology and size of EVs were analyzed using transmission electron microscopy (TEM). The EVs pellet was fixed using 250 µL of glutaraldehyde 2.5% in 0.1 M Na^+ cacodylate buffer (1:1); later, an aliquot was resuspended in 40 µL of sterile PBS filtered using 0.22 µm pore membranes, and subsequently adsorbed on carbon-coated copper grids with formvar mesh (0.3%) at room temperature. The grids were stained for 30 sec using a uranyl acetate solution (2.5%); the excess liquid was then removed. The samples were analyzed using the JEOL JEM-1011 transmission electron microscope (Jeol, Ltd., Tokyo, Japan).

4.2.5. Specific Exosome Markers

Exosome quality was examined using Exo-Check Exosome Antibody Arrays (SBI system biosciences, EXORAY210B-8. Palo Alto, Santa Clara, CA, USA) following the manufacturer's protocol. Exo-Check Exosome Antibody Arrays had a total of nine antibodies against eight known exosome markers (CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5, and TSG101) and one cis-Golgi marker (GM130).

4.2.6. Analysis of Biochemical Biomarkers

We analyzed plasma glucose, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides using the VITROS® 250 System (Ortho Clinical Diagnostics, Raritan, NJ, USA). The triglyceride-glucose index (TyG index) was calculated using the following formula: [fasting triglycerides (mg/dL) × fasting plasma glucose (mg/dL)/2] [39].

4.2.7. Statistical Methods

Regression analyses using mixed effects models were performed using the EV size, Zeta potential, and expression of miRNA-145, miRNA-155, and miRNA-30 as outcome variables. Anthropometric (body weight and height), body composition (visceral fat, total body fat, and fat-free mass), and tumor molecular biomarkers were included as explanatory variables. Regression coefficients and 95% confidence intervals were reported. $p \leq 0.05$ was considered significant. Analysis was performed using STATA (v15.0 StataCorp LP, College Station, TX, USA).

Models were generated using a sequence of univariate and stepwise analyses. Univariate models were constructed and variables with a value of $p \leq 0.2$ and biological plausibility were considered as possible explanatory variables, adjusted variables, or both [154,155]. These variables were considered for the stepwise model procedure. Variables with a p-value ≤ 0.05 were chosen for the final multivariate models along with their AIC (Akaike Information Criteria).

Differences between baseline and end-line expression of miRNAs, anthropometric variables, biochemical biomarkers, and EV characteristics were analyzed using Wilcoxon signed-rank test. Statistical analyses were performed using NCSS 2007 software.

5. Conclusions

Despite finding different EVs sizes in the plasma samples, we could not identify a significant difference before and after the nutritional intervention. However, the association of the Zeta potential (one of the characteristics of EVs) with different tumor biomarkers and body composition data stands out. Here, we showed that the characteristics of the EVs could contribute to explain different pathophysiological processes, as well as the development and progression of the disease. For its part, the expression of miRNA-30 and miRNA-155 contained in EVs was also influenced by changes in the levels of biomarkers of lipid metabolism. Our results add to the little evidence that exists on the miRNA-30, miRNA-155, and the Zeta potential, as a sensitive characteristic of EVs, so this characteristic should not be left aside in the study and search for new biomarkers in breast cancer. More evidence is needed for the use and validation of the Zeta potential and miRNAs in the clinical area.

An important limitation to our work was the sample size; however, our models show statistical significance between EVs characteristics and breast cancer. Characterizing the changes in the EVs under different stress situations will contribute to a better understanding of the development of the disease and to the development of new targeted therapeutic strategies, as well as the development of sensitive biomarkers of early risk, prognosis, or progression of the disease. More studies are required to elucidate these mechanisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24076810/s1.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by ethics committee of the Center for Research in Food and Development (CIAD) (protocol Code CE/05/2015. Date of approval May 2015).

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4. CONCLUSIONES GENERALES

A pesar de encontrar diferentes tamaños de VE en las muestras de plasma, no se logró identificar una diferencia significativa antes y después de la intervención nutricional. Sin embargo, en nuestros resultados se destaca la asociación del potencial Zeta (una de las características de los VE) con diferentes biomarcadores tumorales y datos de composición corporal. Por su parte, la expresión de miRNA-30 y miRNA-155 contenidos en los VE también se relacionaron con biomarcadores tumorales y con biomarcadores del metabolismo de lípidos. Aun cuando la expresión de miRNA-145 no tuvo ninguna asociación con características de VE, se destaca la sobreexpresión de este al final de la intervención nutricional.

Nuestros resultados suman a la escasa evidencia que existe sobre el miRNA-30, miRNA-155 y el potencial Zeta, como característica sensible de las VE, por lo que estas características no deben dejarse de lado en el estudio y búsqueda de nuevos biomarcadores en cáncer de mama. Sin embargo, se necesita más evidencia para el uso y validación del potencial Zeta y miRNAs en el área clínica y nutrición. El uso de las VE como biomarcadores, no sustituiría el uso de los actuales. Más bien, su uso busca una mayor comprensión de los cambios fisiológicos que ocurren antes del desarrollo de complicaciones de salud.

Una limitación importante del trabajo fue el tamaño de la muestra; sin embargo, los modelos de regresión obtenidos muestran una significancia estadística entre las características de las VE y el cáncer de mama. La caracterización de los cambios en los VE bajo diferentes situaciones de estrés contribuirá a una mejor comprensión del desarrollo de la enfermedad, al desarrollo de nuevas estrategias terapéuticas dirigidas, así como al desarrollo de biomarcadores sensibles de riesgo temprano, pronóstico o progresión de la enfermedad.

En este proyecto, se logró demostrar que las características de las VE, con investigación adicional de soporte como biomarcadores, podrían contribuir a explicar diferentes procesos fisiopatológicos, así como ayudar a dilucidar el desarrollo y progresión de la enfermedad, así como los cambios en composición corporal que se presentan durante la enfermedad.

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