Mediterranean Diet and Breast Cancer Biology: Novel Insights into the Role of Extracellular Vesicles

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Abstract

A number of risk factors have been identified in relation to breast cancer (BC). Among these, environmental factors, particularly those related to nutritional status, have been shown to play a significant role in the biology of BC. Thus, there is a growing interest in assessing the potential benefits of diet and life style in the prevention and treatment of BC. Among dietary interventions, the Mediterranean Diet (MD) has been suggested to be the most efficacious in reducing the risk of BC and enhancing patient outcomes[1,2]. Numerous studies have attempted to address the relationship between diet and circulating Extracellular Vesicles (EVs), small membrane-bound particles that represent a novel axis of intercellular communication in BC[3,4,5]. It has been reported that dietary compounds may influence the biogenesis, release, and composition of the endogenous EVs. However, there is still a knowledge gap regarding the accurate phenotyping of diet-induced EVs and their potential role in mediating the effects of nutritional status on BC. Here, we investigated the impact of MD adherence on the composition and function of serum circulating EVs isolated from BC patients. BC patients have been classified by their level of MD adherence (Low: ≤5; Medium: 6–9; High: ≥10), as assessed by the MEDAS questionnaire[6]. We isolated and fully characterized EVs from the serum of BC patients, in accordance with the MISEV 2023 guidelines, by Transmission Electron Microscopy, NanoSight analysis and Immunoblot. The biological effects of BC patient-derived EVs on cell proliferation, stemness, motility and metabolism in two different ERα-positive BC cell lines, MCF-7 and T-47D, were evaluated. The expression profiles of EV-packaged miRNAs were investigated by small RNA-sequencing analysis. NTA analysis revealed a correlation between elevated MD adherence and alterations in the number and dimensions of circulating EVs in BC patients. In addition, we demonstrated that the EVs isolated from BC patients with High MD adherence were less effective in sustaining cell viability and growth in both MCF-7 and T-47D as evidenced by anchorage-dependent (clonogenic assay) and anchorage-independent (soft-agar) assays, respectively. The boyden-chamber transmigration assay revealed that EVs isolated from BC patients with High MD adherence were less effective in promoting the migration of BC cells in the two employed experimental models. Finally, given that migratory potential is among the most significant characteristics of BC stem cells, the mammosphere forming efficiency assay was conducted. We found that EVs isolated from BC patients with High MD adherence exhibited a reduced capacity to form mammospheres in ER+ BC cells compared to EVs isolated from BC patients with Low MD adherence. Accordingly, BC cells treated with High MD adherence EVs showed a reduced cellular bioenergetics as measured by oxygen consumption rate, in terms of basal and maximal respiration, compared to cells treated with Low MD adherence EVs. The analysis of EV-miRNA profile revealed 172 miRNAs, 18 of which were differentially expressed (|fold change|≥1.5) in Low vs High MD adherence EVs. These deregulated miRNAs are mainly involved in controlling proliferation, migration and metabolism in BC. These data demonstrated that High MD adherence modulates the EV composition (e.g. the miRNA profile) and exerts beneficial effects on BC cell development, growth and progression.
Although these results are still preliminary, they underscore the importance of healthy dietary patterns in modulating BC biology and open new avenues for identifying potential therapeutic targets, to be exploited in patients with poor dietary habits.

Aim of the study

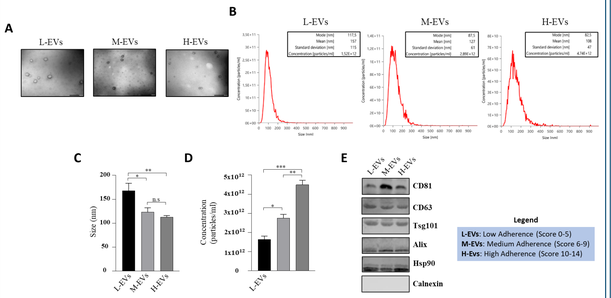
To Investigate the Impact of the Mediterranean Diet (MD) Adherence on the Composition and Function of Circulating Extracellular Vesicles (EVs) in Breast Cancer (BC) Patients



Schematic representation of the study design (A) and BC patient clinical features (B).
EVs were purified from the serum of BC patients using the Exoquick polymeric kit. The EV samples have been mixed in equal amounts (3 samples in each pool) to obtain pooled EVs for each group of patients categorized according to the MD adherence (L-, M- and H-EVs) assessed by MEDAS questionnaire.

Figure 1

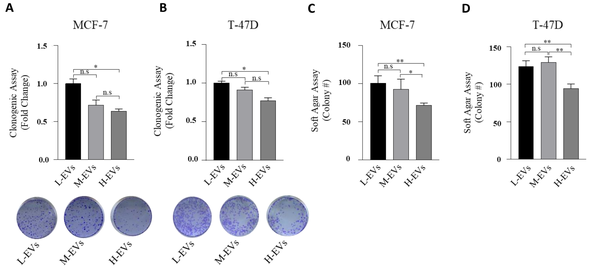
Characterization of EVs Isolated from the Serum of BC Patients with different adherence to the MD



A. Representative images of TEM showing EVs isolated from the serum of BC patients with different adherence to the MD. Scale bar, 100 nm. **B**. Representative size distribution profiles and concentration of serum EVs measured by NanoSight Analysis. The bar charts represent the average values for size (**C**) and particle concentration (**D**) from NanoSight Analysis results of the entire sample of specimens. *p<0.05; **p< 0.01; ***p< 0.001; n.s.=non-significant. **E**. Immunoblot analysis showing expression of the EV hallmarks CD81, CD63, Tsg101, Alix and Hsp90 in equal amount of EV lysates. The specificity of EV isolation was confirmed using endoplasmic reticulum marker Calnexin.

Figure 2

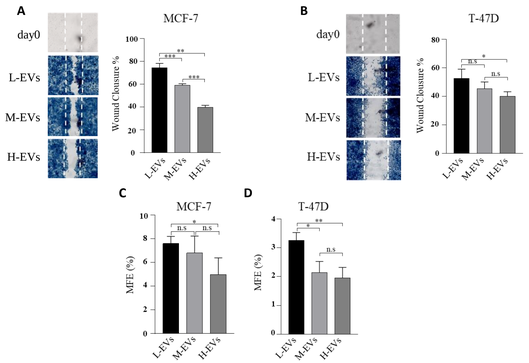
Effects of Circulating EVs from BC Patients with Different Adherence to the MD on Estrogen Receptor-Positive (ER+) BC Cell Growth



Clonogenic assay in MCF-7 (**A**) and T-47D (**B**) cells treated with EVs (2 µg/ml) for 15 days. Colonies were stained with 0.01% (w/v) crystal violet, solubilized and the absorbances were measured at 570 nm. The bar charts represent the fold change in absorbance at 570 nm ± S.D. Lower panel, representative pictures. Soft agar growth assays in MCF-7 (**C**) and T-47D (**D**) cells treated with EVs for 20 days. After 20 days of growth, colonies ≥50 µm were counted. The histograms represent the mean colony number ± S.D. *p<0.05; **p< 0.01; n.s.=non-significant.

Figure 3

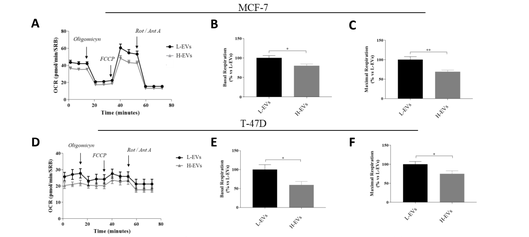
Effects of Circulating EVs from BC Patients with Different Adherence to the MD on ER+ BC Cell Motility and Stemness



Wound healing assays in MCF-7 (**A**) and T-47D (**B**) cells. Fields were photographed immediately after wounding (Day 0) and 24 h later. The bar charts represent the relative percentage of wound closure ± S.D, calculated with Image J software. Left panel, representative pictures. Mammosphere formation efficiency (MFE), a reliable assay for quantifying cancer stem cell activity, was evaluated in MCF-7 (**C**) and T-47D (**B**) cells. The bar charts represent the MFE expressed as percentage ± S.D. *p<0.05; **p < 0.01; ***p< 0.001; n.s.=non-significant.

Figure 4

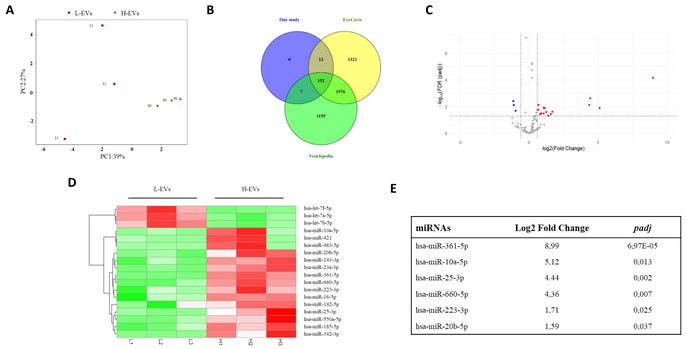
Effects of Circulating EVs from BC Patients with Different Adherence to the MD on ER+ BC Cell Metabolism



Mitochondrial metabolic activity of MCF-7 and T-47D cells untreated (-) or treated with EVs (2 µg/ml) for 48 h. OCR flux (**A and D**), basal (**B and E**), and maximal respiration (**C and F**) are shown. Data were analyzed using Student's t test and are represented as mean ± SEM of 5 biological replicates from 3 independent experiments and normalized to protein content (SRB assay). *p≤0.05.

Figure 5

Profiling of miRNAs in EVs Isolated from the Serum of BC Patients with Low and High MD Adherence



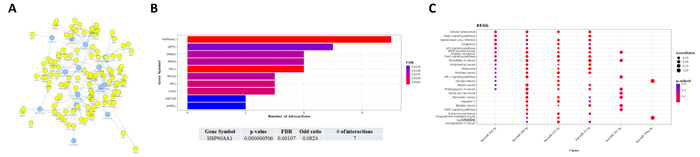
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miRNAs	Log2 Fold Change	*padj*
hsa-miR-361-5p	8,99	6,97E-05
hsa-miR-10a-5p	5,12	0,013
hsa-miR-25-3p	4,44	0,002
hsa-miR-660-5p	4,36	0,007
hsa-miR-223-3p	1,71	0,025
hsa-miR-20b-5p	1,59	0,037

The expression profiles of EV-packaged miRNAs were investigated by small RNA-sequencing analysis. **A**. The 2D principal component analysis (PCA) plot illustrates the distribution of EV-miRNA expression profiles in BC patients with High and Low adherence to the MD. EV pooled samples from the High-adherence group (blue triangles, n = 3) and the Low-adherence group (red circles, n = 3) form distinct clusters in PCA space. **B**. Venn diagram of the known miRNAs among our study, Vesiclepedia and ExoCarta databases. **C**. Volcano plot displaying all identified miRNAs from EVs of in BC patients with High and Low adherence to the MD (red dots for upregulated miRNAs; blue dots for downregulated miRNAs. **D**. Heatmap of the differentially expressed EV-miRNA expression profiles in breast cancer patients with High and Low adherence to the MD. miRNAs with p≤0.05 and |Fold-Change| ≥ 1.5, were considered as differentially expressed between the two conditions. The red and green color scale indicates increased and decreased miRNA expression levels, respectively. **E**. Statistical analyses of the miRNAs identified by sequencing showed six significantly up-regulated miRNAs in High adherence population compared to patients with Low adherence to the MD.

Figure 6

Bioinformatic Analysis of the Differentially Identifed EV-miRNAs



Gene Symbol	p value	FDR	Odd ratio	# of interactions
HSP90AA1	0.0000000506	0.00107	0.0624	7

A. miRNA–target interaction network of all de-regulated identified miRNAs in the comparison L-EVs vs H-EVs constructed using MIENTURNET web tool. Blue dots represent miRNAs, yellow blocks represent miRNA targets. **B**. Number of interactions between miRNAs and target genes predicted by miRTarBase using the MIENTURNET algorithm. **C**. Dot plot of functional enrichment analysis for target genes of selected miRNAs resulting from the enrichment analysis. The Y-axis reports the annotation categories (e.g. KEGG pathways) and the X-axis reports the selected miRNAs. The color of the dots represent the adjusted p-values (FDR), whereas the size of the dots represents gene ratio (i.e. the number of miRNA targets found enriched in each category over the number of total genes associated to that category).

Conclusions

- High adherence to the MD is associated with a modification in the number and size of EVs isolated from the serum of BC patients.
- EVs isolated from the serum of breast cancer patients with High adherence to the MD are less effective in promoting growth, motility, stem cell phenotype and metabolism in ER+ BC cells.
- EVs isolated from the serum of BC patients with High adherence to the MD showed different microRNA expression profiles compared to those derived from patients with Low adherence.
- Among all identified miRNAs, HSP90 emerges as the most frequently targeted gene.
- Gene ontology analysis of the identified miRNA-targets revealed that "Cellular senescence" is the most significant enriched pathway.

Clinical Implications

These findings support the hypothesis that high adherence to the MD exerts beneficial effects on BC biology, mediated by EVs and, hypothetically, it is associated with specific expression patterns of EV-packaged miRNAs.

These results may open new avenues for the identification of potential therapeutic targets, particularly in patients with poor dietary habits.

References

1. *Seiler A, et al, Curr Breast Cancer Rep. 2018 Mar;10(1):14-27*
2. *Turati F, et al, Nutrients. 2018 Mar 8;10(3):326*
3. *Kumar MA, et al. Signal Transduct Target Ther. 2024 Feb 5;9(1):27*
4. *La Camera G, et al, Cancer Lett. 2021 Nov 28;521:155-168*
5. *Barone I, et al. J Transl Med. 2023 Mar 31;21(1):232*
6. *Martínez-González MA, et al. PLoS ONE 2012, 7(8): e43134*

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