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miR-877-5p as a potential link between triple negative breast cancer progression and metabolic syndrome.

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Abstract: Metabolic syndrome (MS) is a risk factor for breast cancer (BC) that increases its aggressiveness and metastasis. The prevalence of MS is higher in triple negative breast cancer (TNBC), which is the molecular subtype with the worst prognosis. The molecular mechanisms underlaying this association have not been fully elucidated. MiRNAs are small non-coding RNAs that regulate gene expression. Aberrant expression of miRNAs in both, tissues and fluids, are linked to several pathologies. The aim of this work was to identify circulating miRNAs in patients with alterations associated with MS (AAMS) that also impact on BC. Using microarray technology, we detected 23 miRNAs altered in the plasma of women with AAMS that modulate processes linked to cancer. We found that let-7b-5p and miR-28-3p were decreased in plasma from patients with AAMS and also in BC tumors; while miR-877-5p was increased. Interestingly, miR-877-5p expression was associated with lower patient survival, and its expression was higher in PAM50 basal-like BC tumors compared to the other molecular subtypes. Analyses from public databases revealed that miR-877-5p is also increased in plasma from BC patients compared to plasma from healthy donors. We identified IGF2 and TIMP3 as validated target genes of miR-877-5p whose expression is decreased in BC tissue and moreover, is negatively correlated with the levels of this miRNA in the tumors. Finally, a miRNA inhibitor against miR-877-5p diminished viability and adhesion capability of TNBC 4T1 cells. These results reveal that miR-877-5p inhibition could be a therapeutic option for the treatment of TNBC.

Keywords: breast cancer; metabolic syndrome; miRNAs.

1. Introduction

Breast cancer (BC) remains one of the most important public health issues in the world[1]. Among them triple negative breast cancer (TNBC) is the molecular subtype with the worst prognosis and the fewest therapeutic options [2,3] .

Non-hereditary factors have a major impact on the risk and progression of BC[4,5]. Metabolic syndrome (MS) is a group of physiopathological disorders characterized by the presence of three or more of the following conditions: abdominal obesity (waist circumference ≥ 35 inches in women)[6], hyperglycemia (≥ 110 mg/dL), blood triglycerides ≥150 mg/dL (women), HDL-C cholesterol < 50 mg/dL (women) and elevated blood pressure (≥

130/85 mmHg)[7]. Previous studies suggest that several components of MS such as abdominal obesity, hyperglycemia, hypertension and dyslipidemia are associated with BC depelopment[8,9]. In addition, MS is a risk factor for BC that increases its aggressiveness and metastasis[10–14]. Interestingly, a retrospective study found that MS was more prevalent in TNBC compared with the other PAM50 molecular subtypes[15]. Several mechanisms have been proposed to explain the association between MS and BC, however the molecular mechanisms involved have not been fully elucidated[16]. As the prevalence of MS increases worldwide, it is critical to understand the mechanisms responsible for the effects of MS on cancer development and progression.

MicroRNAs (miRNAs) are non-coding RNAs of 21-25 nucleotides long that are regulators of gene expression and modulate numerous biological processes important for maintaining homeostasis. In addition to tissue expression, miRNAs can be detected in various body fluids such as blood and urine, making them interesting biomarkers for the diagnosis and prognosis of diseases[17]. Moreover, circulating miRNAs constitute important components in cell communication at both paracrine and endocrine levels[18,19]. In fact, miRNAs aberrant expression, in both tissues and body fluids, has been associated with numerous pathologies, such as cancer and MS[20,21].

The aim of this work was to identify circulating miRNAs in patients with clinical features linked to MS that also impact on BC development and progression and constitute possible therapeutic targets for these patients. Using microarray technology, we detected 23 miRNAs altered in the plasma of women with alterations associated with MS (AAMS) that modulate processes linked to cancer. We found that let-7b-5p and miR-28-3p were decreased in plasma from patients with AAMS and also in BC tumors; while miR-877-5p was increased in plasma from patients with AAMS and in BC tissue. Interestingly, miR-877-5p expression was associated with lower patient survival, and its expression was higher in basal-like BC tumors compared to the other molecular subtypes, so we focused our research on this miRNA. Thus, we identified IGF2 and TIMP3 as validated target genes of miR-877-5p whose expression is decreased in BC tissue and moreover, is negatively correlated with the levels of this miRNA in the tumors.

2. Results

2.1. Circulating miRNAs were altered in plasma of women with AAMS.

To identify the circulating miRNAs expression profile induced by AAMS, patients from Hospital Militar Central (CABA) were recruited and divided into two groups: control and women with AAMS. The later was defined when participants presented two or more of these conditions: BMI ≥ 25.00 kg/m^2, waist diameter ≥ 82 cm or high blood pressure (systolic≥120, diastolic≥80). MiRNAs were isolated from plasma and four samples (2 control and 2 AAMS) were generated by pooling miRNAs from 9 donors and hybridized to GeneChip® miRNA 4.0 Array (Affymetrix). Following data normalization, we established a threshold for identifying significant genes, requiring a false discovery rate value (FDR) < 0.05%, Log Fold Change (LogFC) > 1.5, and a p-value < 0.01. Thus, we identified 23 miRNAs differentially expressed in plasma of women with AAMS compared to control (Figure 1A and Table 1).

Table 1. Differentially expressed miRNAs in AAMS woman compared to control (FSG<0.05%, LogFC>1. 5 and p value<0.01).

Transcript_ID	miRNA	p value	q value	RP values	LOG(FC)
MIMAT0019806	hsa-miR-4706	0	0,000	1,565	3,249
MIMAT0004513	hsa-miR-101-5p	2,17E-06	0,005	6,774	2,136
MIMAT0027400	hsa-miR-6750-5p	2,17E-06	0,005	4,738	2,348
MIMAT0027088	hsa-miR-5189-3p	1,09E-05	0,013	10,029	2,316
MIMAT0004949	hsa-miR-877-5p	1,30E-05	0,012	10,499	2,044
MIMAT0018984	hsa-miR-378h	3,69E-05	0,028	13,581	1,720
MIMAT0026636	hsa-miR-668-5p	5,65E-05	0,037	16,060	1,607
MIMAT0019870	hsa-miR-4740-3p	5,87E-05	0,034	16,807	1,681
MIMAT0000078	hsa-miR-23a-3p	1,52E-04	0,044	24,926	-2,008
MIMAT0025845	hsa-miR-6716-3p	1,04E-04	0,032	21,203	-2,269
MIMAT0004502	hsa-miR-28-3p	9,56E-05	0,031	20,584	-2,285
MIMAT0000074	hsa-miR-19b-3p	7,82E-05	0,028	18,903	-2,342
MIMAT0000256	hsa-miR-181a-5p	5,87E-05	0,023	16,699	-2,394
MIMAT0000421	hsa-miR-122-5p	6,52E-06	0,003	7,366	-3,473
MIMAT0017991	hsa-miR-3613-3p	6,52E-06	0,003	8,144	-3,599
MIMAT0000064	hsa-let-7c-5p	6,52E-06	0,003	8,050	-3,415
MIMAT0018976	hsa-miR-4454	6,52E-06	0,003	9,118	-3,258
MIMAT0000063	hsa-let-7b-5p	2,17E-06	0,001	5,811	-3,629
MIMAT0000440	hsa-miR-191-5p	2,17E-06	0,001	4,559	-3,631
MIMAT0019745	hsa-miR-4668-5p	2,17E-06	0,001	5,091	-3,910
MIMAT0000449	hsa-miR-146a-5p	2,17E-06	0,001	6,055	-3,531
MIMAT0001639	hsa-miR-409-3p	2,17E-06	0,001	5,696	-3,495
MIMAT0003393	hsa-miR-425-5p	2,17E-06	0,001	6,620	-3,550

To elucidate the biological role of these miRNAs, we identified the KEGG pathways to which the the validated target genes of these miRNAs belong using DIANA miRPath. We found that these miRNAs participate in several processes linked to cancer such as cell cycle, proteoglycans, transcriptional misregulation and pathways in cancer (Figure 1B). In particular, we found that AAMS-downregulated miR-23a-3p, -19b-3p, -181a-5p, -122-5p, -425-5p, -28-3p, -146a-5p, let-7b-5p and AAMS-upregulated miR-101-5p and -877-5p showed the strongest association so we focused on these miRNAs for further studies (Figure 1C).

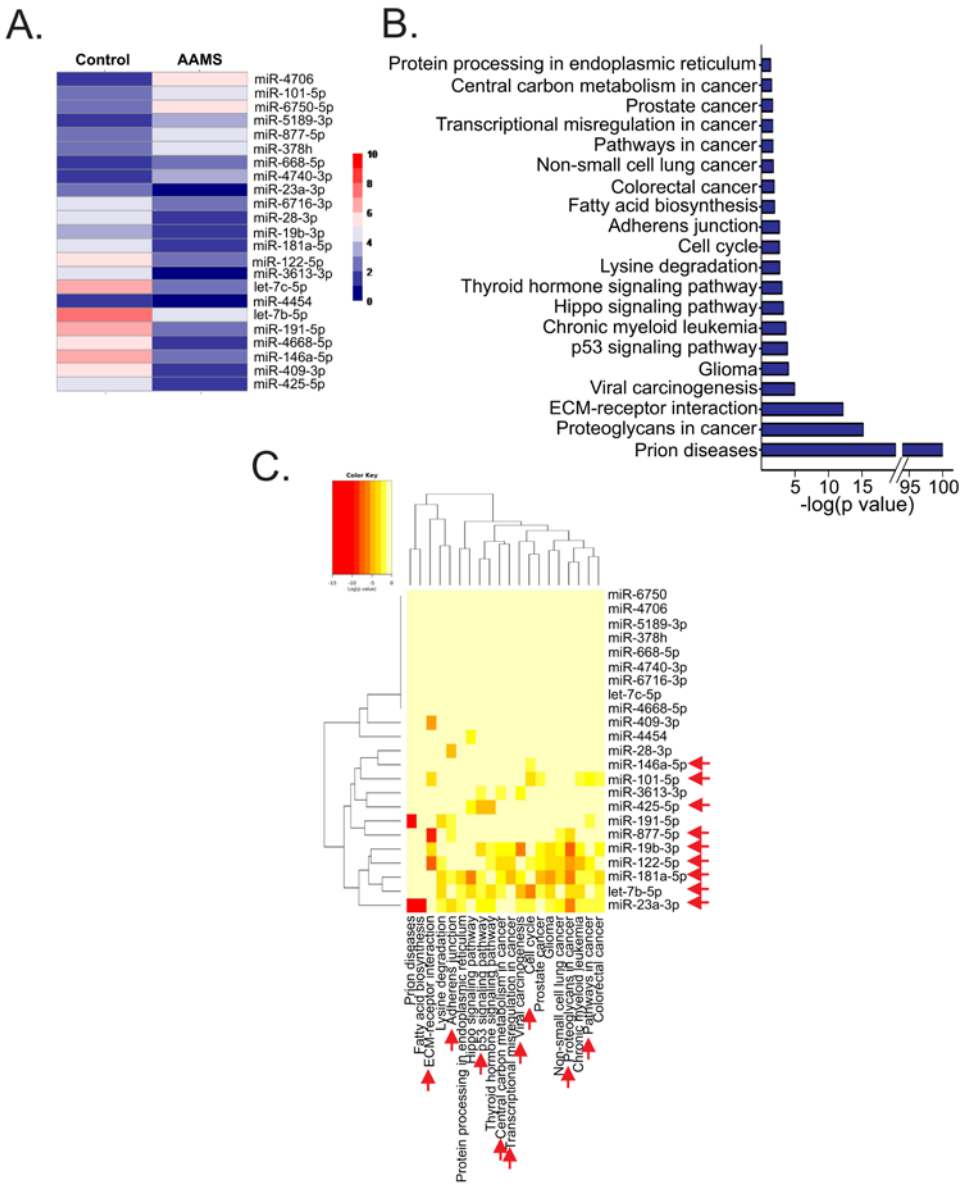

Figure 1. The expression of circulating miRNAs with functions related to cancer was altered in the plasma of women with AAMS. (a) MiRNAs were isolated from plasma of women with AAMS or control and hybridised to GeneChip®miRNA 4.0 Array (Affymetrix). Heatmap depicting the differentially expressed miRNAs (FSG<0.05%, LogFC>1. 5 and p value<0.01) is shown. (b) Histogram depicting the KEGG pathways regulated by the validated target genes from these miRNAs determined through DIANA miRPath. The top 20 most significant pathways are shown. (c) Heatmap showing the miRNAs and their KEGG pathways. Red arrows indicate KEGG pathways relevant to cancer and the miRNAs with stronger association to these pathways.

2.2. MiR-28-3p, -101-5p and let-7b-5p were diminished while miR-181a-5p, -425-5p and -877-5p were increased in primary tumors of BC patients.

Our hypothesis was that circulating miRNAs of women with AAMS could impact on BC development and progression. We assessed the role of those miRNAs in BC using

public databases. Thus, we compared their expression levels in primary tumor (PT) versus NAT from BC patients in TCGA BRCA. We found that miR-28-3p, -101-5p y -let-7b-5p were diminished in PT compared to NAT while miR-181a-5p, -425-5p and -877-5p were increased (Figure 2A). Interestingly, miR-28a-3p and let-7b-5p expression levels, which are diminished in AAMS patients, were also diminished in PT compared to NAT, and miR-877-5p expression levels, which are augmented in AAMS patients were increased in PT compared to NAT.

Next, we studied the expression of these miRNAs according to PAM50 molecular subtypes in tumors from the TCGA-BRCA. These miRNAs were differentially expressed in tumors of BC patients according to PAM50 subtype (Figure 2B). Additionally, miR-877-5p expression was increased in the basal-like subtype, which is the most aggressive BC subtype, and let-7b-5p levels were diminished in basal-like tumors compared to luminal B (Figure 2B).

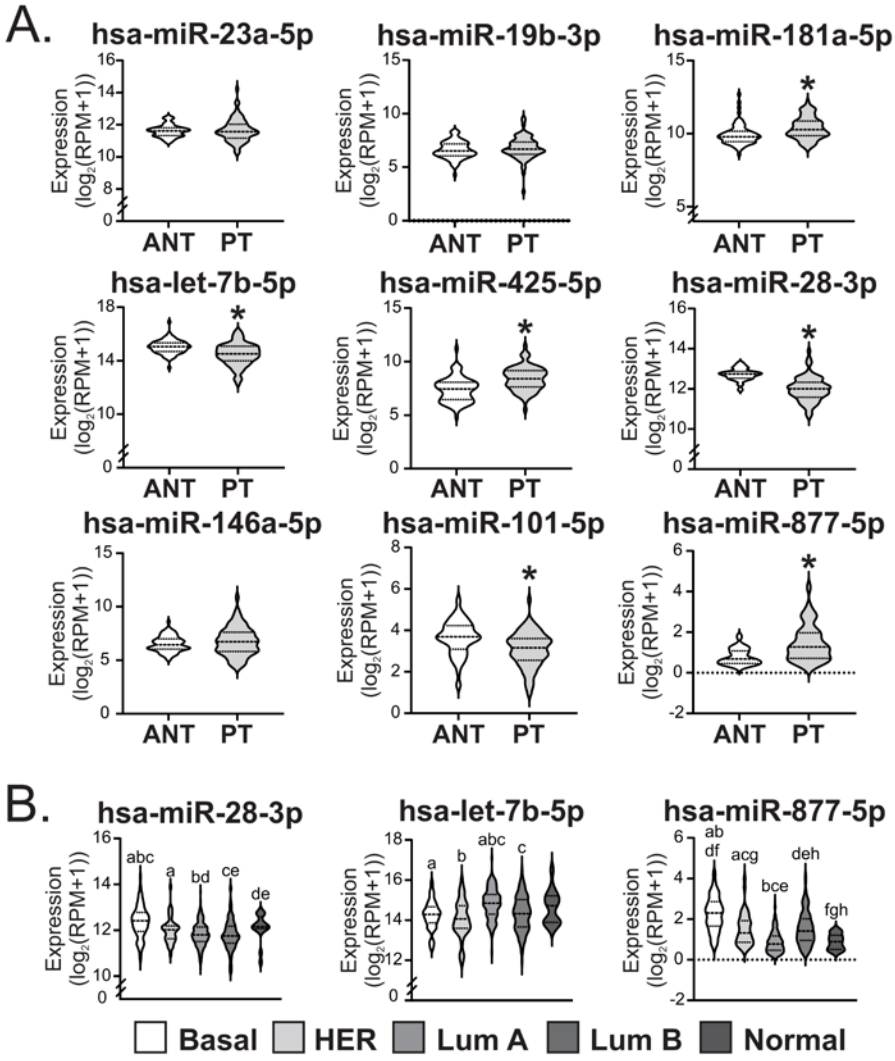

Figure 2. MiR877-5p, -28-3p, and let-7b-5p expression levels were altered in primary breast tumors from patients and their expression are dependent on the PAM50 subtype. (a) Expression levels of AAMS-modulated miRNAs were determined in primary tumors

(PT) and adjacent normal tissue (ANT) of patients from TCGA BRCA data – Illumina HiSeq. Significance was determined by paired T-test or Wilcoxon Signed Rank Test, when normality failed (*, p<0.05). (b) Expression levels of miR-877-5p, miR-28-3p and let-7b-5p were determined in BC tissue from the different PAM50 molecular subtypes using TCGA BRCA data –Illumina HiSeq available in UCSC Xena tool. Significance was analysed by one-way ANOVA or Kruskal-Wallis rank sum test,when normality failed. Letters indicate a p value < 0.05.

2.3. The miR-877-5p expression was correlated with diminished overall survival of BC patients.

We then examined the role of miR-28-3p, let-7b-5p and miR-877-5p expression in the overall survival, diseases specific survival, progression free interval and relapse free interval of patients with BC from TCGA-BRCA – IlluminaHi Seq using UCSC Xena tool (Figure 3A-D). We found that miR-877-5p expression correlated with poor overall survival in BC patients (Figure 3A). Hence, we focused on miR-877-5p for further studies.

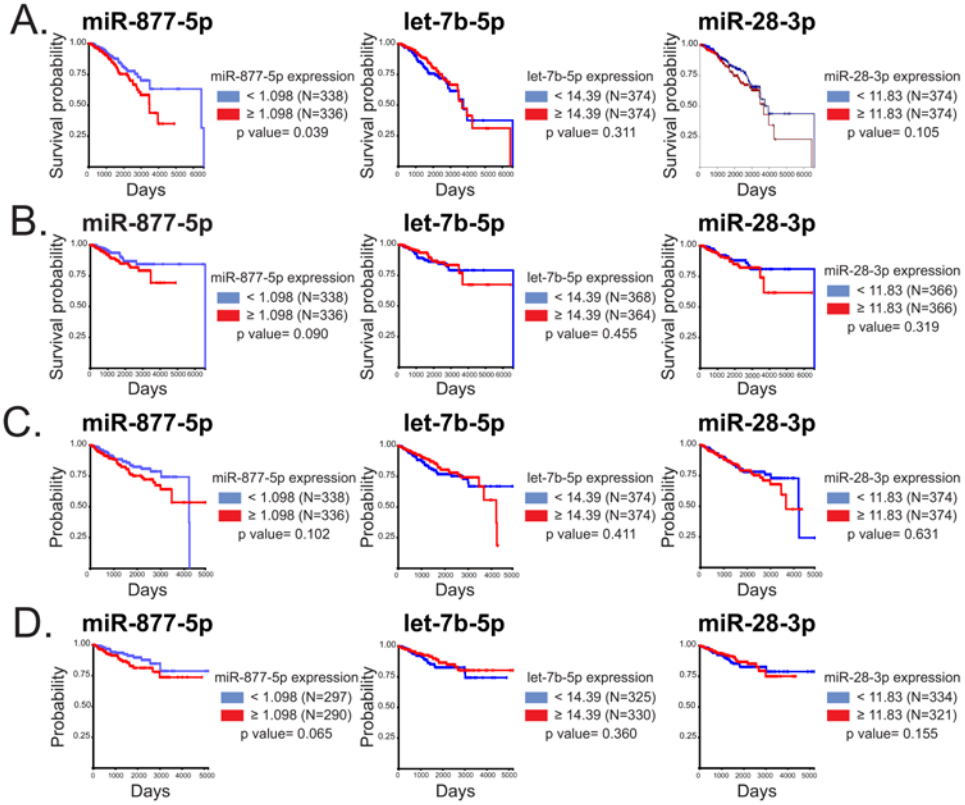

Figure 3. miR-877-5p expression was correlated with a diminished overall survival of patients. The impact of let-7b-5p, miR-28-3p and miR-877-5p in: (a) overall survival, (b) disease specific survival, (c) progression free interval and (d) disease free interval of patients with BC was assessed in TCGA BRCA data – Illumina HiSeq using UCSC Xena tool. Log-Rank test was done to determine significance.

Also, considering TNBC is the molecular subtype with the worst prognosis and the fewest therapeutic options [2,3], we explore miR-877-5p expression levels in PAM50 basal-

like tumors from TCGA BRCA dataset, which represents around 84% of TNBC [22,23]. We found that miR-877-5p expression was diminished in PAM50 basal-like breast tumors compared to NAT (Figure 4), which was reasonable considering the expression of this miRNA was increased in PAM50 basal-like tumors compared to the other molecular subtypes (Figure 2B).

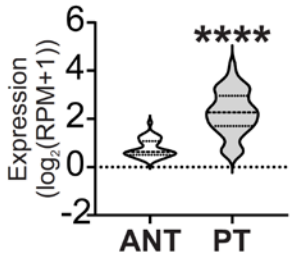

Figure 4. MiR877-5p expression levels were decreased in primary tumors from patients with PAM50 basal-like BC. Expression levels of miR-877-5p were determined in primary tumors (PT) and adjacent normal tissue (ANT) of patients with PAM50 basal-like BC from TCGA BRCA data – Illumina HiSeq. Significance was determined by Mann-Whitney test (balanced N=41; **** $p < 0.0001$).

2.4. miR-877-5p was increased in plasma of AAMS patients and BC patients.

To validate our microarray findings, we performed miRNA RT-qPCR analysis of miR-877-5p expression using the same cohort of 9 women with AAMS and 9 women without AAMS, as shown in Figure 1. Our miRNA RT-qPCR experiments demonstrated a significant increase in miR-877-5p expression in plasma from patients with AAMS compared to the control group (Figure 5A), providing additional support for the microarray results.

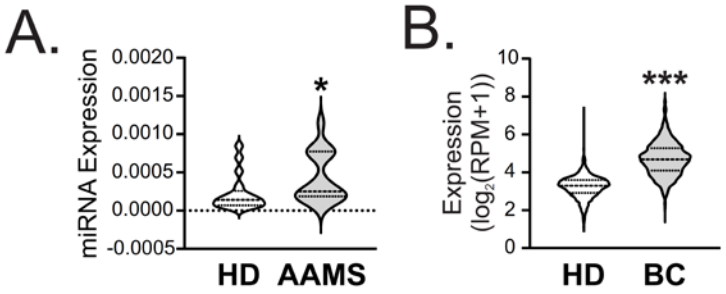

Figure 5. miR-877-5p expression was increased in plasma from patients with AAMS and from patients with BC. (a) miR-877-5 expression in plasma from women with AAMS or control (N=9) was determined by stem-loop RT-qPCR. Data was normalised to spike in cel-miR-39 and control group. Significance was evaluated using Mann-Whitney Wilcoxon signed rank test (*, $p<0.05$). (b) miR-877-5p expression in women with BC and healthy donors was analysed in the GSE73002 dataset (balanced N=1280). Significance was evaluated using Mann-Whitney Wilcoxon signed rank test (*, $p<0.05$).

Moreover, we assessed miR-877-5p expression in blood samples from breast cancer patients using the GSE73002 dataset. Our analysis revealed a significant increase in miR-877-5p expression in serum from patients with breast cancer compared to healthy donors (balanced N=1280) (Figure 5B). These results provide additional support for our previous findings and suggest that miR-877-5p may be a useful biomarker for breast cancer as well as AAMS.

2.5. miR-877-5p expression was inversely correlated with IGF2, ERBB2, TIMP3 and COL6A2 levels in breast tumors.

To underscore the molecular mechanism by which miR-877-5p could impact on BC development and/or progression, we identified the validated target genes of miR-877-5p that are involved in pathways related to cancer using Diana miRPath (Table 2).

Table 2. DIANA miRPath v3 validated target genes of miR-877-5p that are included in KEGG pathways related to cancer

KEGG pathway: Proteoglycans in cancer (hsa05205)		
Gene Name	Gene Ensembl id	Tarbase Methods
ERBB2	ENSG00000141736	HITS-CLIP
ITGB1	ENSG00000150093	HITS-CLIP
KRAS	ENSG00000133703	PAR-CLIP
VAV2	ENSG00000160293	HITS-CLIP
AKT2	ENSG00000105221	PAR-CLIP
IGF2	ENSG00000167244	HITS-CLIP
TIMP3	ENSG00000100234	HITS-CLIP
RAC1	ENSG00000136238	HITS-CLIP
FGF2	ENSG00000138685	PAR-CLIP
FAS	ENSG00000026103	HITS-CLIP
GAB1	ENSG00000109458	PAR-CLIP
PDPK1	ENSG00000140992	HITS-CLIP
ITPR2	ENSG00000123104	PAR-CLIP
RPS6KB1	ENSG00000108443	CLASH
KEGG pathway: ECM-receptor interaction (hsa04512)		
Gene Name	Gene Ensembl id	Tarbase Methods
ITGB1	ENSG00000150093	HITS-CLIP
ITGB8	ENSG00000105855	HITS-CLIP
COL6A2	ENSG00000142173	HITS-CLIP
LAMC1	ENSG00000135862	Multiple
TNC	ENSG00000041982	HITS-CLIP
COL4A1	ENSG00000187498	Multiple

KEGG pathway: Adherens junction		
Gene Name	Gene Ensembl id	Tarbase Methods
ERBB2	ENSG00000141736	HITS-CLIP
WASL	ENSG00000106299	HITS-CLIP
RAC2	ENSG00000128340	HITS-CLIP
NLK	ENSG00000087095	Multiple
RAC1	ENSG00000136238	HITS-CLIP

Next, we determined if the expression of miR-877-5p negatively correlates with its targets. Results showed that the expression of ERBB2, TIMP3, COL6A2 and IGF2 negatively correlated with weak to moderate correlation coefficient (r_s) (Figure 6A). Additionally, using the STRING database, we identified interactions among all the genes (Figure 6B) which is reasonable considering these genes are involved in related pathways such as Proteoglycans in cancer (ERBB2, TIMP3 and IGF2) and ECM-receptor interaction (COL6A2). Moreover, we found that the expression of TIMP3 and IGF2 were diminished in the PT of BC patients from the BRCA-TCGA compared to NAT (Figure 6C).

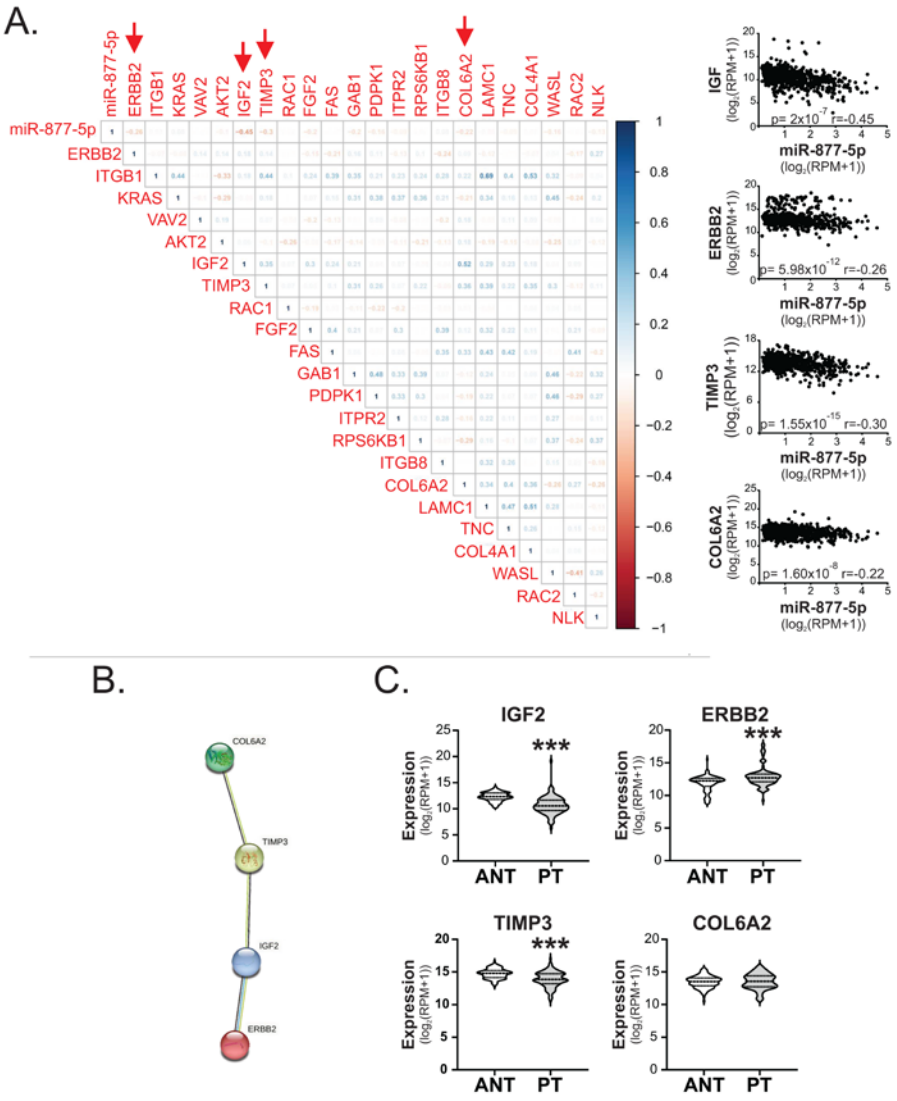

Figure 6. miR-877-5p expression levels were correlated with IGF2, ERBB2, TIMP3 and COL6A2 levels in mammary tumors. (a) Spearman test was performed to analyse correlation between miR-877-5p and its validated target genes in primary tumors of BC cohort TCGA BRCA data–Illumina HiSeq. Matrix correlation and dot plots of genes with low and moderate correlation are shown. (b) Interaction network of the 4 genes with low to moderate correlation. (c) Expression levels of these genes in primary tumors (PT) and adjacent normal tissue (ANT) of patients from TCGA BRCA data – Illumina HiSeq. Significance was determined by paired T-test or Wilcoxon Signed Rank Test, when normality failed (*, $p<0.05$).

These results suggest that miR-877-5p/TIMP3/IGF2 expression in BC tissue and/or plasma could be a relevant molecular axis for BC development and progression, with high relevance in patients with AAMS.

2.6. miR-877-5p inhibition decreased viability and adhesion of TNBC cells.

Finally we examined if miR-877-5p modulated cellular properties related with BC growth and progression of the TNBC 4T1 cell line. The transient transfeccion of 4T1 cells

with a miR-877-5p inhibitor diminished expression levels of this miRNA after 24 and 48 hours (Figure 7A,B). We found that the inhibition of miR-877-5p caused a decrease in viabilitability (Figure 7C) and adhesion capability of 4T1 cells (Figure 7D).

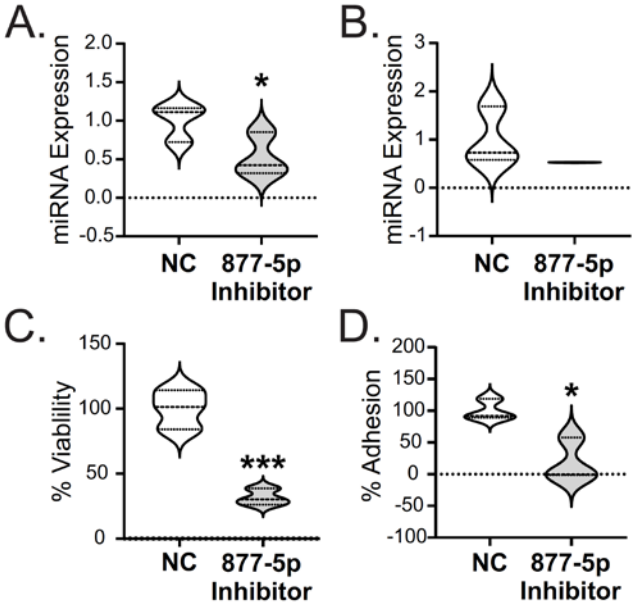

Figure 7. miR-877-5p inhibition decreased viability and adhesion in a model of TNBC. 4T1 cells were transfected with miR-877-5p inhibitor (877-5p inhibitor) or NC5 negative control (NC). (a) miR-877-5p expression was determined by RT-qPCR 24 hours post-transfection or (b) 48hours post-transfection. Data were normalized to U6 expression and control cells. (c) Twenty four hours post-transfection cells were incubated with medium containing 1% FBS and cell viability was determined by MTS assay 48 hours later. (c) Cell adhesion was determined after 24 hours post-transfection. For each experiment three biological replicates were performed with 4 technical replicates Significance was determined by T-test (*, $p<0.05$; ***, $p<0.001$).

Overall, our findings demonstrate that miR-877-5p regulated TNBC cells viability and adhesion. Moreover, our results show that miR-877-5p inhibition could posses therapeutic potential for the treatment of TNBC. Also miR-877-5p/TIMP3/IGF2 expression in BC tissue and/or plasma of patients could be a relevant molecular axis for BC development and progression, with high relevance in patients with AAMS.

3. Discussion

In this work we provided new possible counterparts in the interaction between BC and AAMS based on miRNA detection. Dysregulation in miRNA expression has been linked to several pathologies, including cancer and metabolic disorders[20,21]. In fact, some studies have reported that MS induces aberrant expression of miRNAs in the liver, adipose tissue and blood[24–26]. We hypothesize that circulating miRNAs of women with AAMS could impact in BC development and progression and, hence, could represent important small molecules for prognosis and treatment of this disease. In this study, we performed the first step in highlighting this issue by identifying altered miRNAs in the

plasma of women with AAMS, which also have functions on BC. Thus, we demonstrated that some of the circulating miRNAs in patients with AAMS have functions related to cancer (AAMS-downregulated miR-23a-3p, -19b-3p, -181a-5p, -122-5p, -425-5p, -28-3p, -146a-5p, let-7b-5p and AAMS-upregulated miR-101-5p and -877-5p) and some of them are also dysregulated in BC tissue (Figure 8). Interestingly, we found that let-7b-5p and miR-28-3p are decreased in plasma from patients with AAMS and are also decreased in breast tumors; while miR-877-5p is increased in both pathologies (Figure 8).

Figure 8. A cluster of circulating miRNAs dysregulated in plasma of woman with AAMS has relevance on BC. Review diagram of the new insights presented in this paper. This diagram was performed using Canva free on line resource (https://www.canva.com/).

The Let-7 miRNA family is known to be involved in carcinogenesis and tumor progression, among other processes. Let-7b is known as a tumor suppressor, which plays an important role in the activation of mammary stromal fibroblasts and their connection with tumor cells. Let-7b levels have been found to be lower in tumor-associated fibroblasts (CAFs) than fibroblasts from adjacent normal tissue[27]. In addition, in samples of breast tissue, a greater expression of this miRNA is correlated with a better clinical prognosis in patients with ER+ BC[28]. Low levels of let-7b-5p indicate a low rate of disease-free survival and total survival in patients with basal-like BC, according to an integrative study in preneoplastic breast tissue[29]. Concerning miR-28-3p, there are no studies investigating its functions in BC, but a tumor suppressor role was suggested in esophageal squamous cell carcinoma, since it is down-regulated in tumor tissue[30]. MiR-877-5p has been proposed as a tumor suppressor in hepatocellular and gastric cancer[31–34]. In BC context, a recent study suggested that miR-877-5p could have an inhibitory role in the epithelial to mesenchymal transition of Mcf-7 cells by inhibiting the expression of FGB, which induce EMT [35]. Although this report in not in line with our work, miR-877-5p role in BC is a hardly unexplored field. In our study, we found that miR-877-5p was associated with lower survival in BC patients, and that its expression is inversely correlated with overall survival in BC patients.

We also found that miR-877-5p is increased in basal-like breast tumors compared to the other molecular subtypes. This is interesting since a retrospective study found that MS was more prevalent in TNBC compared with the other BC subtypes[15]. Also, we showed that miR-877-5p expression is inversely correlated with overall survival in BC patients, so we find it to be an interesting miRNA for BC associated with metabolic disorders. Hence, we focus our studies on this miRNA. Moreover, using public databases, we demonstrated that miR-877-5p is also increased in plasma from BC patients compared to plasma from healthy donors (Figure 8).

To provide a possible molecular mechanism for miR-877-5p's role in BC, we identified IGF2 and TIMP3 as validated target genes of miR-877-5p whose expression is decreased in BC tissue and is negatively correlated with the levels of this miRNA in the tumors (Figure 8).

The Tissue inhibitor of metalloprotease 3 (TIMP3) binds directly to the extracellular matrix via the proteoglycan heparan sulfate[36]. Since it controls the activity of metalloproteases, it is considered a suppressor of angiogenesis, invasion, and metastasis, as well as an inhibitor of apoptosis[37]. It has been reported that a decrease in TIMP3 expression, among other genes, associated with an increase in CD44 may constitute an indicator of the invasiveness of BC ER+ or ER-[38]. In addition, TIMP3 overexpression in hormone receptor-positive BC cells reduces tumor growth and invasion while inducing apoptosis in ER+ tumor cells[37]. One study revealed that TIMP3, which is frequently hypermethylated in infiltrating ductal carcinomas, is associated with increased tumor grade and nodal metastasis, along with increased expression of ER, progesterone receptor (PR), and Her2[39]. Lastly, it has been observed that TIMP-3 is expressed by fibroblasts in BC, and this expression is associated with a higher rate of distant metastases[40]. On the other hand, a large amount of evidence suggests that the insulin/insulin-like growth factor (IGF) pathway is highly involved in BC[41]. However, there are few studies that have evaluated the role of this growth factor in TNBC, being mostly described in ER+ tumors, where it promotes tumor growth and progression[42]. It has been observed that the CAFs present in BC metastatic sites exert a pro-tumorigenic role, in part exerted by the high production of IGF2[43]. Particularly, regarding the study of this molecule in TNBC, there are controversial results. It has been described that *in vitro* stimulation of MDA-MB-231 cells with IGF2 increased their migratory capacity[44]. In addition, it has been shown *in vivo*, in TN mammary tumor specimens, that IGF2, together with ERβ1, is significantly expressed in TNBC[45]. However, recently, through an *in silico* analysis of gene enrichment with mutations in metastatic TNBC tumors, using the TCGA and METABRIC databases, IGF2 was described as one of the deleted genes[46].

Finally, in addition to the study of miR-877-5p expression in clinical datasets, we demonstrate that miR-877-5p inhibition caused a decrease in the viability and adhesion capability of TNBC 4T1 cells. All together our results suggest that miR-877-5p could impact in TNBC development and progression, thus miR-877-5p inhibition could be an interesting therapeutic approach.

4. Materials and Methods

Patients' recruitment and samples processing.

For volunteers' recruitment, an ethics protocol was established with Hospital Militar Central (CABA, Argentina). Healthy women were asked to sign an informed consent (IC) to enrol them in the protocol and complete a survey on risk factors associated with BC. Women of any age with no history of cancer were eligible. A small sample of peripheral blood was taken and placed in a sterile RNAse-free tube with EDTA as an anticoagulant. In addition, other patient data were requested, such as age, weight, height, waist diameter, blood pressure, biochemical data (blood glucose, cholesterol, triglycerides), and medication history; which were completed on separate forms by the clinician. The plasma from participants was obtained by centrifugation of blood collected with EDTA, and retained in a -80 ° C freezer exclusively for laboratory use and locked.

Volunteers were divided into two groups, the control group and the AAMS group, when presented two or more of these conditions: body mass index (BMI) ≥ 25.00 kg/m^2, waist diameter ≥ 82 cm or high blood pressure (systolic ≥ 120, diastolic ≥ 80).

RNA isolation.

Total RNA isolation was performed using Tri Reagent (Molecular Research Center) as previously described [47,48]. Before extraction 40 fmol of cel-39 synthetic miRNA was spiked to 400 μl of plasma.

miRNA microarrays.

Four samples (2 control and 2 AAMS) were generated by pooling miRNAs from 9 donors. They were hybridized to the GeneChip® miRNA 4.0 Array (Affymetrix). Data normalization and analysis were performed using Expression Console™ Software 1.3.1 and Affymetrix® Transcriptome Analysis Console (TAC) Software. A false significant gene (FSG) < 0.05%, Log fold change (Log FC) > 1.5 and p value < 0.01 were used to identify differentially expressed miRNAs.

Functional enrichment analyses

Functional enrichment analysis was performed as previously described[49]. To investigate the molecular functions of differentially expressed miRNAs in plasma of patients with AAMS factors we used DIANA miRPath v3 tool (https://dianalab.e-ce.uth.gr/html/mirpathv3/index.php?r=mirpath) [50] for obtaining a list of experimentally validated target genes derived from DIANA-TarBase v7. For functional enrichment analysis we employed KEGG pathways and Gene Ontology (GO) resources. The results were merged, selecting Pathways Union in order to obtain both a heat map and a histogram. The top 20 of the statistically significant terms (p-value < 0.01) were selected. The heat maps returned by DIANA miRPath v3 was used to analyze the relevance of each miRNA in each pathway.

TCGA Dataset Analysis

miRNA and gene expression analyses from TCGA were performed similar to what has been described previously [51]. Briefly, clinical-pathological data (PAM50 molecular subtype), mature miRNA and gene expression of BC and Normal Adjacent Tissue (NAT) of patients were obtained from TCGA Breast Cancer (TCGA-BRCA) cohort and from Normal Mammary Gland (NMG) tissues from the GTEx project available in the UCSC

Xena bioinformatics tool (https://xena.ucsc.edu/)[52]. The miRNA-Seq (IlluminaHiSeq_miRNASeq) and RNA-Seq (IlluminaHiSeq) data was downloaded as log2 (RPM+1) values. Seventy-five BC samples were paired with 75 NAT, excluding patients without paired samples. To determine miRNA and gene expression levels based on PAM50 molecular subtypes and to analyse survival curves we utilized all available tumor samples present in the TCGA-BRCA. The Shapiro-Wilk test, the Levene F test, and the box plot were used to assess data normality and variance homogeneity. Paired Sample t-test was applied for data that fulfilled the requirements using Sigma Plot 12.0. Otherwise, the Wilcoxon Signed Rank test was performed. When three or more groups were analysed, one-way ANOVA followed by Tuckey test was performed for data that met the requirements. Otherwise, Kruskal-Wallis rank followed by Dunn's Test was performed. Kaplan Meyer curve analysis was performed using UCSC Xena tool and Log-Rank test was done to determine significance.

Correlation matrix

Validated target genes of miR-877-5p that are involved in processes related to cancer were determined by KEGG using DIANA miRPath v3 and their expression levels were downloaded from TCGA BRCA data available in UCSC Xena as described above. Using the Hmisc R package, we generated a correlation matrix for this miRNA and their target genes, applying the Spearman correlation coefficient. Genes with a negative correlation with miR-877-5p expression (p-value < 0.05) and correlation coefficient rho < -0.2 were selected for further analysis.

miRNA RT-qPCR

miRNAs were retrotranscribed using stem-loop method as previously described[47,48] with some modifications. Briefly, 4 µl of total RNA in the case of plasma samples, were preheated in 14 µL containing 0.07 µM of stem-loop primer at 70° C during 5 min. Then, retrotranscription was performed in a final volume of 20 µL using M-MLV Reverse Transcriptase (Promega) and incubated in a TC 9639 Thermal Cycler (Benchmark) for 30 min at 16° C, 60 min at 42° C and 2 min at 70° C. qPCR was performed with FastStart Universal SYBR Green Master (Roche) using a final volume of 10 µL, 0.1 µM primers and 1 µL of cDNA. Reactions were incubated at 50° C for 2 min, 95° C for 10 min, 40 cycles of 95° C for 15 s, annealing temperature for 15 s and 60° C for 1 min. All reactions were run in duplicate. The expression levels of miRNAs were normalized to cel-39 levels. Primer sequences for miRNA RT-qPCR are as follow: RT-miR-877-5p Fw STEM, GTCTCCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGGAGACCCCTGC; RT-cel-miR-39-3p Fw STEM, GTCTCCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGGAGACCAAGCT; RT-cel-miR-39-3p Fw, CGGGGTCACCGGGTGTAAATC; RT-miR-877-5p Fw, GGGCGGGTAGAGGAGATGGC and RT-Stemloop Rv, TGGTGCAGGGTCCGAGGTATT.

Cell culture and transfections

4T1 cells were grown in RPMI medium (GIBCO) supplemented with 10% of fetal bovine serum (FBS) and antibiotics.

For miR-877-5p inhibition, 4T1 cells were plated in 60mm plates with 80% confluence and transient transfected using 50nM of miR-877-5p inhibitor or NC5 negative control (IDT Technologies) by polyethylenimine methodology (PEI 25kDa linear - PolySciences INC) with PEI:RNA ratio 5:1.

Cell viability assay.

4T1 cells were transfected as described above. After 24 hours, cells were harvested and 4000 cells per well were plated in 96-wells culture plates and grown in RPMI supplemented with 1% FBS and antibiotics during 48 hours. Cell viability was assayed by MTS (Cell-Titer-96-wells Aqueous non-Radioactive Cell-Proliferation Assay, Promega) according to manufacturer instructions [53].

Cell adhesion assay.

4T1 cells were transfected as described above and 24 hours post-transfection cell adhesion assay was performed as previously [47]. Briefly, 12×10^3 cells per well were incubated for 45 min at 37° C in 96-wells culture plates with 200 μL of complete medium. Then, cells were washed with PBS, fixed with 100 μL of methanol and stained with 100 μL of 0.5% crystal violet. Excess of colorant was washed with water and crystal violet was resuspended in 60 μL of 10% methanol and 5% glacial acetic acid solution. Absorbance at 620 nm was determined using an ELISA Multiskan FC (Thermo Scientific).

Statistical analysis

Unless indicated for each experiment section, statistical analysis was performed using Sigma Plot 12.0 based on "n" values corresponding to independent experiments. Data normalization and homogeneity of variances was assessed using Shapiro-Wilk test, and Levene, F test or box plot respectively. A t-test was applied for data that fulfilled the requirements using Sigma Plot 12.0. Otherwise, Non-parametric signed rank test, Mann-Whitney, was performed to test significance for experiments with 2 experimental groups. When one factor and three or more groups were analysed, one-way ANOVA followed by Tuckey post hoc test was performed when the samples in the groups followed a normal distribution and homogeneity of variance. Otherwise, Kruskal-Wallis rank followed by Student-Newman-Keuls test was performed.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Hospital Militar Central (Act number 471, 06/07/2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients.

Data Availability Statement: The research data from microarrays has been submitted to GEO datasets (GSE235355).

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