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Investigating the interplay between the mir-183/182/96 cluster and the adherens junction pathway in early-stage breast cancer

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Although the miR-183/182/96 cluster is overexpressed in breast cancer (BC), little is known about its role in the development of pre-carcinogenic lesions which harbor disrupted adherens junctions (AJ) and may promote BC. Here, we used microRNA and RNA sequencing data from The Cancer Genome Atlas (TCGA) Breast Cancer project to investigate the relationship between the miR-183/182/96 cluster and AJ signaling in early-stage BC. We found that all members of the cluster are significantly overexpressed in early-stage BC, the AJ signaling pathway is enriched for genes down-regulated in early-stage BC, and the AJ signaling pathway is enriched for experimentally validated targets of the miR-183/182/96 cluster. The expression of hsa-miR-182 correlates inversely with the mRNA expression of four of its target genes belonging to the AJ signaling pathway: WASF3, EGFR, MET, and CTNNA3. However, the correlations between hsa-miR-182 and AJ gene expression did not differ significantly between targets and non-targets of hsa-miR-182. This suggests that regulatory effects of microRNAs are less pronounced in cancer, as has been shown by other studies. Furthermore, WASF3, EGFR, and MET are oncogenes that tend to be upregulated in later BC stages, implying that the role of some AJ genes changes with different BC stages.

According to the World Health Organization, breast cancer (BC) is the most commonly occurring cancer worldwide. In women, it is the most diagnosed cancer and the leading cause of cancer-related deaths[1]. Therefore, a better understanding of the etiology of BC is needed.

Most BCs are triggered by lifestyle and environmental factors[2] which can induce DNA damage and some acquired mutations are also capable of altering the epigenome. Non-coding RNAs (ncRNAs) are epigenetic regulators whose expression is often highly altered in cancer[3,4]. Given their roles in fine-tuning gene expression and their involvement in a number of cellular processes, ncRNAs can alter normal cell function.

MicroRNAs are short ncRNAs whose most well-described role is to post-transcriptionally modulate gene expression by binding to the 3'UTR of their designated mRNA targets, destabilizing the mRNAs and inhibiting their translation. Because microRNAs are released into the bloodstream and can be easily retrieved from body fluids, they are promising agents for the diagnosis, prevention, and treatment of several diseases[5].

Tens of microRNAs are deregulated in BC, including the miR-183/182/96 cluster which is a group of genes encoding microRNAs 183, 182, and 96. Members of the miR-183/182/96 cluster are generally overexpressed in cancer[6]. In fact, the cluster is upregulated in BC tissue and cell lines as compared to normal counterparts[7]. The miR-183/182/96 cluster is implicated in a number of BC hallmarks such as proliferation, migration, invasion, angiogenesis, etc[8–10]. However, little is known about the role of these microRNAs during the early stages of BC development.

Early stages of cancer development are associated with a deregulation of intercellular communication, either through changes in the expression levels of proteins in junctional complexes and/or changes in their distribution. Previous work from our lab showed the importance of intercellular communication via gap junctional complexes in the differentiation of ductular and alveolar mammary epithelial cells. The inhibition of gap junctional intercellular communication in alveolar mammary epithelial CID-9 cells disrupted the production of β-casein – a marker of alveolar cell differentiation[11]. Similarly, knocking down connexin-43 (Cx43), a gap junctional protein, in non-neoplastic HMT-3522 S1 ductular mammary epithelial cells, disrupted their differentiation as indicated by their loss of polarity and lumen formation[12]. Notably, Cx43 knockouts exhibiting a pre-

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carcinogenic phenotype also demonstrate elevated miR-183 and miR-182 levels[13], indicating the involvement of these microRNAs in pre-tumorigenic events. Additionally, we showed that the upregulation of miR-183-5p in HMT-3522 S1 cells leads to the disruption of epithelial polarity, loss of lumen formation, and enhancement of invasion and proliferation, which in turn reflects the loss of a differentiated phenotype and the acquisition of a pre-cancerous one, similar to that seen in Cx43 knockouts[14]. Our findings suggest a relationship between microRNAs 183/182, gap junctions, and pre-cancerous phenotype. Additionally, since miR-96 is similar in terms of seed sequence to miRs 183/182 and shares a promoter with these two miRs, we were prompted to investigate the interplay between the miR-183/182/96 cluster and cell-cell communication.

The importance of intercellular interactions in the pathogenesis of breast cancer and the impact that microRNAs have on cell-cell communication poses a need to investigate the role of microRNAs in such early tumor development events, especially considering the understudied role of the miR-183/182/96 cluster within the context of early breast carcinogenic lesions. In this paper, we report that the adherens junction (AJ) pathway is enriched for genes that are targets of miRNA 183, 182 and 96. We also found that the expression of epidermal growth factor receptor (EGFR), Wiskott-Aldrich Syndrome Protein Family Member 3 (WASF3), Mesenchymal Epithelial Transition (MET), and Cadherin-Associated Protein Alpha 3 (CTNNA3) transcripts – which belong to the AJ signaling pathway – negatively correlates with the expression of hsa-miR-182 in early-stage breast cancer. This implicates miR-182 in the disruption of AJ signaling which is expected to interfere with the assembly and signaling of other junctional complexes including gap junctions. Our findings also point to the involvement of miR-182 in the development of early carcinogenic lesions of the breast, making it a potential biomarker for early detection of breast cancer.

Results

Clinicopathological characteristics and variability of patient samples

The microRNA and RNA sequencing data used for this study was obtained from The Cancer Genome Atlas (TCGA) – Breast Cancer project. The 17 selected patients are females whose ages range between 31 and 88, 41.2% of whom are younger than 50. All BC samples are stage I, 17.6% are specified as Stage IA while the rest are unspecified. 82.4% of the samples are infiltrating ductal carcinomas while the rest are evenly distributed between mixed histology, medullary carcinomas, and mucinous carcinomas (5.9% each). The majority of patients are ER positive (76.5%) and PR positive (64.7%). 58.8% are HER2 negative, 11.8% are HER2 positive, while this information is either not available or has not been evaluated for the other samples (Table 1).

To assess the variability within our two sample groups (i.e. normal and cancer tissue from each patient), we built PCA plots of RNA and microRNA expression data. The cancer group is highly variable in terms of both mRNA and microRNA expression (Supplementary Figs. 1, 2), but this can be attributed to the variable histological and molecular characteristics of our patient cohort, in addition to the wide range of patient ages.

Characteristics	Percentages
Stage	
Stage I	82.4%
Stage IA	17.6%
Histology	
Infiltrating ductal carcinoma	82.4%
Mucinous carcinoma	5.9%
Mixed histology	5.9%
Medullary carcinoma	5.9%
Age	
Younger than 50	41.2%
Older than 50	58.8%
HER2 Status	
Positive	11.8%
Negative	58.8%
Not evaluated/not available	29.4%
ER status	
Positive	76.5%
Negative	23.5%
PR status	
Positive	64.7%
Negative	35.3%

Table 1. Clinicopathological characteristics of 17 TCGA BRCA patient samples. Most samples correspond to Stage I breast cancer patients while the rest are specifically Stage IA patients. The majority of samples are infiltrating ductular carcinomas. Most patients are over the age of 50. Most patients are negative for HER2, and the majority of patients are ER positive and PR positive.

MicroRNAs 183, 182, and 96 are concomitantly overexpressed in stage I breast cancer

MicroRNAs 183, 182, and 96 belong to the top 50 overexpressed microRNAs in stage I BC tissue according to FDR with a log2 fold change (logFC) > 1.2 and FDR < 0.05 (Fig. 1a-c). The expression of hsa-mir-183, hsa-mir-182, and hsa-mir-96 is highly correlated ($R > 0.7$ and p-value < 0.05 for all pairwise correlations) (Fig. 1d). However, hierarchical clustering showed a higher correlation between hsa-miR-182 and hsa-miR-183 while hsa-miR-96 clustered closely to hsa-miR-200c and hsa-miR-141 (Supplementary Fig. 3).

The adherens junction pathway is enriched for genes that are targets of microRNAs 183, 182 and 96

To better understand the role of the miR-183/182/96 cluster, we examined the overrepresentation of the microRNAs' experimentally validated mRNA targets in 2815 KEGG pathways[15–17]. Over-representation analysis (ORA) revealed that the mRNA targets of each of the three microRNAs are overrepresented in the adherens junction KEGG pathway hsa:04520 with FDR < 0.05 (Fig. 2a).

The adherens junction pathway is enriched for genes downregulated in stage I breast cancer

Gene set enrichment analysis (GSEA) revealed 31 pathways enriched for genes significantly upregulated and 88 pathways enriched for genes significantly downregulated in stage I BC (Supplementary Tables 1 and 2). Interestingly, hsa:04520 adherens junction KEGG pathway is enriched for genes significantly downregulated in early-stage BC with an FDR < 0.05 (Fig. 2b,c).

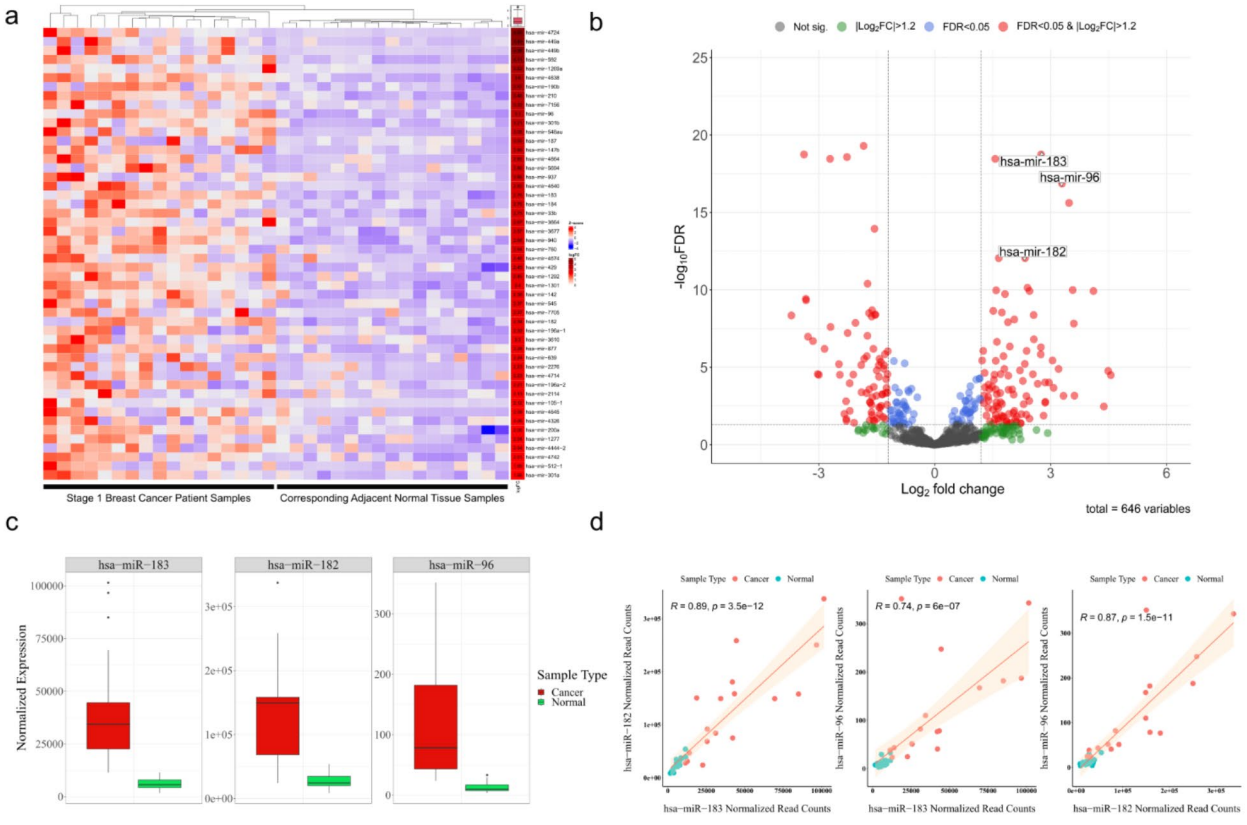

Fig. 1. MicroRNAs 183, 182, and 96 are overexpressed in parallel in stage I breast cancer (**a**) Heatmap of the z-scores of the top 50 upregulated microRNAs in 17 normal breast tissue samples and corresponding stage I breast cancer samples. (**b**) A volcano plot showing the -Log_{10} FDR with respect to the LogFC of 646 microRNAs in primary tissue of stage I BC as compared to normal tumor-adjacent breast tissue. Red: microRNAs with an absolute LogFC > 1.2 and adjusted p-value < 0.05. Blue: microRNAs with an absolute LogFC < 1.2 and adjusted p-value < 0.05. Green: microRNAs with an absolute LogFC > 1.2 and adjusted p-value > 0.05. Grey: microRNAs with no significant difference between cancer and normal. (**c**) Boxplots showing the normalized expression of hsa-miR-183, hsa-miR-182, and hsa-miR-96 in primary tumor and tumor-adjacent normal tissue of stage I breast cancer patients. (**d**) Scatter plots showing the pairwise correlations between microRNAs183, 182, and 96. Pearson's correlation coefficient was calculated. A regression line is shown in dark orange and a 95% confidence interval in light orange.

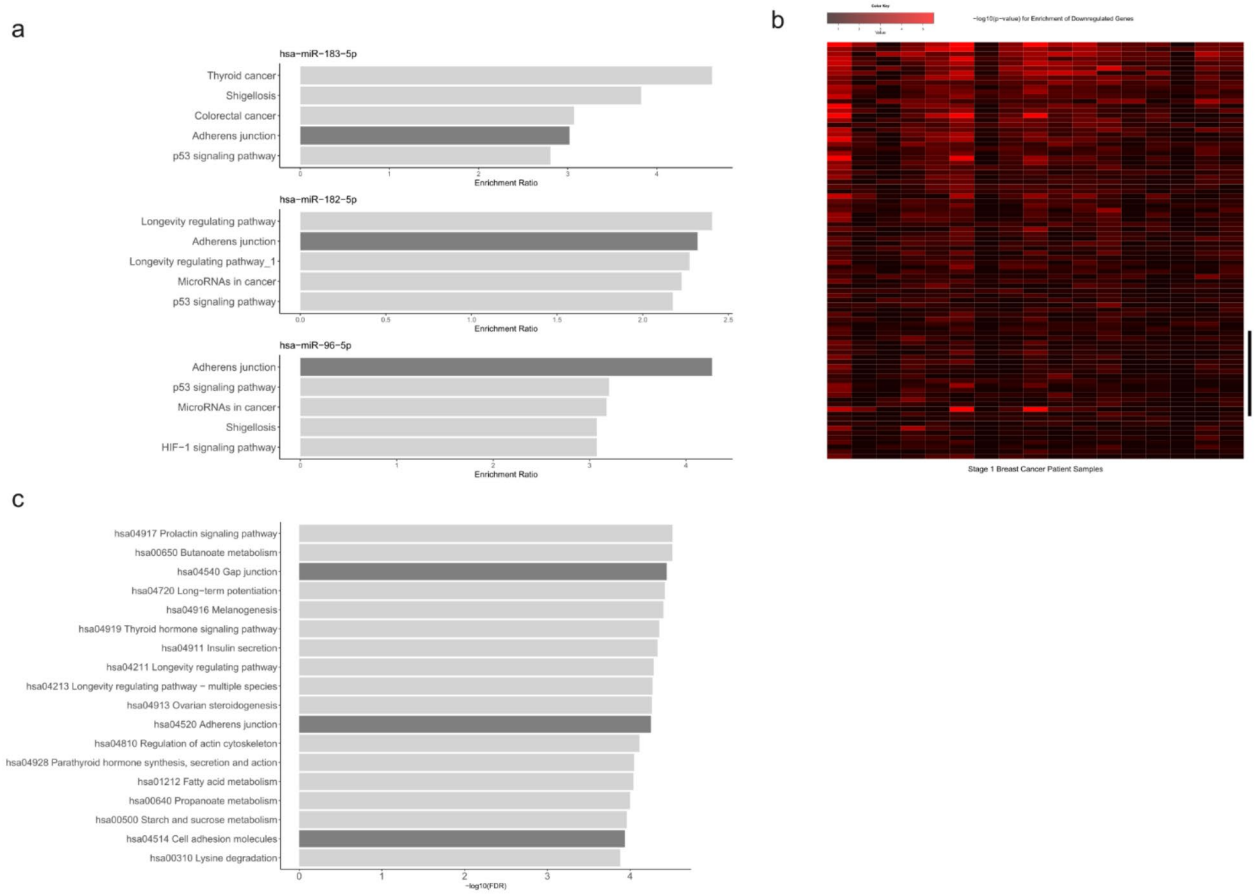

Fig. 2. Targets of hsa-miR-183-5p, hsa-miR-182-5p, and hsa-miR-96-5p are overrepresented in the adherens junction KEGG pathway which is enriched for genes downregulated in stage I breast cancer. (**a**) ORA of KEGG pathways was performed on experimentally validated targets of microRNAs 183, 182, and 96 using WebGestalt. Among the top 15 significant pathways for hsa-miR-183-5p and the top 10 significant pathways for hsa-miR-182-5p and hsa-miR-96-5p, the 5 pathways with the highest enrichment ratios are shown. (**b**) GSEA revealed 88 pathways which are significantly enriched for downregulated genes in early-stage breast cancer. The heatmap shows the -log10(pvalue) of different pathways (rows), ordered from most to least significant, with the columns representing cancer samples. (**c**) Bar plot showing the -Log_{10}(FDR) of selected pathways marked with a black bar in (**b**). Cell communication pathways of interest are shown in dark grey.

WASF3, MET, EGFR, and CTNNA3 are Downregulated and Inversely Correlate with Expression of hsa-miR-182 in Stage I Breast Cancer

hsa-miR-183 targets 14 genes involved in AJ signaling while hsa-miR-182 targets 32 and hsa-miR-96 targets 20. Among these targets, only WASF3, MET, EGFR, and CTNNA3 were significantly downregulated in stage I BC with an absolute logFC > 1.2 and FDR < 0.05 (Fig. 3). Although these genes are experimentally validated targets of hsa-miR-182 only, an AJ pathway target of hsa-miR-182 does not have a higher probability to be downregulated in cancer than a target of any of the other two miRs according to fisher's exact test (Supplementary Fig. 4).

To investigate the association between the downregulated AJ pathway targets and the miR-183/182/96 cluster, we generated pairwise correlations between the expression of the microRNAs and gene transcripts. A significant negative correlation exists between the microRNAs and WASF3/MET/EGFR/CTNNA3 mRNA transcripts (p-value < 0.05 for all twelve pairings) (Fig. 4a, Supplementary Figs. 5 and 6). However, fisher's exact test did not show an association between an AJ gene transcript being a target of the miR-183/182/96 cluster and it being downregulated in BC (Fig. 4b). Among AJ gene transcripts, the correlation between gene and microRNA expression did not differ significantly between miR-targets and non miR-targets (Wilcoxon rank-sum test). This was true for all three microRNAs from the miR-183/182/96 cluster.

Notably, the normalized difference in hsa-miR-182 expression between cancer and normal samples negatively correlates with the difference in EGFR and WASF3 but not MET and CTNNA3. In other words, the more hsa-miR-182 is upregulated, the more the gene transcripts of EGFR and WASF3 are downregulated (Supplementary Fig. 7).

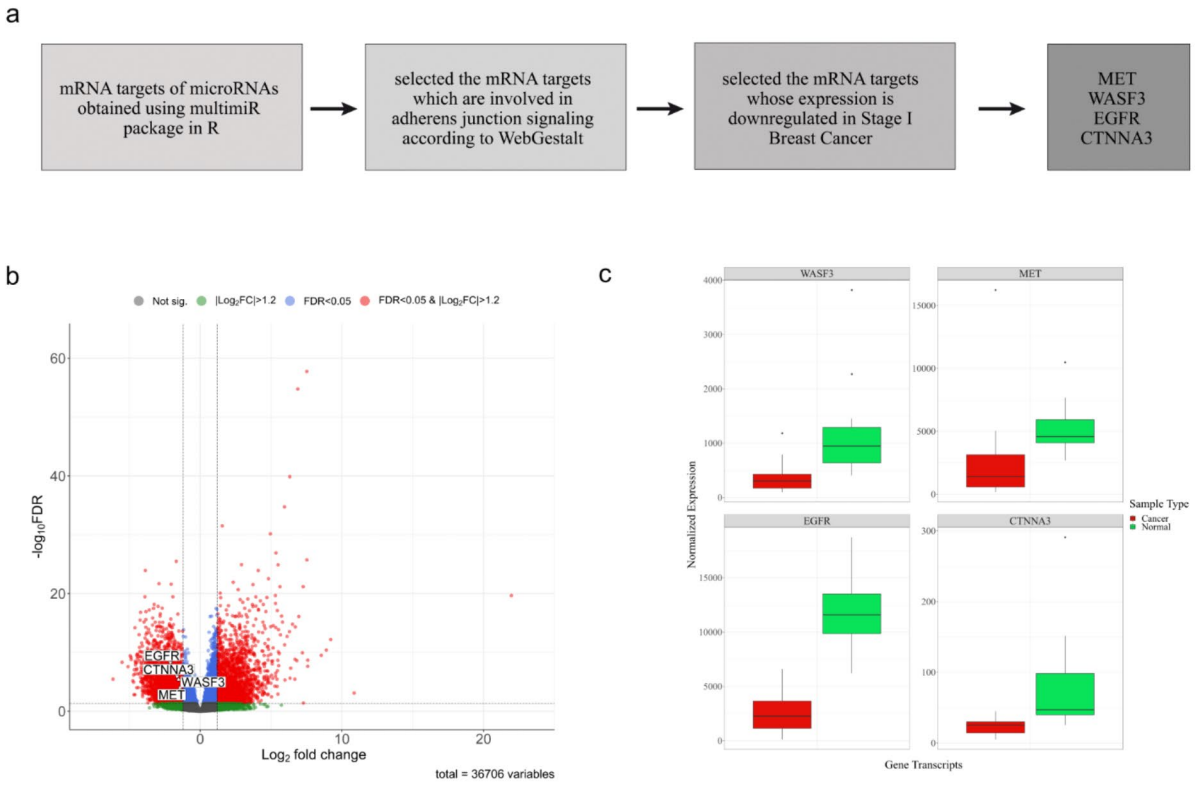

Fig. 3. Adherens junction gene transcripts of MET, WASF3, EGFR, and CTNNA3 are significantly downregulated in stage I breast cancer. (**a**) Experimentally validated targets of microRNAs 183-5p, 182-5p, and 96-5p which also belong to the adherens junction KEGG pathway were selected. WASF3, MET, EGFR, and CTNNA3 were the only downregulated targets, belonging to the adherens junction pathway. (**b**) Volcano plot showing the -Log_{10} adjusted p-values with respect to the Log2 Fold Change of 36,706 genes in primary tissue of stage I breast cancer as compared to normal tumor-adjacent breast tissue. Red: genes with an absolute Log2 Fold Change greater than 1.2 and adjusted p-value < 0.05. Blue: genes with an absolute Log2 Fold Change less than 1.2 and adjusted p-value < 0.05. Green: genes with an absolute Log2 Fold Change greater than 1.2 and adjusted p-value > 0.05. Grey: genes with no significant difference. (**c**) Boxplots showing the normalized expression of WASF3, MET, EGFR, and CTNNA3 in primary tumor tissue of stage I breast cancer as compared to normal tumor-adjacent tissue.

Hsa-miR-200a and hsa-miR-3065-5p are potentially acting in synergy with hsa-miR-182

The relationship of WASF3, MET, EGFR, CTNNA3 expression with hsa-miR-182 expression is non-linear. While all patients with high hsa-miR-182 levels have low expression levels in these genes, some patients exhibiting low hsa-miR-182 levels possess low gene transcript expression as well (Fig. 4a). This is an indication that another regulator or regulatory mechanism is at play. Aside from hsa-miR-182, hsa-miR-200a is the only significantly overexpressed microRNA commonly targeting WASF3, EGFR, and MET in stage I BC (Fig. 5a). Additionally, hsa-miR-200a's expression is significantly negatively correlated with the expression of the three genes (Fig. 5b). On the other hand, CTNNA3 is targeted by 25 microRNAs other than hsa-miR-182-5p, none of which target the other three genes. One of these microRNAs, hsa-miR-3065-5p, is significantly overexpressed in stage I BC and negatively correlates with CTNNA3 (Fig. 5c-d). Since transcripts are usually targeted by several microRNAs and because the association between microRNAs 200a and 3065-5p with their corresponding transcripts mirrors that of hsa-miR-182, we suggest that miRs 182, 200a, and 3065-5p are potential co-regulators of WASF3, EGFR, MET, and CTNNA3 transcript expression.

Next, we aimed to better understand the interplay between hsa-miR-182 and hsa-miR-200a/hsa-miR-3065. Interestingly, the average expression of two microRNAs regulating a common transcript exhibited the strongest negative correlation with the transcript; this is true for all transcripts and their corresponding microRNAs (data not shown). Linear regression that included either WASF3, EGFR, MET, or CTNNA3 expression as response variable and hsa-miR-182 and hsa-miR-200a or hsa-miR-3065 as predictor shows that both microRNAs are inversely associated with EGFR's expression. However, only hsa-miR-200a is negatively associated with WASF3 and hsa-miR-182 is negatively associated with MET. Neither microRNAs are significantly associated with CTNNA3 in the regression analysis (Supplementary Table 3), which can be attributed to the non-linear relationship between the microRNAs and CTNNA3 and/or to the more stringent assumptions of linear regression.

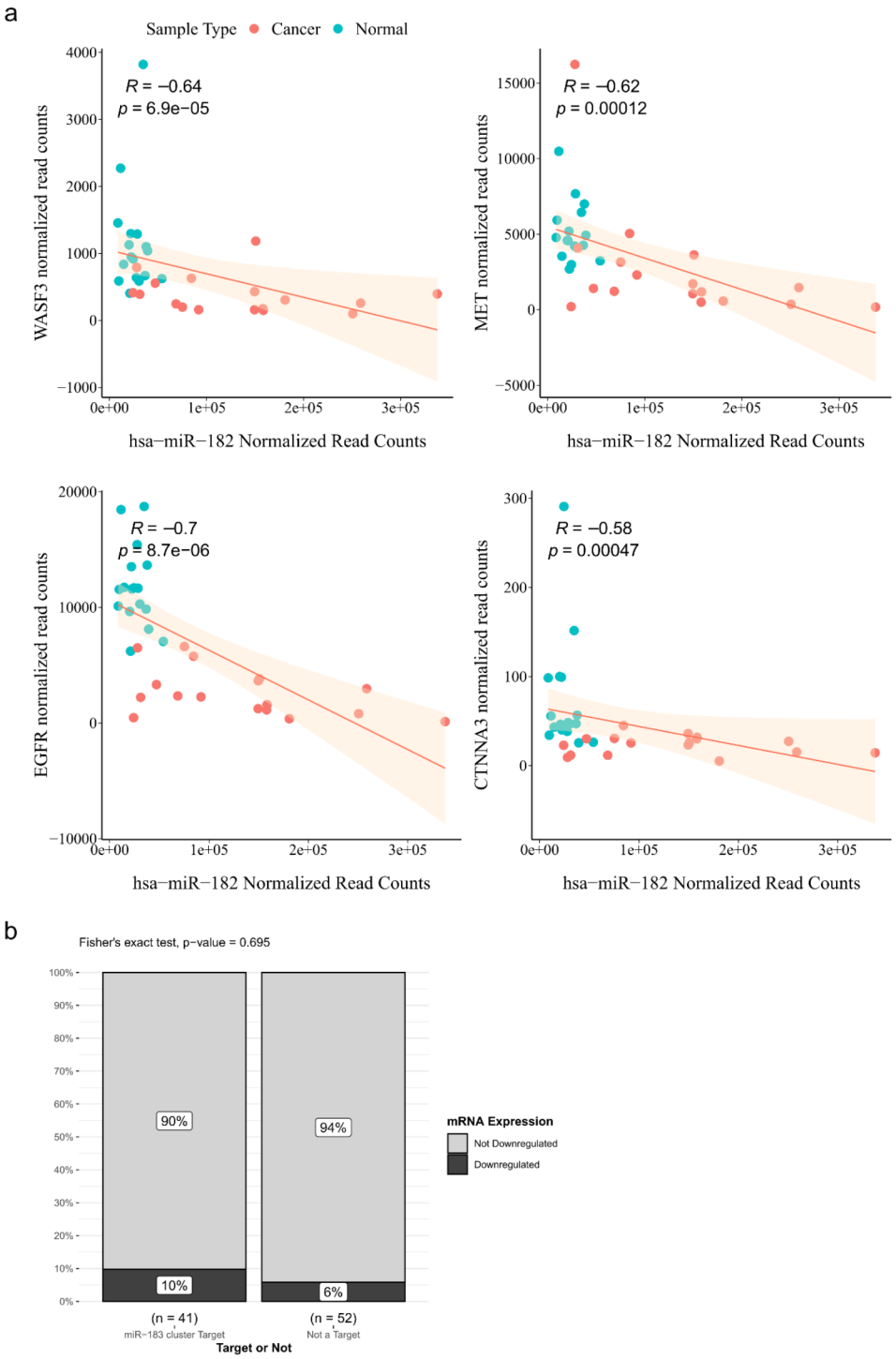

Fig. 4. The upregulation of hsa-miR-182 is significantly correlated with the downregulation of the adherens junction genes WASF3, MET, EGFR, and CTNNA3. (**a**) Scatter plots showing the normalized read counts of hsa-miR-182 versus the normalized read counts of WASF3, MET, EGFR, and CTNNA3 in normal (blue) and stage I breast cancer tissue (red). Spearman's correlation coefficient was calculated for each. A regression line is shown in dark orange and a 95% confidence interval in light orange. (**b**) No statistically significant association is found between being a target of the miR-183/182/96 cluster and mRNA downregulation of adherens genes. 93 Adherens junction genes exist in KEGG pathway has:04520. 41 of these genes are miR-183/182/96 cluster targets while 52 are not. 10% of miR-183/182/96 cluster targets are downregulated and 6% of genes that are not targets are also downregulated. Fisher's exact test shows that there is no statistically significant non-random association between being a miR-183/182/96 cluster target and being downregulated (p-value is equal to 0.695).

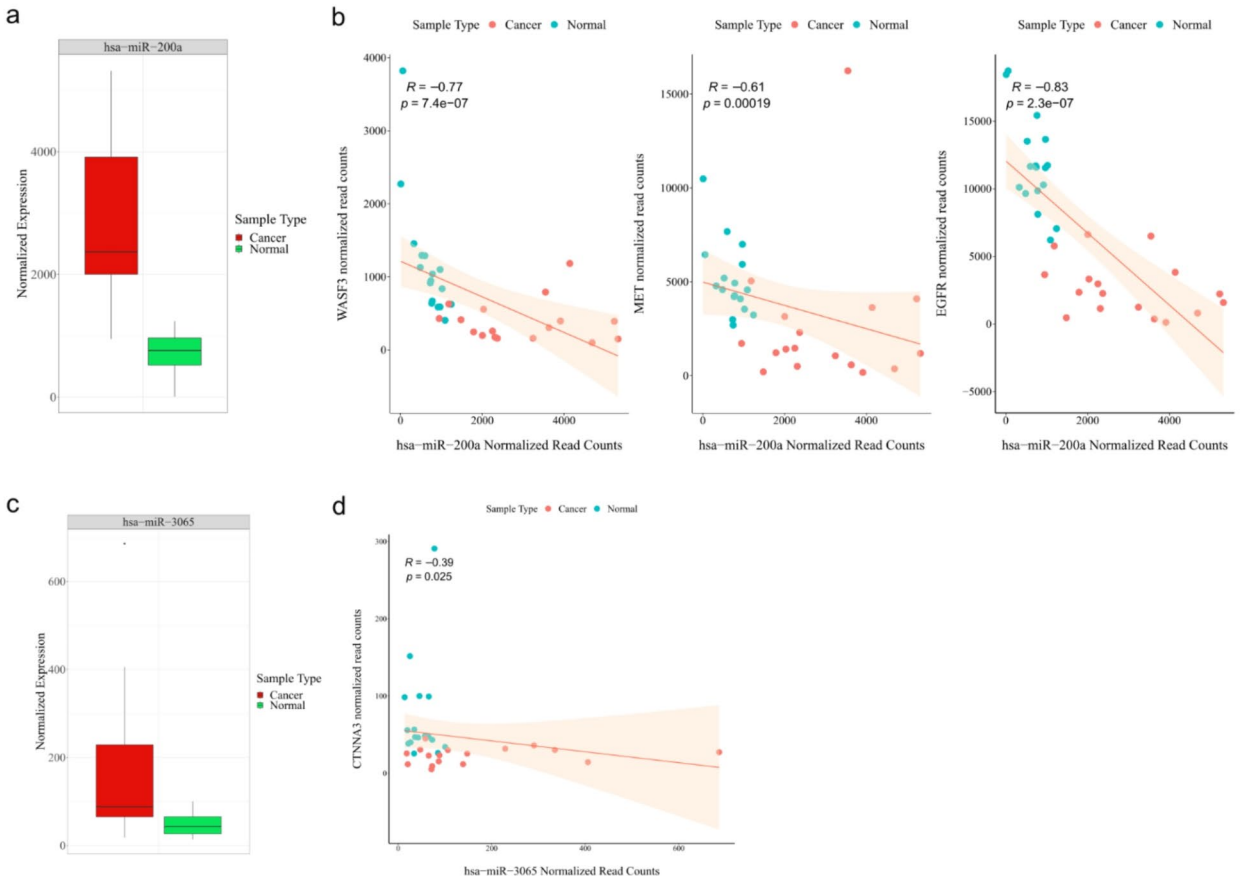

Fig. 5. hsa-miR-200a expression is inversely correlated with the expression of WASF3, EGFR, and MET whereas hsa-miR-3065's expression negatively correlates with the expression of CTNNA3 transcripts. (**a**, **c**) Boxplots showing the normalized expression of hsa-miR-200a and hsa-miR-3065 in stage I breast cancer and corresponding normal samples. (**b**, **d**) Scatter plots showing the normalized read counts of hsa-miR-200a and hsa-miR-3065 against the normalized read counts of WASF3, MET, EGFR, and CTNNA3 in normal (blue) and stage I breast cancer tissue (red). Spearman's correlation coefficient was calculated for each. A regression line is shown in dark orange and a 95% confidence interval in light orange.

We also screened for microRNAs which are associated with the downregulation of our genes at low hsa-miR-182 expression levels but found none which exhibited a statistically significant negative correlation with the transcripts.

Discussion

Here, we report the co-overexpression of microRNAs 183, 182, and 96 in early-stage BC and that the adherens junction pathway genes are enriched in the experimentally validated target transcripts of these microRNAs. Moreover, we found that the adherens junction pathway is enriched for genes downregulated in stage I BC, and that transcripts of four of the pathway's genes – EGFR, WASF3, MET, and CTNNA3 – are downregulated targets of hsa-miR-182. While these findings could suggest that overexpression of hsa-miR-182 down-regulates AJ genes, disrupts AJ signaling and contributes to BC development, we did not find that over-expression of hsa-miR-182 is generally associated with the downregulation of its targets in the AJ pathway.

The structural integrity and normal functionality of the breast depend on regulated interactions between neighboring epithelial cells within the mammary epithelium. These interactions are mainly controlled by polarity proteins and junctional complexes[18]. When cell polarity and intercellular communication are hijacked, normal cell function is lost, and early cancer development events ensue[19]. Our previous work further highlighted the importance of gap junctional intercellular communication (GJIC), particularly Cx43, in the maintenance of a differentiated phenotype in mammary epithelial cells[11,12].

Our results show that AJ pathway genes are enriched in the targets of microRNAs 183, 182, and 96, pointing to a potential mechanism through which these miRs take part in early BC events. We also show that the AJ pathway is enriched for genes that are downregulated in stage I BC. This aligns with the decrease in expression of AJ

proteins (such as CDH1) in parallel with pre-carcinogenic events (such as DCIS) and reduced differentiation[20]. Since cell communication complexes are closely associated in junctional plaques, we propose that the disruption of adherens junctions would create a domino effect, disrupting other intercellular communication mechanisms such as gap junctions and tight junctions[21–24].

The fact that the AJ pathway is enriched for targets of the miR-183/182/96 cluster and for genes downregulated in early BC suggests that the up-regulation of the miR-183/182/96 cluster in early BC is responsible for the downregulation of its targets in the AJ pathway. However, we could not establish that general link. Only targets of microRNA 182 but none of microRNAs 183 and 96's AJ gene targets were significantly downregulated in early BC. Additionally, the downregulation in adherens genes in early BC is not statistically associated with them being targets of microRNAs 183, 182, or 96. This lack of association between expression of microRNAs and their targets is surprising. MicroRNAs and mRNAs tend to be negatively correlated, however, some microRNAs can inhibit translation without substantially impacting mRNA levels. Therefore, it is possible that the regulatory effects of some microRNAs are not visible at the mRNA level and would require proteomics data[25]. Furthermore, the correlation between expression of microRNAs and their targets tends to be reduced in cancer[26].While we could not establish a general signal of the miR-183/182/96 cluster upregulation on AJ gene downregulation in early BC, there are nevertheless, four AJ genes – WASF3, EGFR, MET, and CTNNA3 – that are significantly downregulated hsa-mir-182 targets and negatively correlate with its expression. It is reasonable to assume that the downregulation of these four genes in early BC is at least partially due to the hsa-mir-182 up regulation.

Catenin Alpha 3 / CTNNA3 is a member of the alpha catenin family of cell adhesion molecules which bind to beta-catenin and allow it to attach to the actin cytoskeleton[27,28]. CTNNA3 has been reported to act as a tumor suppressor in different cancers by restoring cell-cell linkage through the E-Cadherin/catenin complex[29–31]. CTNNA3 expression is epigenetically regulated in BC[32]. Additionally, CTNNA3 is a marker of good prognosis in BC and the absence of its transcript has been reported in BC cell lines overexpressing the transcription factor Grainy head-like 2[33]. Therefore, the potential downregulation of CTNNA3, as suggested by our results, is expected to weaken cell adhesion, consequently disrupting the mammary epithelium's homeostasis.

The downregulation of WASF3, EGFR, and MET in stage I BC is surprising since they are considered oncogenic in multiple cancers including BC[34–36]. Nonetheless, these genes are downregulated in TCGA BC patients of all stages according to the UALCAN webtool[37,38]. Additionally, an analysis of a separate microarray dataset from normal and breast cancer tissues (GSE139038) also showed a significant downregulation of WASF3 and MET in early BC (FDR < 0.05), and EGFR is downregulated with a p-value < 0.05 (FDR > 0.05)[39] (data not shown). This variation in WASF3, EGFR, and MET levels is somewhat analogous to the variation in ECM degrading proteins during mammary gland development. Although ECM degrading proteins are elevated during pregnancy, their levels are reduced during lactation and early involution, but increase again as involution progresses[40,41]. This suggests the involvement of these proteins in both mammary gland branching and growth – which is a proliferative phase – as well as its retraction – which is an apoptotic phase. Therefore, it is possible that WASF3, EGFR, and MET are involved in opposing processes, potentially promoting cancer development by either being overexpressed or downregulated. Therefore, we suggest that WASF3, EGFR, and MET's role in BC development is context dependent and could vary with the cancer subtype and stage. This is comparable to other junctional proteins such as Cx43 which acts as a tumor suppressor in the normal breast and whose loss has been linked to cancer initiation and early BC development, whereas its overexpression in later stages of breast cancer is associated with poor prognosis and metastasis[42]. In addition, using the TCGA dataset in UALCAN, we found that the expression of microRNAs 183, 182, and 96 is not statistically different between Stage 1 and later stages of BC (Supplementary Table 4), indicating that the expression of the miR-183/182/96 cluster is likely not reversed in later BC stages. This suggests that other regulators of WASF3, EGFR, and MET alternate during different BC stages.

Although EGFR expression and signaling is associated with BC progression, a recent study by Oshi et al. reported variation in its expression among different BC subtypes. The study highlighted that EGFR's expression was highest in triple negative BC[43]. Interestingly, this study also found that survival was reduced in EGFR low ER-positive/HER2-negative breast cancer patients as compared to EGFR high ER-positive/HER2-negative patients. Notably, our patient sample is predominantly ER-positive (80%). Together, this might indicate that low EGFR levels in ER-positive patients do not indicate reduced malignancy but might lead to further disruption of epithelial homeostasis. Interestingly, a study by Dubé et al. showed that reduced EGFR expression in human epididymal cells resulted in a decrease in GJA1 (Cx43) and its relocalization to the cytoplasm[44]. As such, we propose that the reduction of EGFR levels in early-stage BC potentially results in the disruption of GJIC by re-localizing Cx43, which is a pre-carcinogenic signature of BC[45,46].

MET is a gene coding for a receptor tyrosine kinase whose expression has been associated with poor prognosis in BC. MET's expression reduces survival and increases cancer recurrence in ER-positive/Her2-negative patients[47]. Notably, some studies report that MET expression inversely correlates with E-cadherin levels[48]. This is not concomitant with our data, since E-Cadherin (CDH1) levels were relatively unchanged in stage I BC (log2FoldChange = 0.68 and FDR > 0.05), unlike MET whose expression was downregulated by 2.75 folds. E-cadherin has also been reported to inhibit receptor tyrosine kinases such as MET[49]. Aside from its role as an oncogene, MET is an important player in normal mammary gland morphogenesis[50]. Interestingly, the transcript levels of c-MET and its ligand (hepatocyte growth factor / HGF) vary during different stages of mammary gland development; their expression is low during pregnancy, absent at the onset of lactation, and restored upon involution[51]. This variation in MET levels is somewhat analogous to the variation in ECM degrading proteins during mammary gland development. Although ECM degrading proteins are elevated during pregnancy, their levels are reduced during lactation and early involution, but increase again as involution progresses[40,41]. This suggests the involvement of these proteins in both mammary gland branching and growth – which is a proliferative phase – as well as its retraction – which is an apoptotic phase. As such, it would not

be surprising for MET to be involved in opposing processes, possibly promoting cancer development by either being overexpressed or downregulated. One study reported that miR-182 reduces the resistance of breast cancer cells to trastuzumab by targeting and downregulating MET[52], which points to MET's context-dependent role in BC.

As for WASF3, it is commonly expressed in high grade breast cancers and known to drive invasion and metastasis[53]. Interestingly, a study by Qin et al. aimed to investigate the impact of WASF3 depletion on breast cancer metastasis found that WASF3 null mice exhibited normal mammary gland function and development[54]. Qin et al. assessed mammary gland structure through whole mounts that show normal mammary branching and alveolar formation. Nonetheless, molecular changes were not assessed, and the WASF3 null mice's vulnerability to breast cancer development was not investigated. Therefore, more research is necessary on WASF3's involvement in homeostasis of the mammary gland and breast cancer development.

Although hsa-mir-182 expression negatively correlates with the mRNA levels of the four genes, the relationship between the microRNA and the mRNAs is not linear because some patients display both low hsa-miR-182 and target gene expression (Fig. 4a). One explanation could be that other regulators, including other microRNAs, could suppress the genes' expression levels. Our results show that hsa-miR-200a is potentially one microRNA that is influencing EGFR, MET, and WASF3's expression as it is significantly overexpressed in stage I breast cancer and negatively correlates with the genes' transcript levels. This is in line with a number of other studies that have reported elevated miR-200a levels in breast cancer[55]. Findings regarding miR-200a's role in breast cancer development and progression are inconsistent. Whereas some studies reported that the overexpression of miR-200a promotes resistance to cell death mechanisms and induces chemoresistance in breast cancer cell lines[56,57], other studies showed that miR-200a decreases invasiveness in triple negative breast cancer cells[58]. As for CTNNA3, our data suggests that its lowered expression is associated with elevated hsa-miR-3065 levels. There are no current studies on the role of this microRNA in breast cancer. Although a recent study linked its high expression to reduced overall survival in colon cancer patients[59], another study reports that this microRNA can exhibit both tumor suppressor and oncomiric effects in melanoma cells[60].

Taken together, our results show that hsa-miR-182 is overexpressed in early-stage breast cancer, and its expression is associated with a downregulation in four adherens junction pathway genes WASF3, EGFR, MET, and CTNNA3. As such, we propose that hsa-miR-182, in synergy with other microRNAs such as hsa-miR-200a and hsa-miR-3065-5p, disrupts adherens junction signaling by altering the expression levels of the previously mentioned genes. Since cell communication complexes and their corresponding signaling pathways are interdependent, we expect that the loss of adherens junction signaling hubs such as EGFR, MET, WASF3, and CTNNA3 could disrupt gap junctional intercellular communication and tight junction functionality and localization, therefore leading to reduced differentiation and promoting breast cancer development. However, our results are correlational and require further experimental validation. Some suggestions would be performing luciferase assays to validate the interaction of hsa-miR-182 with its targets and confirm its functional consequences. In addition, it would be valuable to use hsa-miR-182 mimics in ductal mammary epithelial cells and assess EGFR, WASF3, MET, and CTNNA3 mRNA and protein levels, in addition to co-localization of hsa-miR-182 and gene transcripts. Finally, we suggest an assessment of the integrity of junctional complexes in a 3D system treated with hsa-miR-182 mimics using immunofluorescence and scrape loading assay.

Methods

Overrepresentation analysis (ORA)

Experimentally validated microRNA targets were obtained using the multimiR package database version 2.3.0[61]. Overrepresentation analysis (ORA) of KEGG pathways was performed on the obtained targets using the functional enrichment analysis webtool WebGeStalt 2019[62] with "protein-coding genome" as the reference gene set. A false discovery rate and p-value of 0.05 was used as a cut-off. microRNA target genes involved in AJ signaling were retrieved from WebGeStalt for further analysis.

TCGA data acquisition and pre-processing

Breast cancer RNASeq and miRSeq TCGA data was downloaded from the Genomic Data Commons (GDC) using the TCGAbiolinks package on R version 4.2[63]. On February 2023 1,097 breast cancer cases were available in the TCGA-BRCA project but 1207 sequenced samples were available including replicates. The clinical data corresponding to the TCGA-BRCA project was also retrieved using TCGAbiolinks. We only used sequencing data of primary cancer tissue or normal tumor-adjacent tissue obtained from Stage I breast cancer patients. We selected patients that had both normal and cancer samples, and those that had both microRNA and mRNA sequencing data. One outlier was removed based on a preliminary PCA plot. The final sample size was $n = 17$ normal breast tissue and $n = 17$ stage I primary breast cancer tissue.

Rnaseq and mirseq analysis

Read counts were filtered so that only mRNAs or microRNAs with more than 10 reads in total were kept. Differential expression analysis was performed using the DESEq2 package (built under R version 4.2.2)[64]. Normalized read counts produced by the median of ratios method using DESeq2 were utilized for visualization purposes.

Gene set enrichment analysis (GSEA)

Using the GAGE package in R[65], we performed gene set enrichment analysis to assess which KEGG pathways are enriched for genes that are significantly downregulated in stage I breast cancer tissue as compared to normal. Library size normalization was used for this analysis.

Correlation analysis
Normalized read counts were used to build pairwise correlation plots between the microRNAs themselves, and between microRNAs and selected mRNAs. Using the ggpubr package, Pearson's correlation coefficient was used for microRNAs and Spearman's correlation coefficient was computed for microRNAs vs. gene transcripts.

Association testing
Fisher's Exact Test was performed to test, among AJ genes, for an association between being a target of the microRNA183/182/96 cluster and being downregulated. Additionally, the following test was performed for each microRNA in the cluster: a correlation coefficient was calculated between the microRNA expression and gene expression of each gene in the AJ pathway. These correlation coefficients were then compared between genes that are targets and non-targets of the respective microRNA using the Wilcoxon rank-sum test.

Linear regression
The expression of WASF3, EGFR, and MET was regressed against hsa-miR-182 and hsa-miR-200a expression as predictors in separate univariate regression analyses. In addition, the expression of CTNNA3 was regressed against hsa-miR-182 and hsa-miR-3065 expression as predictors.

Principal component analysis (PCA)
To assess the variability within the patient groups, principal components of the normalized miRNA and RNA sequencing data were retrieved using the stats package. PCA plots and factor loadings were plotted using ggplot2.

Hierarchal clustering
A microRNA-microRNA distance matrix was generated by subtracting the pairwise correlation of microRNA expression from one. The stats package was used to perform hierarchal clustering.

Data availability
All data used belongs to and is publicly available at The Cancer Genome Atlas Breast Cancer project.

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Author contributions

H.D., R.T., and T.N. designed and conceptualized the project and wrote the manuscript. T.N. performed data processing, analysis, and visualization. N.M. and A.K. assisted with some data processing and visualization. H.D. and R.T. mentored T.N. throughout the writing process. All authors critically revised all drafts and approved the final version for submission.

Declarations

Competing interests
The authors declare no competing interests.

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