CIRCULATING miRNA-21 LEVELS IN BREAST CANCER PATIENTS BEFORE AND AFTER CHEMOTHERAPY AND ITS ASSOCIATION WITH CLINICAL IMPROVEMENT

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DECLARATION

I hereby declare that thesis entitled **"Circulating miRNA-21 levels in breast cancer patients before and after chemotherapy and its association with clinical improvement"** embodies the original work carried out by me.

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CERTIFICATE

This is to certify that the thesis entitled **"Circulating miRNA-21 levels in breast cancer patients before and after chemotherapy and its association with clinical improvement"** is the bonafide work of **Dr. Sanchi Sukhija** carried out under our guidance and supervision, in All India Institute of Medical Sciences, Jodhpur

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Dr. Sanchi Sukhija

DEDICATED TO MY PARENTS AND

DEPARTMENT OF PHARMACOLOGY

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LIST OF ABBREVIATIONS

AC-T	Adriamycin/Doxorubicin, cyclophosphamide - Taxanes
BCBM	Breast Cancer Brain Metastasis
BRCA	Breast Cancer gene
EGF	Epidermal Growth Factor
EMT	Epithelial Mesenchymal Transition
ER	Oestrogen Receptor
Her2neu	Human epidermal growth factor receptor 2
IDC	Invasive Ductal Carcinoma
LZTFL	Leucine Zipper Transcription Factor Like 1
MCF 7	Michigan Cancer Foundation 7
miR-21	Micro RNA-21
miR	Micro- ribose nucleic acid
NACT	Neo Adjuvant Chemo Therapy
NFκβ	Nuclear Factor Kappa beta
Onco miR	Oncogenic miRNA

PCR	Pathological Complete Response
PDCD4	Programmed Cell Death Protein 4
PR	Progesterone Receptor
PTEN	Phosphate and tensin homologue
RECK	Reversion Inducing Cysteine rich protein with Kazal motif
STAT3	Signal Transducer Activator of Transcription 3
TGF	Transforming Growth Factor
TIMP3	Metalloproteinase Inhibitor 3
TNBC	Triple Negative Breast Cancer

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SUMMARY

Breast cancer is the most frequent type of cancer in women, and many patients experience recurrences and metastasis. As per few published studies, the pathology of many solid tumors including breast cancer involves overexpression of microRNA-21 (miR-21). MicroRNAs (mi-RNAs) are naturally occurring 15–29 nucleotide long non-coding RNA molecules that influence gene expression in a variety of biological roles. They have key regulatory roles in the development, differentiation and apoptosis of normal cells, as well as seen to play role in pathogenesis of cancer cells, affecting carcinogenesis and metastatic potential.

There have been few studies related to increase in miR-21 levels in breast cancer but we don't know how it relates with clinical outcome. At present, there is very limited information existing regarding effect of chemotherapy particularly neoadjuvant chemotherapy on miR-21 expression in breast cancer and relationship of this with the clinical improvement in such patients. Hence, this study was planned to study the effect of neoadjuvant chemotherapy (NACT) on miR-21 expression in metastatic breast cancer and its relationship with the clinical outcome.

For this study, the breast cancer patients of age 18-90 were recruited. After taking informed consent, patients were assessed for RECIST 1.1 clinical scoring and blood sample were collected for measurement of miR-21 level. The patients were followed up for 3 cycles of neoadjuvant and palliative chemotherapy and post treatment clinical score assessment was done. Post treatment blood sample was also collected for measurement of miR-21 level. Effect of chemotherapy on miR-21 level and relationship between miR-21 level and clinical improvement was assessed by suitable statistical tests. After neoadjuvant chemotherapy, expression of miR-21 was significantly increased by 5.65-fold. Whereas, when patients were analyzed for their clinical response using RECIST 1.1 scoring before and after chemotherapy it was seen that there was significant decrease in the tumor size overall after chemotherapy treatment as compared to baseline proving the efficacy of this chemotherapy regimen. When other clinical parameters were analyzed including complete hemogram, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, albumin and creatinine, it was found that there was significant decrease in mean of post-chemotherapy protein and albumin when compared with pre-chemotherapy mean although they were within the normal range proving the mild adverse effect of AC

regimen on liver. Further, hemoglobin and indirect bilirubin were also found to be significantly decreased after chemotherapy. However, the correlation between change in individual expression of miR-21

and clinical scores using RECIST 1.1 was not significant between the groups. The findings of this study will help us to understand the effect of AC regimen on miR-21 and relationship of miR-21 with clinical parameters.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in India and around the world, as well as the leading cause of cancer-related death (1). Breast cancer is the most frequent type of cancer in women, and many patients experience recurrences and metastasis. As per the 2020 epidemiology, there were 22,61,419 new cases of female breast cancer and 6,84,996 deaths worldwide in 2020 (2). It accounts for 1 in 4 cancer cases and 1 in 6 deaths due to breast cancer reaching first rank among 110 countries for incidence and mortality (2). In India, the age adjusted rate of breast cancers in females is 25.8 per 1,00,000 women and death due to breast cancer is 12.7 per 1,00,000 women which makes it the cancer with high incidence and mortality (1). The age-adjusted incidence rate for breast cancer was found to be 41 per 100,000 women for Delhi, followed by Chennai (37.9), Bangalore (34.4), and Thiruvananthapuram (33.7) (3). Looking at the mortality and morbidity associated with the breast cancer, it is important to understand various factors which affects its pathogenesis so that early diagnosis and treatment can be done.

Breast cancer is a heterogenous disease with many subtypes. Proper therapy course for each patient is decided according to the category of breast cancer based on molecular biomarkers (4). Multistep epigenetic and genetic changes in the breast cancer cells leads to the invasion of these cells into the surrounding tissues (5). miRNAs (microRNAs) were found to play an important role in the pathogenesis of breast cancer, including its invasiveness. miRNAs belong to the class of small and non-coding RNA molecules having 15-29 small nucleotides (6). The dysregulation of miRNAs affects the biological processes of the cells like increased proliferation, decreased apoptosis and enhances the metastatic potential. The importance of miRNAs alterations in cancer was further enlightened by Josie et al. (2014) where he observed that miRNAs could alter various cancer specific mechanisms like alteration in target binding site, post transcriptional editing, etc. (7,8).Various miRs are expressed unusually in different types of cancer like head, neck, lungs, colon and stomach when compared with the normal tissues and work as either oncogene or tumor suppressors in breast cancer (9). Of all the miRNAs involved in various types of cancer, miR-21(microRNA-21), appeared to be overexpressed in different cancers including breast cancer (10). miR-21 which is located on chromosome 17q21.3 is one of the most overexpressed miRNA and it was seen that it suppresses multiple target genes (11). Target genes of miR-21 which played chief roles in the oncogenic action were recognized as PTEN (phosphate and tensin homolog), PDCD4 (programmed cell death protein 4), STAT3 (signal transducer activator of transcription 3), RECK (reversion-inducing cysteine rich protein with kazal motifs) (6,12).

In the study by Abdel Hamid et al. (2021), there was a link between miR-21 and chromosome 17 in breast cancer. It was discovered that miR-21 was increased by 12.9 fold in breast cancer tissues compared to normal tissues, and the upregulation was linked to high tumor grade and poor prognostic features (11). Whereas, chromosome 17 monosomy and trisomy was found in several percentage of breast cancer patients which positively correlated with higher miR-21 expression showing their potential for prognostic markers in future (11). Najjary et al. (2020) studied the oncogenic role of miR-21 in breast cancer and demonstrated that miR-21 acts as an oncogene and its inhibition leads to cell cycle arrest, increased chemosensitivity, apoptosis and decreased invasiveness (13). Further, Huang and colleagues confirmed that upregulated miR-21 levels were associated with more aggressive tumor phenotype (14). Erson et al. (2009) silenced miR-21 in MCF7 cancer cells to identify the targets of miR-21 and they identified 58 recognized protein targets of miR-21 and all of them had stable miRNA levels in miR-21 silenced cells further gaining deserved attention for their role in normal as well as cancer cells (15). The expression of miR-21 is regulated by STAT3 and NFkB (nuclear factor kappa B) transcription factors which further play a critical role in cell proliferation, invasion, and tumor invasion processes (16). Even Chunfu Zhang et al. (2016) explained the oncogenic role of miR-21 by its action on its target STAT3, and how the therapy with STAT3 activation inhibitor stattic decreased the cell proliferation and further invasion of the tumor (17). Bautista et al. (2020) investigated the role of miR-21 as a diagnostic, prognostic marker, and therapeutic target for many types of cancer, and it was discovered that miR-21 is overexpressed in various malignancies, implying that miR-21 downregulation may have beneficial impacts on cancer treatment (12). Downregulatory effects of its target genes for cancer therapy further needs more evidence (12). Petrovic et al. (2017) concluded that decrease of TIMP metalloproteinase inhibitor 3 (TIMP-3) mRNA with overexpression of miR21 might be responsible for the invasion of breast cancer process and it was found that there was a positive correlation between the two in In-situ tumors, whereas in case of PR+ tumors, negative correlation was seen, proving that molecular subtypes is an important factor to decide the therapy for cancer subtypes (4).

miRNAs are considered to have strong potential for improving diagnosis and prognosis of cancer and therefore can be used as blood-based biomarkers because they correlate with various stages of metastatic process like progression of disease, EMT (Epithelial-mesenchymal transition), intravasation, extravasation, local invasion, therapeutic response and patient's survival (19,20). There has been evidence supportive of miRNA based staging

and typing of cancers since every type of cancer has unique miR expression at different stages of cancer (19). Just with the expression of other cancer-related genes, miR expression can be altered by chromosomal amplification/deletion, promoter methylation, and transcription factor activation (21). miR expression varied in line with status of HER2 (human epidermal growth factor 2) that is HER2+ or HER2- in breast cancer , as it was found that miR-21 and miR-125b were remarkably elevated in HER2- samples as compared to HER2+ samples in breast cancer and this explained their oncogenic role in breast cancer (22). Pfeffer et al. (2015) identified novel miR-21 target genes as IGFBP3 and FBX011 and observed that their levels were inversely related with miR-21 levels proving their role as tumor suppressors. It was concluded that increased miR-21 appearance was related to poor patient staging and low survival by suppressing these target genes, proving prognostic role of miR-21 (16). miRNAs were also seen to be associated with brain metastasis by regulation of their target genes, when studied miR-21 levels were upregulated in breast cancer brain metastasis. The challenges in breast cancer brain metastasis are to develop potential early detection markers which can improve the prognosis of patients. Therefore, miRNAs present in plasma and CSF seems to be promising biomarkers for providing severity of the disease and also its prognostic role (19). In meta-analysis by Wang et al. (2017), miR-21 expression was correlated with characteristics in breast cancer patients and it was seen that upregulated miR-21 levels were associated with lymph node metastasis, ER/PR (Estrogen /Progesterone receptor) status and HER2/nue expression, whereas age, size of tumor had no significant association thus miR-21 may serve as a novel predictor for prognosis because patients with elevated miR-21 levels had poor clinical characteristics indicating poor overall survival (23). In the meta-analysis by Xiaonan Fu et al. (2011), role of miR-21 was seen in various cancers including breast cancer and it was found that miR-21 play several roles in the pathogenesis of breast cancer like increased cancer cell proliferation, further invasion, inhibiting tumor suppressor genes and also its downregulation can lead to apoptosis and decreased proliferation, overall elevated miR-21 levels were directly related with poor prognosis but this needs further larger studies to support this data (24). miR-21 levels were clinically correlated with early and advanced breast cancer by using American Joint Committee Cancer staging (AJCC) and was seen by Asaga et al. (2011) that it was independent of ER/PR status and age of the patients. There was a direct correlation among upregulated miR-21 levels and staging of tumor. In addition, it was seen that miR-21 further provokes in-vitro and in-vivo cell invasion processes, therefore it might have potential, for detection and advancement of cancer (25). Overall, miR-21 was indicated to play number of roles in tumor development

processes, therefore it was put forward that miR-21 could be a diagnostic, prognostic marker and therapeutic target for various types of carcinomas (12,23). As, this miRNA is involved with tumorigenesis, it may be affected by the therapy, which ideally should decrease the level of miR-21 as a surrogate effect of the therapy, clinical condition and prognosis .

The effect of therapy on miR-21 is explored in few small studies. In a study by Khalighfard S et al. (2018), miR -21, miR-155 and miR-10b were compared before and after the chemotherapy and radiation therapy and all of these miRNAs were found to be downregulated after the therapy hinting the role of these miRNAs as biomarkers for disease control (26). They also closely correlated the miR expression levels with clinic-pathological features and found that miR-21 and miR-155 were also related with ER receptor status as 70 % patients diagnosed were overexpressing ER. The upregulation of ER receptor status has been found to be an important aggravating factor for mammary gland proliferation in early stages of cancer (26). In a similar study, the serum miR-21 expression was assessed in histo-pathologically confirmed breast cancer cases with controls before and after NACT and it was seen that there was 8.9 mean fold increase in expression of miR-21 as compared to controls before therapy and increased levels were also found to be associated with the poor survival of the patients. miR-21 expression was found to be significantly suppressed after the NACT and therefore can be considered as a primary choice of treatment in future (27). Feng et al. (2016), observed that there was decreased drug resistance, if miR-21 was inhibited. Therefore, the potential of miR-21 targeted therapy along with other chemotherapy drugs can be explored in future (28). On the contrary, as seen by Sales et al. (2020), there was no change in miR-21 expression levels after NACT despite complete pathological response as compared to controls (29). Al Khanbashi et al. assessed miR-21 expression at different times while undergoing 4 cycles NACT consisting of AC-T regimen. He observed that, there was no change in miR-21 between different cycles of NACT (30). Leticia De Mattos et al. (2015), demonstrated increased expression of miR-21 after chemotherapy indicating increase in the metastatic potential. It was observed that chemotherapy-induced DNA damage causes an increase in miR-21 expression via NF-kb activation, which leads to increased breast cancer cell invasion (31). Therefore, the effect of therapy particularly drug therapy on miR-21 is not clear, as evidence of no effect of chemotherapy on miR-21 level is also available (29). Looking at the uncertainty of the effect of neoadjuvant chemotherapy on miR-21 expression in breast cancer patients and due to conflicting results and paucity of data for correlation between miR-21 and improvement of clinical scores, it is difficult to consider this miR-21 as

prognostic and therapeutic biomarkers for breast cancer. To fulfill this uncertainty, this study was designed with the aim of evaluating the effect of the neoadjuvant chemotherapy on miR-21 levels and to see the relationship between its levels and clinical condition of the patients.

REVIEW OF LITERATURE

Breast cancer is the malignant most common non-skin cancer originating from breast tissues and comprises of 10.4% of overall cancer incidence in women (32). It is a crucial public health concern worldwide and is the second most common cause of death (33). Around 1.5 million new cases are diagnosed yearly with mortality reaching up to 460,000 annually due to metastasis and chemo-resistance (34). 2021 data calculated by American Cancer Society estimated the new breast cancer cases as 284,200 including both sexes and 44,130 estimated deaths (33). In India, however, breast cancer was graded 1st among females, with Globocon 2012 predicting that India, together with the United States and China, accounts for around one-third of the worldwide breast cancer burden. Between 2008 and 2012, the incidence and mortality of breast cancer increased by 11.54 percent and 13.82 percent, respectively. According to projections for 2020, the number of people will reach 1797900 (1).

Breast cancer is a term that refers to a set of diseases that arise from the breast. BRCA1 (BReast CAncer Gene 1) and BRCA2 (BReast CAncer Gene 2) gene mutations are the most common cause of breast cancer (35). Age, hormone status, family history, and gene alterations are all risk factors for breast cancer (36). Breast cancers are divided into distinct types based on their invasiveness and the location of the primary tumor. They can also be characterized based on tumor staging, status of hormone receptor (ER, PR, HER2), lymph node status, and histologically (36). Breast cancer is marked by metastasis, which is accountable for 90% of cancer deaths. Epithelial Mesenchymal Transition (EMT) plays a significant role in the mechanism of metastasis (36). EMT is a biological process in which epithelial cells lose their cell identity and take on a mesenchymal phenotype. (37). It is linked to enhanced tumor invasion and metastasis in carcinomas because it allows for higher cell proliferation in surrounding tissues as well as diffusion into multiple organs, resulting in secondary tumors/metastasis. More mesenchymal markers like N-cadherin and Vimentin are expressed during the EMT transition, while epithelial markers like E-cadherin are expressed less (38). According to data, some miRNAs are seen to play an important role in breast cancer invasion and metastasis (35).

MicroRNAs (miRNAs) are non-coding RNA molecules with 18-25 nucleotides that modulate gene expression by partnering with the target mRNA's 3'-UTR (three prime untranslated region) (38). More than 700 miRNAs have been found experimentally, with 1900 miRs in the human library (28). These molecules have been shown to have a role in a variety of physiologic and pathological pathways, including cell proliferation, invasion, and metastasis

in breast cancer, through influencing gene expression (32). miRNAs are expected to play an important role in gene expression regulation by being a key layer of post-transcriptional control (28). They were found to have various roles like controlling expression of proteins by changing their MRNAs. They have been seen to play centric role in cancer development pathway, regulating molecular pathway in various cancers by pointing various tumor suppressors and oncogenes, angiogenesis, EMT, chemotherapy resistance and sensitivity (39).

A number of miRNAs have been discovered to have a significant role in cancer invasion and metastasis. More than half of miRNAs are found on vulnerable sites where processes like deletion and amplification of human malignancies occur, according to Bautista Sanchez et al. (2020). When the quantities of miRNA and mRNA were compared to predict cancer prognosis, it was discovered that miRNAs play a substantial impact. Depending on their function, miRNAs can be classified as oncogenes (tumor genes) or tumor suppressors (12). For example miR-21, located on chromosome 17 (17q.23.1) is the oncogene since it acts by targeting genes which further decreased the cancer potential like Programmed Cell Death Protein-4 (PDCD4), tropomyosin 1, serpin peptidase inhibitor and Phosphatase and Tensin Homolog on chromosome 10 (PTEN) therefore, inversely related to the levels of these targeted genes (12). One of the first miRNA found to be upregulated was miR-21 in all types of malignancies (38).

miR-21 expression levels in tumor tissues have been found to be much greater than in paired non-tumoral neighbouring specimens across several years, and its downregulation has decreased tumor development and progression in breast, glioma and colon (27,40). Mingli et al. (2012) looked into the role of miR-21 in EMT transition and discovered that when antagomir-21 was transfected into cancer cells to downregulate miR-21, it reversed EMT transition and cancer cell phenotype by inactivating the P13K-AKT and ERK 1/2 pathways by targeting PTEN, a tumor suppressor gene (38). Han et al. (2012), explained the mechanism of how miR-21 increases the invasion and metastasis by targeting PTEN which is also an antagonist of phosphatidylinositol 3-kinase (P13K) by detaching the 3’UTR phosphate of phosphatidylinositol 3,4,5-triphosphate (PIP3) (38). MiRNAs are capable to function as both oncogenes and tumor suppressors. They're also connected to cancer's intrinsic resistance to various types of therapy, and they're implicated in tumor growth and metastasis, according to Pfeffer Sr et al. (2015) (16). Kumarswamy R et al. (2011) studied the role of miR-21 in breast cancer and discovered a ten-fold increase in miR-21 fold expression

in mice not treated with doxycycline compared to those who were, indicating that upregulating of miR-21 can lead to a pre-cancerous phenotype, making miR-21 a true oncogene (41). Later when miR-21 was inhibited, tumor returned back to normal in some days demonstrating how tumor can get addicted to oncogenic miRNAs, therefore targeted therapeutic modalities can be developed seeing the dependence of various cancers on miR-21 for conservation of malignant phenotypes (41). miR-21 upregulation and downregulation has been seen to be associated with oncogene and tumor suppressor gene respectively (32). Si et al. (2007) employed the double delta Ct method to assess the levels of miR-21 in breast cancer tissues and normal tissues from 157 persons, finding that breast cancer tissues overexpressed in contrast to normal tissues. They also used anti-miR21 oligonucleotide to check if it inhibited cell growth in vitro and in tumors, validating miR-21's role as an oncogene (42). Bao Song et al. studied MiR-21 regulation by targeting tissue inhibitor of metalloproteinase 3 expression (TIMP3), and found TIMP3 to be a functional target for miR-21 for cell proliferation, invasion, and subsequent metastasis in their article. Furthermore, miR-21 inhibited TIMP3 levels in breast cancer patients, indicating that it plays a role in cell invasion. As a result, the expression of miR-21 in invasion and metastasis was found to be regulated by TIMP3 expression (14).

It was seen by Arisan ED et al. (2021), that miR-21 plays a significant role in EMT in the cancer aetiology (43). He worked on cancer cells MDA-MB-231 and knocked out the miR-21 from the cells, which further led to the downregulation of EMT due to inhibition of mesenchymal markers. This concludes that miR-21 regulates the EMT and can be considered as a capable target to control the cancer stemness (43). Rodriguez Martinez A et al. (2019) looked at miRNA profiles to see if they could be used as a diagnostic or prognostic tool in 53 breast cancer patients who were detected with localised breast cancer and had undergone NACT. They discovered that exosomal miR-21 and miR-105 expression levels were higher in metastatic than non-metastatic patients and healthy people before NACT. Six of them were then re-examined using PET-CT scans, and it was discovered that they had developed metastasis. Blood samples taken before and during neoadjuvant therapy were used to extract circulating tumor cells and serum exosomal miRNAs, indicating their potential as biomarkers (5). The predictive relevance of serum miRNAs (miR-21 and miR-125b) for the response to NACT and the prognosis of breast cancer was revealed by Liu b et al. (2017) (44). miR-21 and miR-125b are novel, non-invasive prognostic markers for NACT response and prognosis in breast cancer patients, according to the study, which comprised 118 early stage breast

cancer patients and 30 healthy women (40). In the study by Nina Petrovic et al. (2014), 39 Serbian women having intraductal, invasive and mixed breast cancers were evaluated for miR-21 levels and it was seen that miR-21 levels did not vary among the types of cancers but they differed among age, size of the tumor and grade of the tumor. It was concluded that increased miR-21 were associated with ER+ and PR+ status as compared to ER- and PR- status (45). Anwar SL et al. (2019) reported that circulating miR-21 fold change expression was notably upregulated in breast cancer patients than in healthy women, and that this was further reduced after surgery and chemotherapy, demonstrating miR-21 expression as a promising biomarker for monitoring treatment and clinical outcome in breast carcinoma (46). The number of miR-21 levels was not markedly different between histopathological grades and clinical stages at the time of diagnosis; however, it was shown to be higher in above 40 age group. Patients with high circulating miR-21 levels, on the other hand, had a low progression-free survival rate, indicating that miR-21 may play a prognostic role (46). It was discovered that various factors such as miR-21 upregulation, downregulation of miR-21's direct target, smad-7 and the actions of epidermal growth factor (EGF) and transforming growth factor (TGF), and their correlation with each other for breast cancer invasion were assessed (47). It was seen that hinderance of smad7 by upregulated miR-21 levels led to more aggressive tumor type due to enhancement of EGF and TGF dependent invasion and metastasis (47).

miR-21 levels were 6.2 times higher in the recurrent group, whereas they were only 2.7 times higher in the primary group, according to Motamedi et al. (2019), demonstrating their importance in breast cancer detection and prevention in patients with recurrences leading to metastasis (48). By finding two targets of miR-21, PDCD4 and maspin, Zhu et al. (2008) discovered that miR-21 not only plays a function in tumor growth but also in breast cancer invasion and metastasis. He studied MDA-MB-231 metastatic breast cancer cells and discovered that these targets reduced cell invasion in these cells and were inversely related to miR-21 levels, indicating that miR-21 regulates tumor suppressor genes. As a result, miR-21 could be used as a capable target for breast cancer treatment in the future (49). Wang et al. (2019) found the different target of miR-21, LZTFL1 (Leucine zipper transcription factor like 1) and concluded that suppression of miR-21 led to decreased invasion due to LTZFL1 mediated EMT inhibition but in vivo study displayed that LZTFL1 axis along with miR-21 plays role in metastasis, therefore targeting miR-21 or LZTFL1 for cancer therapy still needs more promising information and exact mechanism of LZTFL1 in EMT still needs to be

determined (50). Xie Y et al. (2019) published a review that attempted to apply bioinformatics tools to acquire a better knowledge of miR-21's mechanism in breast cancer. The researchers discovered that the expression of Mitogen Activated Protein Kinase 10 (MAPK10) was much lower in breast cancer tissues when compared to adjacent tissues, and that it was inversely associated to miR-21. In response to treatment with a miR-21-5p mimic, MAPK10 expression was reduced. By reducing MAPK10, the authors discovered that miR-21 increases breast cancer cell proliferation, migration, and invasion while suppressing apoptosis (51). Ranjana K et al. (2020) studied the role of miRNAs in brain metastasis through breast cancer brain metastasis (BCBM), to make the treatment of brain metastasis easier (19). miRNAs being crucial as oncogene and further inhibiting tumor suppressor genes play a significant role in brain metastasis. Starting from dissemination from primary sites to surviving in the circulation to further entering brain, miRNAs have been seen to be involved in the early steps of the spread. However they did not have enough data regarding the survival of miRNA in the new domain that is brain, their entrance by crossing the blood brain barrier or how do miRNAs regulate the metabolism of brain for survival of breast cancer cells (19).

miR-21 as a Diagnostic Marker

The availability of diagnostic, prognostic, and predictive biomarkers to guide appropriate treatment for various types of breast cancer is crucial. miRNAs have been discovered to be valuable biomarkers for a range of malignancies, including breast cancer, where miR-21 level was seen to be higher, indicating a role in breast cancer detection (34). Due to their ease of isolation and structural stability under diverse sample processing circumstances, they are being recognised as interesting diagnostic, prognostic, and predictive biomarkers in several malignancies, including breast cancer (52). Gao et al. (2013) explored the potential diagnostic role of miR-21 by comparing it to other breast cancer markers CA153 (cancer antigen 15-3) and CEA (carcinoembryonic antigen). They discovered that miR-21 has a much better sensitivity for early breast cancer diagnosis than CEA and CA153, which are the most commonly used breast cancer markers (53). Bautista Sanchez et al. (2020) identified miR-21 as a sex independent diagnostic biomarker since in his study on 76 samples of breast cancer, even the male breast cancer tissues expressed upregulation of miR-21. He also compared miR-21 to other breast cancer markers such as CEA and enolase, and discovered that miR-21 was more sensitive and specific than the other markers, implying that more studies with

bigger sample sizes are required to determine the relevance of miR-21 as a diagnostic marker (12). In the study by Alba Rodriguez et al. (2019), miR-21 levels were correlated with the clinical pathological characteristics in localized breast cancer patients and it was seen that miR-21 expression levels were directly proportional with the size of tumor and inversely proportional with the Ki-67 expression during the Neoadjuvant Chemotherapy (NACT). Some of these patients, later were diagnosed with metastasis indicating the low sensitivity and specificity of present modalities and highlighting favorable role of miR-21 as a diagnostic marker (5). Furthermore, Sales et al. (2020) discovered a link between miR-21 levels and clinic-pathological variables, with higher levels of miR-21 in all types of breast cancer subtypes, regardless of previous chemotherapy, tumor stage, or lymph node status, demonstrating its utility as a diagnostic marker in the future. miRNA could act as a new cancer diagnosis and treatment in future due to its two mechanisms which is either overexpression of miRNA that reduces protein products of tumor suppression genes or loss of tumor suppression expression of miRNA which might lead to elevated levels of oncogenic protein (29,50).

Role of miR-21 as Prognostic Marker

By comparing and analysing the miR-21 expression levels in breast cancer patients and normal tissues using real-time PCR (q-PCR), Li-Xu et al. (2008) studied the potential relevance of miR-21 as a prognostic marker with numerous other miRNAs in 113 breast cancer patients. When statistical analysis was done, it disclosed that overexpression of miR-21 had worst prognosis independent of other factors like ER, PR and age of the patient proving it as an independent predisposing factor whereas, levels of miR-21 were increased up to two-fold in breast cancer patients as compared to the normal tissues. Upregulation of miR-21 has also been linked to advanced tumor stage, metastasis of lymph nodes, and a 5-year survival rate of 86.54 percent in patients with downregulated miR-21 expression, which was significantly higher than that of patients with upregulated miR-21 expression, indicating its role to be used as a prognostic marker (54). Fu et al. (2011) conducted the earliest meta-analysis for miR-21 with the goal of predicting the function of miR-21 in patient survival in diverse malignancies. A higher level of miR-21 may be responsible for a patient's bad prognosis, according to 17 studies gathered for analysis. The hazard ratio of miR-21 expression for overall survival (OS) was 1.69 (P=0.033), indicating poor survival in diverse carcinomas, whereas the hazard ratio for relapse-free survival was 1.48 (P=0.004), implying

that miR-21 may be used to predict survival in general malignancies (24). Positive in-situ staining of miR-21 was found in malignant epithelial cells in advanced stages of Invasive Ductal Carcinoma (IDC) tissues, and further upregulated miR-21 was associated with positive lymph node status in malignant tissues, demonstrating miR-21 potential to be used as a prognostic tool in the future (55).

miR-21 expression was linked to breast cancer stage and prognosis, with the findings indicating a clear link between miR-21 expression and tumor stage, with advanced stage patients having 79.17 percent a greater expression than the early-stage cases who had just 47.19 percent. Therefore, Yan et al. (2008) took all previous data together and concluded that single miRNA has multiple target genes. Due to which different miRNAs target the same gene and further block the number of genes regulating cell proliferation and apoptosis (54). But mechanism of miR-21 in breast cancer further needs more information and evidence. Xin Zhou et al. (2014) did a meta-analysis to investigate the significance of miR-21 as a prognostic marker and discovered that elevated miR-21 levels were inversely connected with the overall survival and recurrence-free survival of patients, although further evidence from large populations is needed (24).

Chunfu Zhang et al.(2016) suggested that miRNAs can regulate malignant changes by targeting various genes. In case of miR-21 it was STAT3 by western blotting he determined if miR-21 levels control the expression of STAT3 (17). Levels of miR-21 were expressed in increasing order from normal through benign to malignant tumor, further increasing cell proliferation, invasion and metastasis with overexpression of miR-21 proving its oncogenic role (17). It was concluded that increased cell proliferation, colony formation and invasion was downregulated by STAT3 inhibitor, Stattic proving the dual role of miR-21 (56). First, by increasing its expression in breast cancer tissues, and then by serving as a tumor gene, it acts as a dual response controller (17). Guinian Wang et al. (1993) observed the expression of miR-21 in Chinese women with breast cancer to see if it had any predictive value, and discovered that upregulated miR-21 levels were linked to a poor prognosis and survival, indicating that miR-21 could be used as a new prognostic marker in the future (57). miR-21 was seen as a prognostic marker by Marta Sereno et al. (2020) in which he demonstrated that extracellular matrix was remodelled by metalloproteinase 3 (MMP) which was further promoted by miR-21 by hindering the tissues inhibitor of MMP3, secreted by breast cancer cells in large levels further degrading extracellular matrix facilitating the emigration of malignant cells and local invasion (58). Kelly et al. (2015) investigated miR-21 expression

and tissue distribution in malignant and benign breast tissues to assess its relevance in breast cancer. It was observed that miR-21 levels were significantly related with lymph node status of the patient showing its role as a valuable prognostic marker. Further when benign types of breast tissues were assessed like Fibrous dysplasia (FD), Fibroadenoma (FA) and Stromal Fibrosis (SF), it was found that there was decrease in levels or very low level of miR-21 in benign tissues except in SF. These findings imply that a modest number of miR-21 functions may be blamed for abnormal proliferation in healthy tissues. Future research into the significance of miR-21 in benign breast diseases, as well as whether the levels are transient or persistent, will be exciting (55). In malignant and normal/benign breast cancer patients, Hui wang et al. (2019) investigated the molecular mechanism and therapeutic significance of miR-21 by looking at another novel target of miR-21, Leucine zipper transcription factor-like 1 (LZTFL1). They found that knocking down LZTFL1 increased the activity of miR-21 inhibitors, which reduced cell proliferation, invasion, and metastasis, implying that miR-21 plays a role in tumor progression, growth, and spread by inhibiting tumor suppressor genes, and that targeting the LTZFL-1-EMT pathway could be a thoughtful strategy for breast cancer therapy (50). It was also seen that miR-21 pathways were part of anti-angiogenesis process of hormonal therapy. Therefore miR-21 levels can be used to interpret the malignancy and to decide the treatment (50). Yuanwen Chen et al. (2019) studied the role of miR-21 along with another miRNA and correlated with hormone receptors status in breast cancer subtypes and observed that miR-21 was directly related with HER2/neu and disease progression and inversely related with ER/PR receptor status showing that these receptors serve as potential clinical biomarkers for different cancer subtypes (59).

miRNA expression in breast cancer patients can influence their responsiveness to or resistance to systemic treatment (50). McGuire et al. (2020) investigated a number of miRNAs as potential indicators of NACT response in locally advanced breast cancer patients. He collected blood samples at the time of diagnosis and compared NACT response to clinical features, discovering that individuals with lower miR-21 expression responded to NACT better than those with higher expression. miR-21 has been discovered to be an independent predictor of therapeutic response in all breast cancer patients, as well as having prognostic potential as circulating biomarkers, which can help us distinguish between patients who react to NACT and those who will benefit the most in the future (60). In addition, an intrinsic breast cancer subtype associated with significant variation in miR-21 and miR-145 expression levels was found to be a predictor of NACT response, suggesting that miR-21

could be used as a prognostic marker (60,61). In meta-analysis and systemic review by Xiaonan Fu et al. (2011) prognostic role of miR-21 was evaluated for various cancers where they observed that miRNAs were taken as capable biomarkers for prognosis because they expressed themselves in the cancer tissue in such a way that is entirely different from expression in normal tissue. miRNAs had more steady expression than mRNAs and further, it was easier to measure miRNAs by RT-PCR (54). In this review, they found that miR-21 was remarkably but not strongly related to the survival of the patients. Alba Rodriguez et al. (2019) investigated the effect of trastuzumab as a NACT on miR-21 levels in HER2-positive and HER2 (-) breast cancer patients, finding that HER2 (+) patients showed a decrease in miR-21 expression after trastuzumab treatment, despite the fact that both groups of patients had similar miR-21 levels at the time of diagnosis (46). Based on previous research, this suggests that HER2/neu may block the Mitogen Activated Protein Kinase (MAPK) pathway, resulting in decreased miR-21 expression. This draws attention to miR-21's potential as a prognostic marker (5). Ji Guang Han et al. (2017) looked at serum levels of miR-21 to determine if it could be used as a diagnostic marker, and found that miR-21 levels were elevated in both tissue and blood samples of breast cancer patients, demonstrating their importance in cancer progression and stage. This suggests that miR-21 can be employed as a prognostic and diagnostic biomarker, as well as a predictor of tumor progression. As a result of its status as an oncogenic microRNA, miR-21 can be employed as a therapeutic target for breast cancer (8). It was also seen that miR-21 expressions were more as compared to the CEA and CA153 proving miR-21 diagnostic sensitivity more for breast cancer patients (8). In a study by Bautista Sanchez et al. (2020), miR-21 was linked to disease-free survival and overall survival, and overexpression of miR-21 was related to poor prognosis, demonstrating its utility as a prognostic marker for a variety of cancers (12). According to Campos-Parra et al. (2017), miR-21 can be employed as a predictive marker as well as a diagnostic and prognostic marker, since patients with elevated levels of miR-21 and HER2 positive status demonstrated resistance to NACT, including trastuzumab, resulting in poor clinical outcomes (12,62). Resistance was found to be developed due to BCL2 (B-cell lymphoma 2) and other target gene expressions. Hence, more studies and data are required to consider it as a predictive marker in the future (12). As a result, it was found that elevated miR-21 levels were directly related to the worse prognosis in numerous malignancies, including breast cancer, and that further clinical data is needed to explore miR-21 in breast cancer treatment (24). From sample collection and processing to data analysis, the development of a reliable and conclusive panel of circulating miRNAs for diagnosis, prognosis and prediction of breast

cancer appears daunting (52). One of the major drawback is the scantiness of miRNAs which hampers their detection in serum or plasma (52). Secondly, they have been seen in abundance in serum than in plasma and also detecting miRNAs in plasma lead to excluding large number of samples due to the presence of haemolysis. Mostly miRNAs are detected using q-RT-PCR which is cost-effective as well as sensitive but cannot detect novel miRNAs. Further, there is also a need for control/housekeeping gene for normalization of expression levels of miRNA (52).

Chemotherapy and Breast Cancer

Chemotherapy has always been a preferred therapy for all malignancies including breast cancer. However, long term use of chemotherapeutic drugs and disturbance of miRNAs can lead to chemoresistance. The mechanism involved in resistance includes mutations in gene, DNA methylation and histone modification further leading to disturbance in miRNAs mechanisms and gene mutations (63). For example, Mata et al. (2010) investigated the role of miRNAs in chemoresistance in breast cancers and observed that majority of primary breast cancers were ER+ subtypes and responded to anti-oestrogen treatment i.e., tamoxifen but as disease progresses ER receptor loses its expression and further losing its sensitivity towards tamoxifen leading to tamoxifen resistance (63). As a result, antago-miRNAs or anti-sense oligonucleotides are being researched for the breast cancer treatment alone or in combination with conventional therapy since they appear to be more promising than other medications.

Neoadjuvant Chemotherapy response to breast cancer was assessed using RECIST 1.1 by Kitajima et al. (2018) in which he took 32 patients and assessed them before and after NAC using MRI and FDG-PET/CT and he observed that sensitivity and specificity of RECIST1.1 was 28.6% and 94.4% respectively whereas Regression Free survival (RFS) was slightly longer in pathological complete response (pCR) as compared to the ones who did not (64). M. Spielmann et al. (1999) investigated the neoadjuvant effect of the docetaxel-cisplatin (DC) regimen in advanced breast cancer patients. The DC regimen has been shown to be an effective alternative or substitute for single docetaxel therapy in patients with anthracycline-resistant advanced cancer. Although the majority of patients experienced neutropenia, other toxic side effects such as anaemia, asthenia, and fluid retention were reduced with DC treatment as compared to docetaxel alone (61).

The oligonucleotide analogues of miRNA have the ability to boost the susceptibility to chemotherapy medicines by upregulating the amount of particular miRNA that are depleted in breast cancer (34). Yang et al. (2020) concluded that combining miRNA with standard therapy could improve breast cancer prognosis (34). Despite the fact that inhibition of miR-21 has been found to be helpful in the treatment of breast cancer, there is currently a scarcity of information on miRNA inhibitor delivery devices. As a result, using the poly (L-lysine) modified polyethyleneimine (PEI-PLL) approach, Shiqian Gao et al. (2017) studied two inhibitors of miR-21, namely miR-21 sponge and anti-miR-21 oligonucleotide (AMO), in the breast cancer cell line MCF-7 (44). They discovered that inhibiting miR-21 using miR-21 inhibitors/PEI-PLL slows down the G1 phase of the cell cycle, increasing the expression of tumor suppressor genes including PDCD4, and initiating the apoptosis pathway. Worth mentioning, the inhibitors of miR-21 i.e., PEI and AMO were found sensitizing breast cancer cells MCF-7 to the chemotherapy drugs, doxorubicin and cisplatin proving their novelty for breast carcinoma therapy by miR-21 inhibition (65).

Al Khanbashi et al. (2016), explored the profile of four miRNAs including miR-21 in serum and plasma of LABC patients to see the response of NACT on miR-21 and its clinical correlation and he concluded that there was no noticeable variation in miR-21 levels after chemotherapy or clinical features instead, miR-21 levels were increased after Adriamycin/cyclophosphamide therapy (30). Further, past studies shows that only triple negative breast cancer (TNBC) subtypes shows positive correlation with miR-21 levels and poor survival of patients (66). Ugur Gezer et al. (2014) investigated the effect of NACT on various miRNAs, including miR-21, in breast cancer patients, observing miR-21 levels at baseline and after the fourth cycle of chemotherapy. It was notable to see that patients with stage II tumors had much higher levels of miR-21 than patients with advanced stages of tumor, and when samples were taken after the fourth cycle, the levels of miR-21 declined in patients who had smaller tumors, proving that significantly demonstrated miRNAs are distressed by chemotherapy in early stages of tumor (67). The ability of silencing miR-21 to further modify the sensitivity of tamoxifen and fulvestrant was investigated in a study by Xin Feng et al. (2016), and it was discovered that inhibiting miR-21 in cancer cells (MCF-7) by its inhibitor boosted the sensitivity of ER (+) breast tumors. The indicators of increased sensitivity were an increase in autophagy of cells , upregulated beclin-1 and LC-3 dots through. As a result, it was established that inhibiting miR-21 enhances cell apoptosis through inhibiting the mTOR pathway, resulting in increased sensitivity of ER (+) cells to

chemotherapeutic treatments (68). Leticia De Mattos-Arruda et al. (2015) observed that miR-21 plays a significant role in cancer chemotherapy drug resistance by acting through EMT. Its level increased after neoadjuvant chemotherapy. They further illustrated that miR-21 affects trastuzumab and chemotherapy by activating P13K pathway through triggering an IL6/STAT3 mediated signalling loop. Sales et al. (2020) investigated expression of miR-21 after NACT in a cohort study and discovered that miR-21 expression was not different between breast cancer patients with pathological complete response (pCR) and healthy controls, implying that miR-21 may be restored in response to NACT (29). This suggests that miR-21 levels before and after NACT can be utilised to determine whether miR-21 is involved in drug resistance and to identify patients with HER2+ drug resistance who may benefit from future pharmacological treatments (31).

Seeing the chemotherapy drug resistance leading to metastasis and relapse requires urgent treatment for various cancers. Therefore, the role of miRNAs in chemoresistance was studied by Peter Magee et al. (2015) and evidence was found regarding use of miRNA as a curative tool in the management of cancer along with anti-cancer chemotherapy drugs. One example includes phase 2a trial of Miravirsen, an anti-sense oligonucleotide that encapsulates miR-122 and hampers its role in hepatitis C infection (69). No adverse effects were found during the trial proving its efficacy for promising results. Further, there are still many limitations to overcome before miRNA can be used as a therapeutic tool which includes delivery system. Viral vectors and few chemical changes can help with this problem but still it can be concluded that miRNAs along with traditional chemotherapy treatment can be used for management of cancers needing further data in future (69).

Future Therapeutic role of MicroRNAs

As we know miRNAs either work as tumor suppressor gene or oncogene therefore, there are only two ways for using miRNA as a therapeutic tool, the first one is either by inhibition of oncogenic miRNA which further includes silencing of targeted miRNA profiles or secondly by miRNA replacement therapy which includes either introducing some miRNAs to decrease oncogenesis or to increase the sensitivity to the chemotherapy systemic treatment (70). In an in vivo study, miR-21 displayed the phenomena of "oncomiR addiction" in which when miR-21 was inactivated with anti-sense/antagomirs, then full tumor regression was seen in a few days (71). Since, boosting miR-21 expression can lower chemotherapeutic drug sensitivity, therefore, miR-21 could be used as a future way to prevent chemotherapy drug resistance

(72). However, further research regarding the complex regulatory pathways that further influence miRNA function and tumor-specific effect is needed in order to comprehend miRNAs as therapeutic targets in the future (73). miR-21, a potential onco-miRNA in breast cancer, was investigated for its role as a predictor of cancer severity by correlating it with various clinicopathologic features in 15 patients. It was discovered that miR-21 fold change expression was elevated significantly in contrast to normal samples, but there was no significant variation in terms of tumor size or receptor status, but there was a direct correlation of miR-21 levels with stage of cancer, lymph node involvement, and HER2 status concluding that upregulated miR-21 expression is related with a more aggressive phenotype and plays an important role in progression of cancer, and that miR-21 could be a novel therapeutic target for breast cancer (74). Dai et al. (2017) studied the mechanism of transforming growth factor (TGF-b1) in breast cancer and discovered that TGF-b1 promotes the PTEN axis, which upregulates miR-21 in a dose and time-dependent manner, leading to metastasis and chemo resistance. As a result, TGF-b1/miR-21/PTEN were discovered to play crucial in breast cancer and might be a feasible target for future breast cancer therapies (75). Wengong et al. looked into the role and mechanism of action of microRNAs in chemotherapeutic drug resistance, and found that miRNAs cause drug resistance by targeting genes of drug resistance or changing genes involved in death of cell cycle cell proliferation. Combining miRNA with conventional treatment appears to be a realistic strategy, given that how miRNAs affect resistance (39). For example, Wu et al. combined miRNA-27b with a few medications and discovered that, via activating p53 and inhibiting CYP1B1, miRNA was able to boost the impact of anti-cancer therapies, demonstrating synergism. Some studies, such as the co-encapsulation of miRNA-34a with doxorubicin for breast cancer treatment, attempted to combine miRNA with medicines in nano carriers. As a result, when drug resistant regulatory miRNAs are paired with chemotherapy medications, they demonstrate synergism, which can improvise the results of chemotherapy drugs and reverse the resistance of drug. This concludes that when drug resistant regulatory miRNAs are combined with chemotherapy drugs, they show synergism which further can improvise chemotherapy drugs effects and reversal of resistance to drugs (39).

miR-21 has been found to be closely associated with and regulated by hypoxia through HIF-1-alpha (76). It is not yet clear how miR-21 changes dynamically in a tumor during treatment and thus what is its effect in the final outcome. The present proposed study can enlighten us about same. Interestingly miRNA can be interrupted by molecular biology method.

AIM AND OBJECTIVES

AIM

To evaluate the levels of miRNA 21 before and after neoadjuvant and palliative chemotherapy in locally advanced and metastatic breast cancer patients undergoing ACT therapy.

OBJECTIVES

1. To evaluate the levels of miRNA 21 before and after neoadjuvant and palliative chemotherapy in locally advanced and metastatic breast cancer patients.
2. To evaluate the clinical scores before and after neoadjuvant and palliative chemotherapy in locally advanced and metastatic breast cancer patients.
3. To study the relationship between change in miRNA 21 levels with change in clinical scores in patients of locally advanced breast cancer.

MATERIALS AND METHODS

Study Design

This is a single group Quasi Experimental study which was conducted in Department of Pharmacology in association with Department of Biochemistry, Department of Radiation and Surgical Oncology and Department of Pathology.

Study Setting

Patients attending the outpatient and inpatient services in department of Radiation Oncology, in AIIMS Jodhpur were enrolled for this study.

Sampling Population

Pre-chemotherapy and Post-chemotherapy samples of Breast cancer patients (n=29) planned for Neoadjuvant Chemotherapy were collected in Radiation Oncology Department of AIIMS, Jodhpur from February 2020 to August 2021. Patients fulfilling the inclusion and exclusion criteria were included in the study.

Sample Size calculation

Looking at the preliminary nature of the study and feasibility issues, it was decided to fix the sample size to 30 patients.

Patient Inclusion-Exclusion criteria

Patient Inclusion Criteria

- Females ≥ 18 - ≤ 90 years
- Clinically diagnosed as large operable and locally advanced breast cancer and planned for systemic neoadjuvant chemotherapy followed by surgery
- Histo-pathologically confirmed IDC [Invasive Ductal Carcinoma]
- Metastatic breast cancer planned for palliative chemotherapy
- Karnofsky performance score of more than or equal to 60 [Ref.(77)]

Patient Exclusion Criteria

- Any contraindication for systemic chemotherapy
- Uncontrolled hypertension
- History of Diabetes
- History of any other malignancy treated or untreated

- Pregnancy
- Patients who were operated upfront for breast cancer
- Patients with diagnosis of DCIS/LCIS (Ductal carcinoma In situ/ Lobular carcinoma In situ)
- Metastatic breast cancer patients planned only for hormonal therapy

Ethics statement

The study was approved by the Institutional Ethics Committee of All India Institute of Medical Science, Jodhpur (AIIMS/IEC/2020/2059).

Work plan

This was a single group Quasi Experimental study with no new interventions involved. All the interactions with the participants and investigations are the current guidelines of care. All the patients diagnosed with Breast cancer planned for neoadjuvant chemotherapy were screened and offered to be a part of the study. The applied neoadjuvant chemotherapy regimen was Adriamycin/cyclophosphamide with or without taxol's. In these patients, effect of chemotherapy on miR-21 and clinical condition was assessed using standard guidelines. Pre-chemotherapy and Post-chemotherapy samples of patients (n=29) were collected in Radiation Oncology Department of AIIMS, Jodhpur from February 2020 to August 2021.

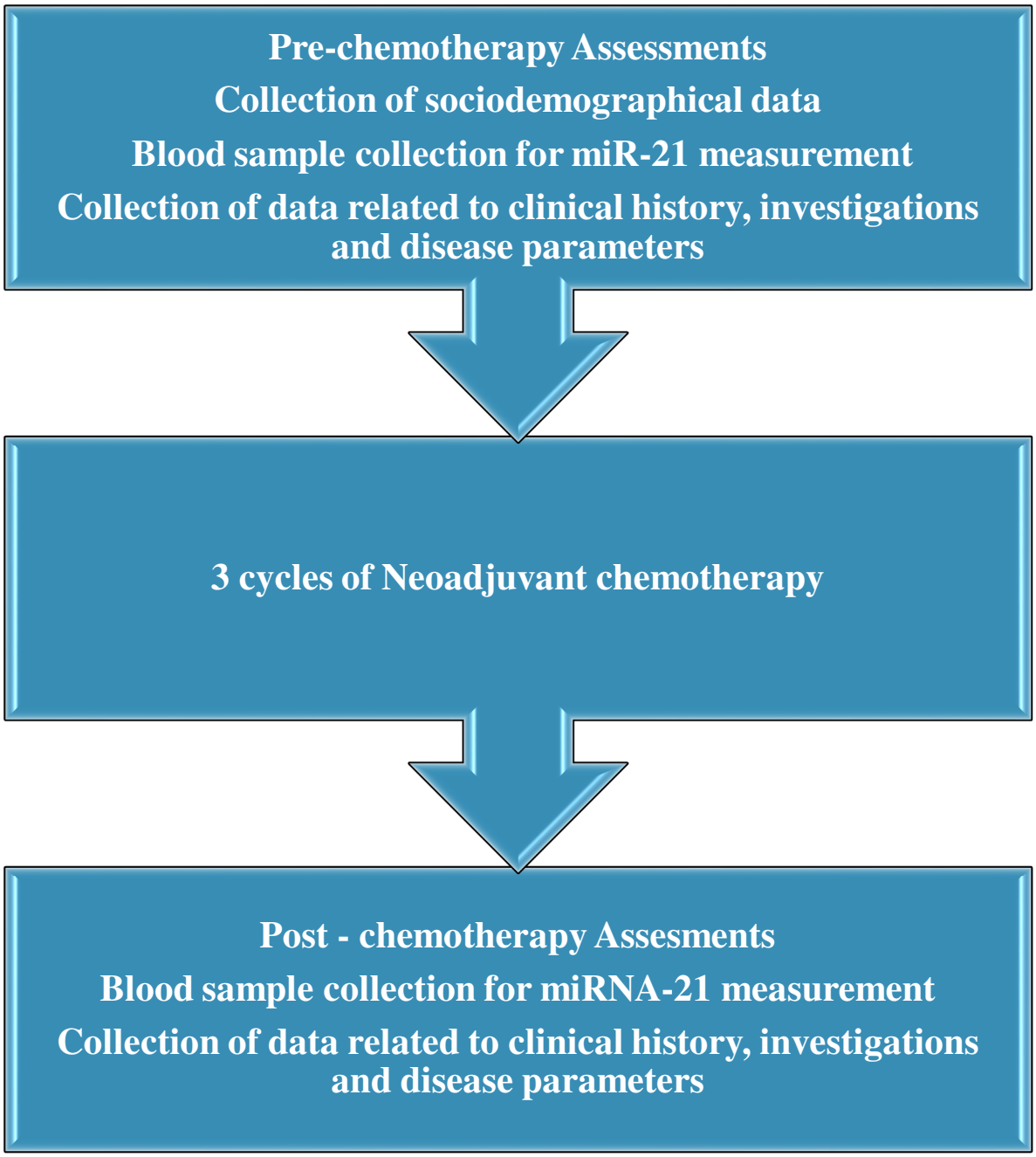


Figure 1. Work Plan

On the first visit to the hospital baseline data for assessment of different variables were taken which includes-

1. Socio-demographic Characteristics- Name, Patients Hospital ID, Age, Gender, baseline data, TNM staging, hormone receptor status, tumor histological type and diagnosis
2. Clinical History- Any comorbidities, Past malignancy, Weight, Height, Body mass index
3. Investigations- Blood sample in EDTA vial was taken for miR-21 isolation

The patients were interviewed about their standard medical history and were evaluated clinically according to the standard guidelines for chemotherapy. This data was captured in the Institute's Computerized Patient Management System (CPMS). All the details of patient's investigations including CT scans, baseline investigations were entered and saved in CPMS. Informed consent was served, explained and signed before initiating the study. After which, the primary baseline samples of Pre-chemotherapy, were obtained from 29 patients and were assessed for fold change expression of miR-21. For 29 patients who underwent chemotherapy, the follow-up samples were obtained after three cycles of chemotherapy and were analyzed for the changes in the miR-21 expression after neoadjuvant chemotherapy. The venous blood sample of about 2-3 ml from each patient was collected in the EDTA vial and was kept at 4^0 Celsius which was processed within 24 hours for RNA extraction (**Figure 2**). RNA was converted further to cDNA using standard procedures in our Institute. The cDNA was stored and preserved in - 80^0. The expression of miR-21 was done using SYBR-Green method.

Besides usual routine investigations being done according to the chemotherapy protocol following samples were taken for the thesis study:

Sample 1 – Baseline sample: Week 0, Day 0, Before starting neoadjuvant chemotherapy.

Sample 2- Follow up sample: Sample was collected after 3 cycles of chemotherapy, the gap between each cycle was 21 days i.e., after 42 days of first sample, week 6-7.

SCHEMATIC DIAGRAM SHOWING THE STEPS OF GENE ANALYSIS AND CLINICAL ASSESSMENT

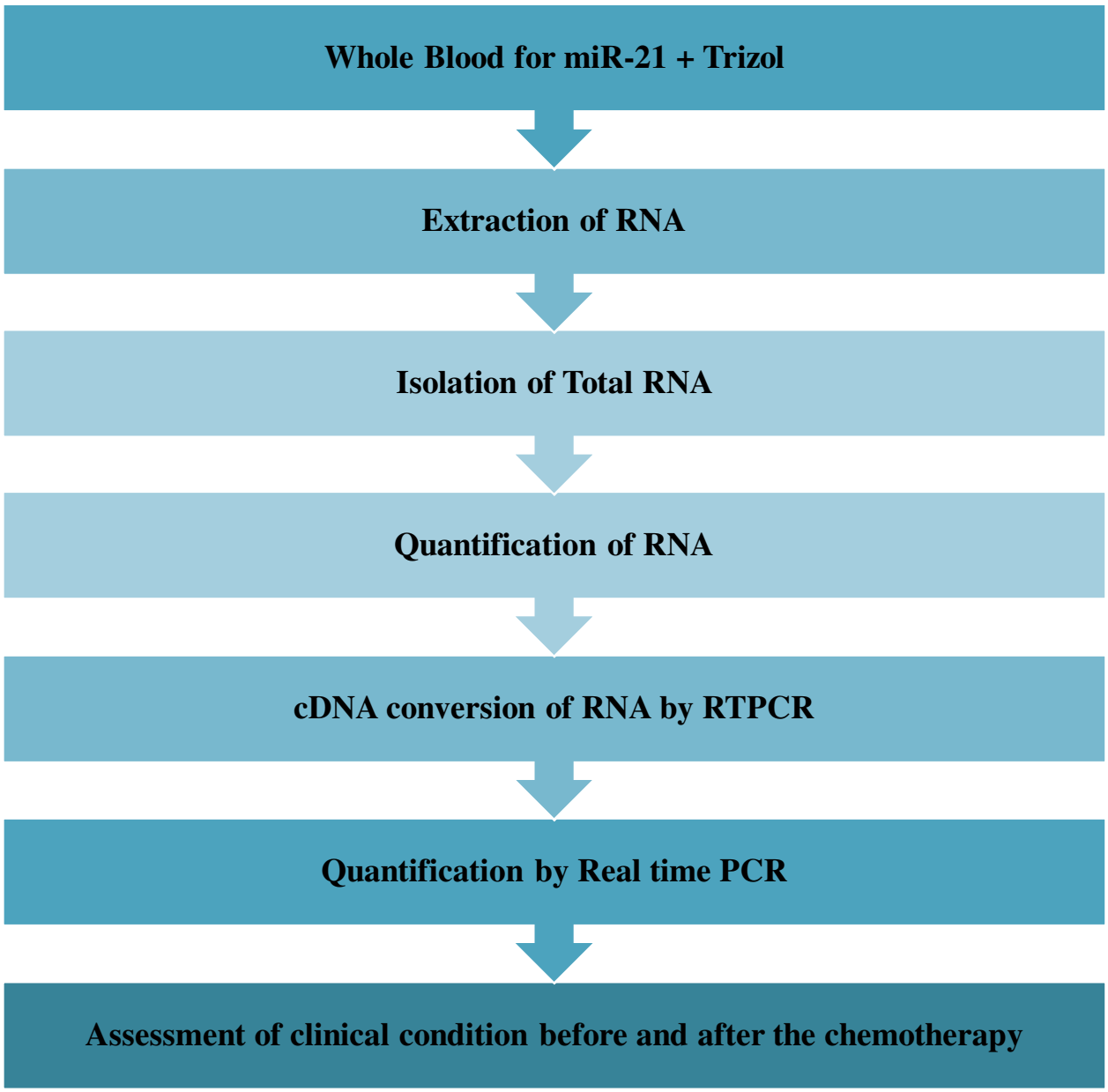


Figure 2. Work Flow

Isolation of Total RNA

As per the institution standard protocol, 2-3ml of venous blood was taken from patient in EDTA vial and kept at 4°C which was processed within 24 hours (1). In order to get RNA from whole blood, RBCs were lysed by adding RBC lysis buffer in the blood volume in the ratio of 3:1. The sample was kept to stand for 15 mins at room temperature (RT), before carrying out the centrifugation. The centrifugation was carried out at 1400 rpm on RT for 15 mins. The supernatant was discarded and pellet was dissolved with 1ml of RBC lysis buffer again. The sample was kept for 5 mins and then centrifuged at 3000 rpm for 2 minutes at RT after which the pellet was dissolved in 1ml of chilled PBS. This was followed by centrifugation at 3000 rpm for 2 minutes at RT. The pellet was dissolved in 100 µl of trizol per ml of blood. The sample was left to stand out for 5 minutes followed by addition of 200ul of chloroform. The sample was then shaken vigorously and left on ice for 15 minutes, following which centrifugation was done at 12000g for 15 minutes at 4^0C. After centrifugation, the upper aqueous layer was carefully pipetted out and transferred to a newly labelled vial. Equal volume of chilled isopropanol is added in a ratio of 1:1 and vortexed and incubated on ice for 20 minutes. It is then centrifuged at 12000g for 12 minutes at 4^0 C. After centrifugation, the supernatant was discarded and the pellet was dissolved in 1 ml of chilled 75% ethanol followed by vortex and centrifugation at 12000g, for 5 minutes at 4^0 Celsius followed by similar ethanol wash. The pellet then obtained was air dried followed by addition of 30-50 µl of diethyl pyrocarbonate (DEPC) water. The sample was then incubated at 60^0C for 10 minutes and stored at -80^0 C.

Quantification of RNA

After extraction of RNA from individual samples, the total RNA was quantified by assessing the concentration and purity by UV spectroscopy.

A260/A280 purity ratio - Ratio of absorbance at 260 nm absorbance at 280 nm. An A260/A280 purity ratio of ~1.8 is generally accepted as "pure" for DNA (~ 2.0 for RNA).

A260/A230 purity ratio - Ratio of absorbance at 260 nm to absorbance at 230 nm. An A260/A230 purity ratio between 1.8 and 2.2 is generally as "pure" for DNA and RNA.

A low A260/A230 ratio indicates contamination with the wash solutions, phenols or proteins.

Figure 3: Microplate reader for RNA quantification

cDNA conversion of RNA by RTPCR

RNA samples were taken out from minus 80^0 C and thawed over ice. Reverse transcription was performed using Eppendorf nexus Gradient master cycler, Qiagen miRCURY LNA RT Kit (339340). The kit consists of 5x miRCURY RT Reaction Buffer, RNase-free water, 10x miRCURY RT enzyme mix and Template RNA (1).

After quantification, the RNA as well as cDNA concentration of 500ng, 250ng and 100ng were fixed according to the amount of RNA present in the blood samples. For the 10µl reaction mixture, 2µl miRCURY RT Reaction Buffer, 10x miRCURY RT Enzyme mix, Template RNA (3ng/ul) were added and volume was made up with RNAse free water.

The reaction mixture was incubated for 60 mins at 42^0 C in thermo cycler. Reaction mixture was again incubated for 5 mins at 95^0 C to inactivate Reverse transcriptase enzyme. The converted cDNA was kept at -80^0 C for qPCR.

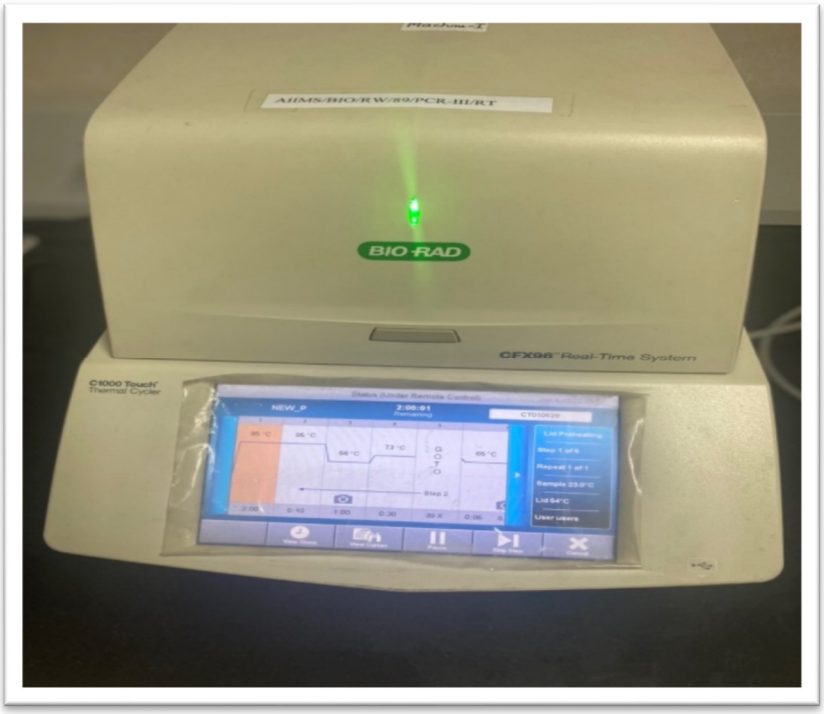


Figure 4(a): RT-PCR machine

Figure 4(b): Centrifuge machine

Quantification by Real time PCR (qPCR)

After cDNA conversion, qPCR was carried out by placing the reaction strips in an automated and temperature-controlled cycles consisting of denaturation, annealing and elongation/extension using thermal cycler (1). Primers used for qPCR were miR-21 QIAGEN miRCURY LNA Primer (3555396), and for internal control RNU6 (3450123) along with QIAGEN miRCURY SYBR Green PCR kits (339345).

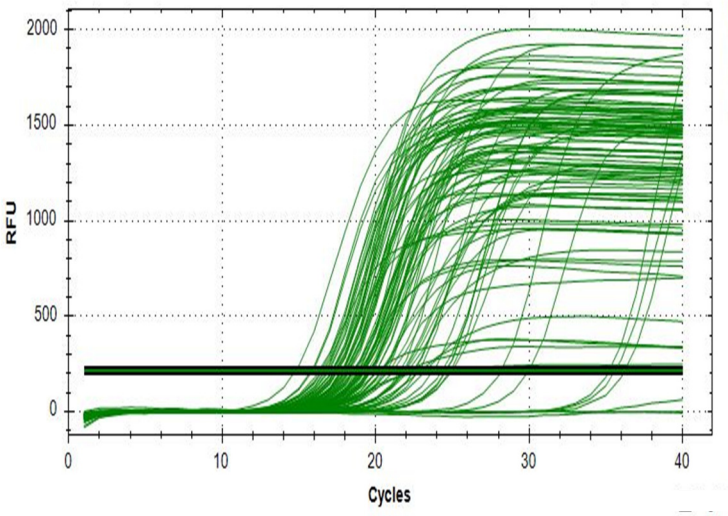
For 10µl reaction, 5µl of 2x miRCURY SYBR Green master mix, 1 µl of PCR primer mix, 1 µl cDNA template was taken and then volume was made up with RNAse free water.

Steps carried were PCR initial heat activation at 95^0 C for 2 minutes, followed by a 3-step cycling process which includes denaturation at 95^0 C for 10 seconds, annealing at 56^0 C for 60 seconds and extension at 72^0 C for 30 seconds. These steps were repeated for 39 times. After this, melt curve was generated using temperature of 65 ^{0}C for 5 seconds and 95 ^{0}C for 5 minutes.

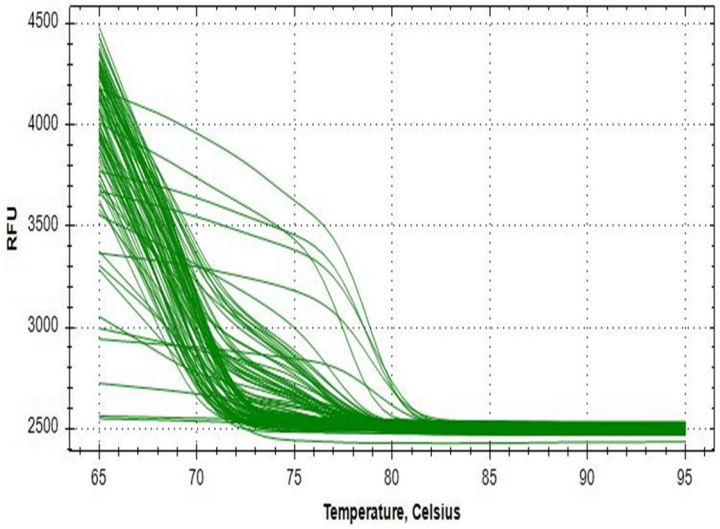
The normalization process utilized the $2^{-(\Delta\Delta ct)}$ method. The delta Cq values of the Pre-chemotherapy patients were calculated by subtracting mean Cq values of the control RNU6B (Qiagen) from the mean Cq values of the cases i.e., target miR-21 (2). Similarly post-chemotherapy delta Cq values were calculated. A subsequent double delta Cq value was calculated by subtracting delta Cq of pre-chemotherapy from post-chemotherapy. The fold change expression level of miR-21 was calculated using $2^{-(\Delta\Delta ct)}$ (78).

A positive reaction in PCR assay is determined by the accumulation of fluorescent signal. The Cq i.e., cycle threshold is the number of cycles needed for this fluorescent signal to pass the threshold i.e., surpass background level. Cq values are inversely related to the amount of nucleic acid. Therefore, higher the Cq level, the lesser the nucleic acid present in the sample. Ct values of less than or = 29 are considered strongly positive reactions and contain plenty nucleic acid, 30-37 indicate moderate amount of nucleic acid, whereas 30-40 are considered weak reactions.

a) Amplification Curve



b) Melt Curve



c) Melt Peak

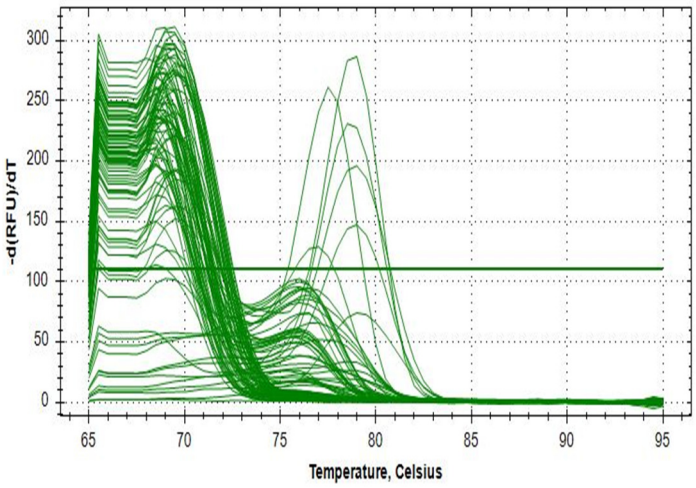


Figure 5 : Real time PCR Expression curve a) amplification curve, b) melt curve, c) melt peak.

Assessment of clinical condition before and after the chemotherapy

All patients were clinically staged using American Joint Cancer Congress (AJCC) 8th edition at the time of diagnosis and clinical response after 3 cycles of Neoadjuvant Chemotherapy was obtained by measurements from CT Scan Imaging (4). Response on primary lymph nodes and target lesions was also obtained from CT images. Further response was evaluated using clinical and radiological findings and finally graded according to the RECIST 1.1 criteria (Table 1) in standard terms as Partial Response (PR), Complete Response (CR), Stable Disease (SD) or Progressive Disease (PD) (4).

Table 1. RECIST CRITERIA

RECIST 1.1	
CR (Complete Response)	Disappearance of all lesions and pathologic lymph nodes
PR (Partial Response)	> or = 30% decrease SLD no new lesions no progression of non-target lesions
SD (Stable Disease)	no PR, no PD
PD (Progressive Disease)	> or = 20% increase SLD* compared to the smallest SLD in study or progression of non-target lesions or new lesions

The RECIST criteria was measured by comparing the sum of the longest diameters (SLD) of the target lesions (long axis > or = 10mm), maximum of 5 for the study in CT scan of post therapy with the baseline and the smallest SLD during treatment. Lymph nodes could be used as target lesions if short axis diameter (SAD) was >15mm. Lymph nodes <10mm were considered as normal, between 10-14mm were considered as pathological but not as target

lesions. Non target lesions were defined as lesions <10mm, non-measurable like ascites, pleural fluid.

Statistical analysis

Descriptive statistics was reported in the form of mean, standard deviation (SD), frequency and percentages. Before and after miR-21 levels were compared by paired t-test. Before and after clinical scores were compared by paired t-test. Relationship between change in individual gene fold expression of miR-21 and change in clinical scores was measured by Pearson correlation. Individual gene fold expression was calculated using ΔΔCt method. In this method, average of all pre-chemotherapy ΔCt values was calculated. Further, average of pre chemotherapy Δ Ct values was subtracted from individual post chemotherapy Δ Ct value to calculate ΔΔCt value for each individual. Gene fold expression of each individual was calculated by using formula 2 to the power negative ΔΔCt [$2^{-(\Delta\Delta ct)}$]. Statistical analysis was done by Statistical Package for the Social Sciences (SPSS) version 23. P value < 0.05 was considered significant.

RESULTS

Total 30 subjects were recruited in the study. One patient was lost to follow up as she shifted to other centre for the further treatment. Different parameters of the patients included in the primary and secondary analysis are mentioned in the [**Table 2]**. Majority of the patients were from the age group 41-50 years. Average age of patients included in the study was 51.3 years (range 30 years to 73 years). Majority of patients were negative for hormonal status. Half of the patients included in the analysis were having right invasive breast cancer and remaining half were having left invasive breast cancer.

Table 2: Characteristics of the subjects included in the study (n=30)

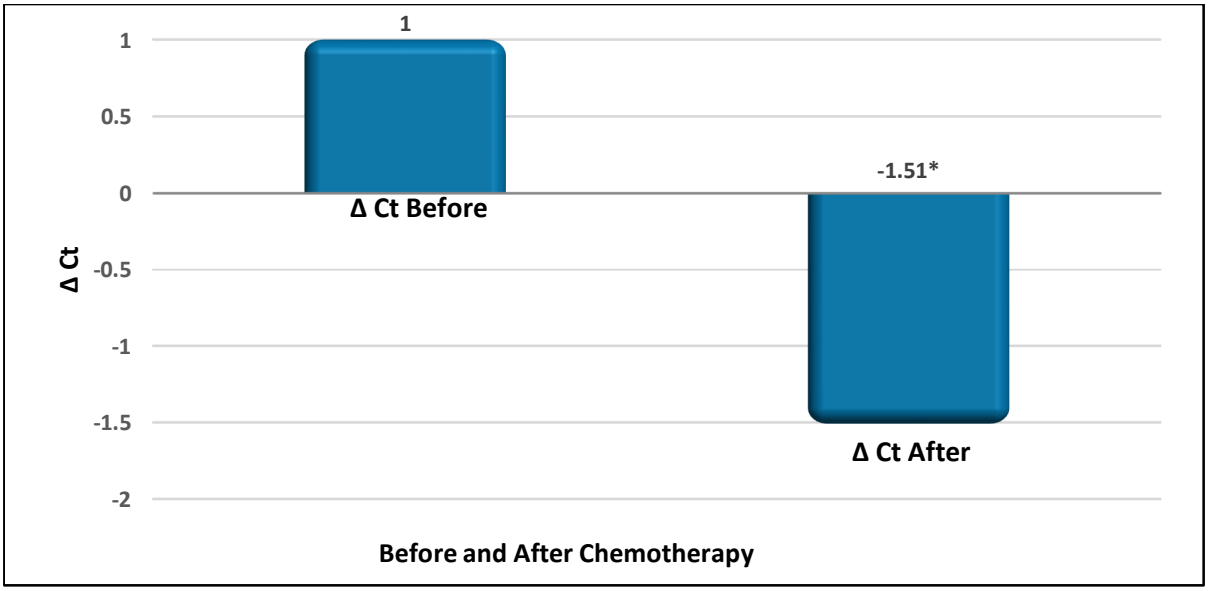
Parameters	**Subgroup**	**Frequency (%)**
Age in years (n=30)	30-40	4 (13.3)
	41-50	12 (40)
	51-60	7 (17.5)
	61-70	6 (20)
	71-80	1 (3.3)
Hormonal Receptor Status (n=29)*	ER Positive	9 (31)
	ER Negative	20 (68.9)
	PR Positive	4 (13.7)
	PR Negative	25 (86.2)
	HER2nue Positive	9 (31)
	HER2neu Negative	20 (68.9)
Types of Breast Cancer (n=30)	Right Invasive Breast Cancer	15 (50)
	Left Invasive Breast Cancer	15 (50)

*One subject withdrawn from the study before the assessment of the other parameters.

Evaluation of miR-21 before and after Neoadjuvant Chemotherapy

In five patients the quantifiable amount of RNA was not detected, hence only 24 patients were included in the analysis for primary objective. Δ Ct value for pre-chemotherapy and post-chemotherapy was calculated subsequently. Δ Ct = no of cycle threshold required to calculate the expression of miR-21. The lesser the no of cycles, higher the expression of miR-21 and vice-versa.

Mean value of delta Ct pre-chemotherapy was 1 (SD=3.30) and the mean value of delta Ct post-chemotherapy was – 1.51 (SD= 3.28). This difference was statistically significant (p=0.015) and negative Δ Ct value post-chemotherapy shows that Δ Ct decreased after chemotherapy as shown in **[Figure 6]**. Based on the ΔCt difference of pre and post chemotherapy, ΔΔCt value was calculated and subsequently fold change expression post-chemotherapy was calculated using formula $2^{(-(\Delta\Delta ct))}$. It was observed that, post chemotherapy, the expression of miR-21 was upregulated to 5.65-fold chain as shown in **[Figure 7].**



*p=0.015

Figure 6. Change in delta Ct post-chemotherapy in comparison to pre-chemotherapy

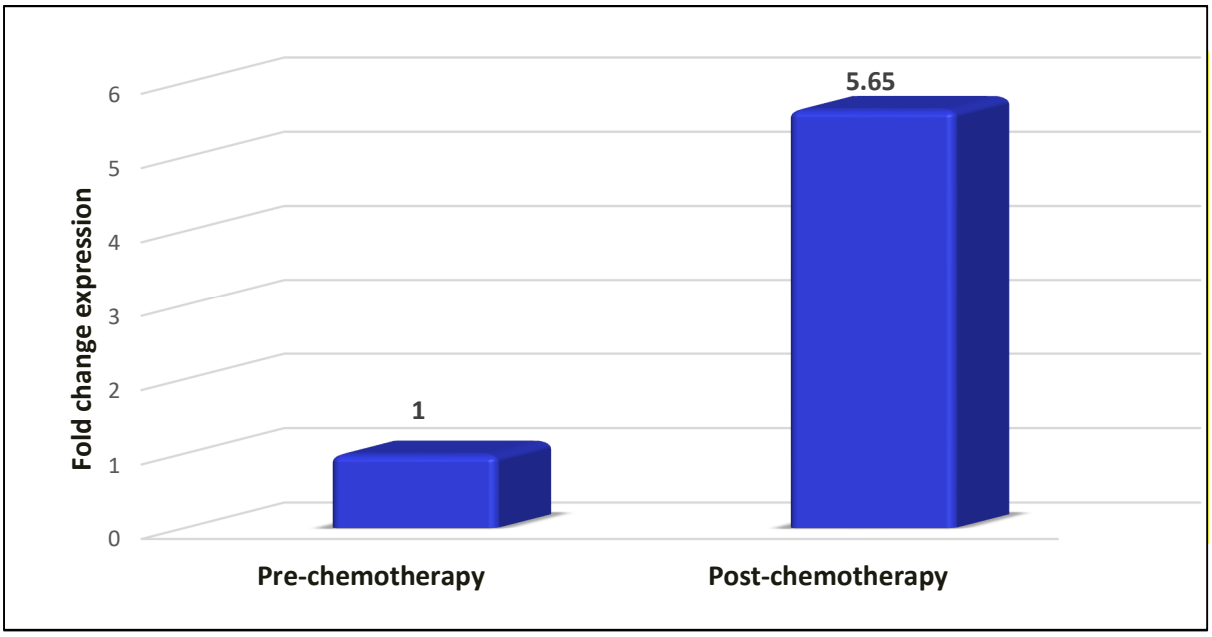


Figure 7 : Fold change expression of miR-21 post-chemotherapy

Evaluation of clinical parameters before and after neoadjuvant chemotherapy

Different clinical parameters like RECIST score, Protein, Albumin, Haemoglobin, Total Leukocyte count (TLC), Neutrophils, Platelets, Creatinine, Indirect Bilirubin, Direct Bilirubin, Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) after chemotherapy were compared with the pre-chemotherapy values **[Table 3]**.

RECIST score was decreased significantly after the chemotherapy (p=0.046). There was also significant decrease in mean of Protein (p=0.041), Albumin (p=0.018), Haemoglobin (p=0.00) and indirect bilirubin (p=0.033) after the chemotherapy although they were within the normal range **[Table 3]**. Whereas, when other clinical parameters, including Total Leukocyte count (TLC), Neutrophils, Platelets, Creatinine, Direct Bilirubin, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) were analyzed there was statistically significant difference **[Table 3].** Out of 29 patients three patient died during the study. After the chemotherapy, there were 7 patients classified as having stable disease (SD), 5 patients were classified as having progressive disease (PD), ten were having partial response (PR) to the therapy and there were 2 cases of complete response (CR).

Table 3: **Clinical parameters before and after neoadjuvant chemotherapy**

Parameters	Pre-chemotherapy Mean (SD)	Post - chemotherapy Mean (SD)	Mean Difference	95 % CI of mean difference	p value
RECIST score (n=24)	78.4 (38.9)	62.8 (54.5)	15.50	0.30 – 30.70	0.046
Protein (n=25)	7.6 (0.5)	7.3 (0.7)	0.28	0.01 – 0.56	0.041
Albumin (n=25)	4.2 (0.2)	4.0 (0.4)	0.21	0.03 – 0.38	0.018
Haemoglobin (Hb) (n=28)	11.8 (1.6)	10.7 (1.4)	1.03	0.57 – 1.49	0.000
Total Leukocyte count (TLC) (n=29)	7355.2 (2171)	7443.1 (2883.7)	-87.93	-1301.68 – 1125.81	0.883
Neutrophils (n=28)	4621.9 (1730.2)	5089.1 (3226.3)	-467.21	-1485.12 – 550.69	0.355
Platelets (n=29)	331379.3 (118407.7)	325310.3 (131824.6)	6068.96	-51207.08 – 63345.02	0.830
Creatinine (n=28)	0.8 (0.1)	0.7 (0.1)	0.01	-0.02 – 0.06	0.371
Indirect Bilirubin (n=28)	0.43 (0.18)	0.35 (0.10)	0.07	0.006 – 0.15	0.033
Direct Bilirubin (n=28)	0.09 (0.06)	0.07 (0.02)	0.02	-0.0016 – 0.045	0.067
Aspartate aminotransferase (AST) (n=28)	29.3 (14.4)	30.3 (13.0)	-0.93	-5.95 – 4.07	0.704
Alanine aminotransferase (ALT) (n=28)	26.9 (18.8)	27.25 (12.0)	-0.33	-6.69 – 6.02	0.915

Evaluation of relationship between change in miR-21 and change in clinical scores

To evaluate the relationship between expression of miR-21 (individual gene fold expression) and difference in pre and post RECIST score, bivariate correlation was done. No significant correlation was observed between miR-21 expression and difference in RECIST score after chemotherapy (r= -0.122, p=0.570) **[Figure 8]**.

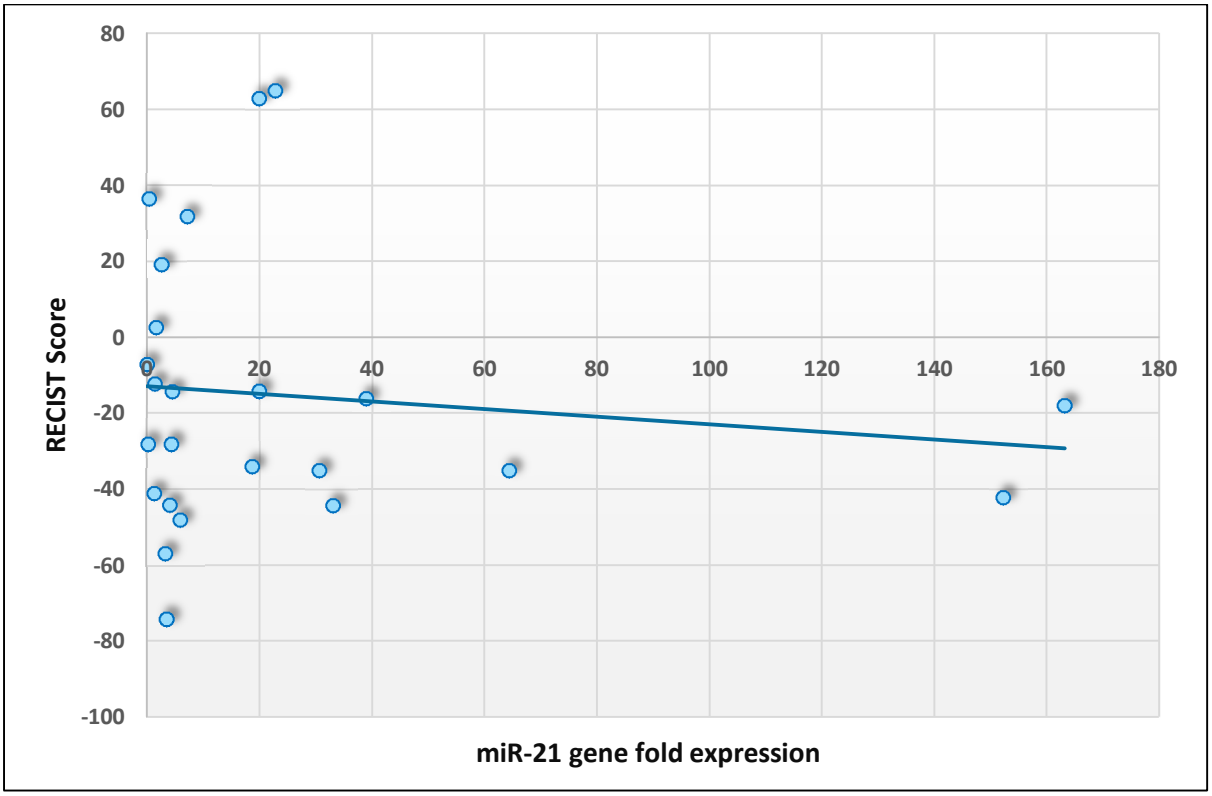


Figure 8: Scatterplot between individual miR-21 expression and difference in RECIST score

DISCUSSION

The present study was conducted to evaluate the expression of miR-21 in breast cancer patients before and after neoadjuvant chemotherapy and to assess the clinical improvement using RECIST 1.1 scoring and other clinical parameters and their clinically correlation with the miR-21 expression. Results observed in our study was that the gene fold expression of miR-21 was upregulated overall after chemotherapy. Our study included total of 30 female subjects, median age was 41-50 years, in which majority of them were hormone receptors negative. Out of 30 patients, 24 patients were followed up, and were assessed for their miR-21 expression after chemotherapy. We found that, the fold change expression of miR-21 was upregulated by 5.65-fold after chemotherapy which was found to be significant. Whereas, when patients were analyzed for their clinical response using RECIST 1.1 scoring before and after chemotherapy it was seen that there was significant decrease in the tumor size overall after chemotherapy treatment as compared to baseline proving the efficacy of this chemotherapy regimen. When other clinical parameters were analyzed including complete hemogram, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin and creatinine, it was found that there was significant decrease in mean of post-chemotherapy protein and albumin when compared with pre-chemotherapy mean although they were within the normal range showing the mild adverse effect of AC regimen on liver. Further, hemoglobin and indirect bilirubin were also found to be significantly decreased after chemotherapy. However, the correlation between change in expression of miR-21 and clinical scores using RECIST 1.1 was not significant between the groups.

As, it is known that breast cancer is the most commonly diagnosed cancer in India and around the world, as well as the leading cause of cancer-related death. When results of our primary objective were analyzed then it was found that serum miR-21 was significantly increased after chemotherapy. It is reported in various studies that miR-21 plays an important role in mechanism of breast cancer by acting on its target receptors as already discussed (79). Al Khanbashi et al. assessed miR-21 expression at different times while undergoing 4 cycles of NACT consisting of AC-T regimen. He observed that, there was no change in miR-21 between different cycles of NACT. In fact, miR-21 expression was further upregulated by the end of AC-T regimen which could have been due to re-establishment of miR-21 as a result of the response to adequate therapy supporting the findings of our study (30). It is known that adriamycin which is an anthracycline acts by forming complexes with DNA by intercalating between base pairs of DNA and also stops the growth of cancer cells by inhibiting enzyme topoisomerase II which is required for DNA synthesis (80). Whereas, cyclophosphamide

which is an alkylating agent acts by converting into its active form which causes cross linking of DNA further decreasing the synthesis of DNA causing its cytotoxic effect (81). But their mechanism of action on miR-21 is still not clear (82). Sales et al. (2020), demonstrated that there was upregulated miR-21 expression after NACT when he compared miR-21 between NACT who achieved a complete pathological response and naïve chemotherapy groups indicating a possible restoration of miR-21 in response to NACT due to an effect of tumor destruction and regeneration of surrounding tissues. He even compared miR-21 in different stages of breast cancer and there was upregulation regardless of past NACT exposure indicating no correlation between miR-21 expression and clinical improvement (29). Leticia De Mattos et al. (2015), demonstrated increased expression of miR-21 after chemotherapy indicating increase in the metastatic potential. It was observed that chemotherapy-induced DNA damage causes an increase in miR-21 expression via NF-kb activation, which leads to increased breast cancer cell invasion favoring the findings of our study (31). On the contrary, Liu et al. observed the expression of miR-21 after 2 cycles of NACT in breast cancer patients receiving chemotherapy and concluded that there was downregulation of miR-21 expression after chemotherapy. The effect of chemotherapy on miR-21 is explored in few small studies, like in a study by Khalighfard S et al. (2018), where miR-21, miR-155 and miR-10b were compared before and after the chemotherapy and all of these miRNAs were found to be downregulated hinting the role of these miRNAs as biomarkers for disease control or early diagnosis (14). Sadaf et al. (2016), also studied the expression of miRNAs including miR-21 after chemotherapy and radiotherapy and demonstrated that miR-21 levels decreased significantly after chemotherapy whereas, there was no significant decrease between chemotherapy and radiotherapy (83).

The occurrence of metastasis, starting with cell proliferation and migration is the primary cause of cancer mortality. Metastasis is a complicated process and is the chief cause of death in solid tumors but knowledge about mechanism of metastasis is still not sufficient (84). Therefore, exploring about biomarkers to assess the metastasis, recurrence and survival of tumor would be extremely beneficial for clinicians. According to Asangani et al, miRNAs being regulators of gene expression in breast cancer are being explored for their role as potential candidates for biomarkers. Common methods used for miRNAs analysis are real time PCR, northern block and microarray-based profiling (84). In our study, we used real time PCR to see the miR-21 expression and it was found that miR-21 gene fold expression was upregulated in breast cancer patients (17). In a study done by Yan Li-Xu et al. (2008)

upregulated miR-21 expression was related to advanced tumor stage, lymph node metastasis, increased chemo-resistance and poor survival of the patients which indicates role of high miR-21 level in poor prognosis (13). Zhang et al. evaluated mir-21 expression in 40 IDC patients who received NACT with non-tumor group and correlated with clinicopathologic features. He found upregulated miR-21 in more aggressive tumor subtypes indicating role of miR-21 as a prognostic marker (85). Prognostic role of miR-21 was supported by another study by Chang et al. who explored various miRNAs and their targets including miR-21 and found that these miRNAs target breast oncogenes by exerting protective phenotype further contributing to survival of patients (86). As, this miRNA is involved with tumorigenesis, it may be affected by the therapy, which ideally should decrease the expression of miR-21 as a surrogate of effect of the therapy, clinical condition and prognosis.

The mechanism of action of chemotherapy regimen drugs on liver and kidney function is still not clear. But this is a well-known fact that chemotherapy reduces the nutrition intake due to most common adverse effects of chemotherapy like nausea, vomiting and also there is an increase energy expenditure in chemotherapy patients due to upregulated metabolism of tumor, all of which further decreases the synthesis of one of the main protein of liver i.e., albumin (87,88). Serum albumin concentration has been studied as a biomarker and is one of the simplest parameter for checking the nutritional status and state of visceral protein (89). Therefore, the significant reduction in albumin is justified with these studies. In a case report by Ala Abudayyeah et al. (2014), it was seen that chemotherapy can lead to hypoalbuminemia quite commonly in cancer patients. They reported the case of 53 years old patient who received chemotherapy including AC regimen which further led to hypoalbuminemia due to depressive effect of chemotherapy (90). In the study by Sharma Ankush et al, (2014) it was seen that chemotherapy affects liver function tests and leads to liver abnormalities and further liver failure (91). In study, by Zikria Saleem et al, (2016) protein, albumin and globulin were assessed after AC regimen of chemotherapy and it was observed that total protein and globulin decreased significantly (92). Further, Lis et al.(2013) concluded that the decreased albumin levels in cancer chemotherapy patients is associated with poor survival of patients while upregulated and normal values were considered as strong prognostic markers (93). However, decreased globulin levels suggest that this chemotherapy regimen has immunosuppressive effect and can be used as a parameter of prognostic value (92). Further, hemoglobin and indirect bilirubin were also found to be significantly decreased after chemotherapy. Chemotherapy induced bone marrow suppression leading to decrease in

hemoglobin is the most common cause of chemotherapy induced anemia. Also, these drugs lead to hemolysis of cells further decreasing hemoglobin. Chemotherapy induced anemia was studied by Emily et al. (2018), and it was found that it is due to multiple factors related to patients and treatment specific including dose and administration timings, age, gender, renal function (94). In another study by Groopman et al. (1999), chemotherapy induced anemia was explored (95). This data suggests that this decrease in parameters could be due to adverse effect of drugs such as Adriamycin/doxorubicin and cyclophosphamide on liver. Whereas, other clinical parameters did not show any significant difference between both the groups.

There was significant decrease in the mean of tumor size post neoadjuvant chemotherapy when patients were assessed using RECIST 1.1 which is supported by the study of Kamal Mohammed et al. (2020), (96). Chunjie Sun et al. evaluated the clinical effects of NACT in treating breast cancer using RECIST 1.1 and concluded that percentage of tumor cells were decreased proving the efficacy of NACT regimen (97). However, the correlation between change in individual expression of miR-21 and clinical scores using RECIST 1.1 was not significant between the groups overall. Similar results were found in previous studies (85). In the study by Al Khanbashi et al. miR-21 levels were assessed in breast cancer patients undergoing neoadjuvant chemotherapy and were clinically correlated with RECIST 1.1 scoring. It was found that there was no significant difference in the correlation supporting the findings of our study (30). Andrea Ritter et al. (2019), assessed the all the stages patients of TNBC patients and their complete pathological response (cPR) using RECIST 1.1 to NACT and no significant correlation was found due to only 8 patients of TNBC indicating towards the small sample size (98). Whereas, Chagpar et al. (2000) observed the accuracy of estimation of decrease in tumor size in patients receiving NACT and found that there was a poor agreement between clinical and pathological measurements (99). Sales et al. also correlated tumor size and miR-21 in NACT patients and did not find any significant correlation between the two groups. It can be attributed due to various reasons including ethnogenetic characteristics of the patients, small sample size and miRNA normalization method which is a sensitive process (29). The no-correlation between change in miR-21 and clinical score may be because of the various reasons including small sample size in majority of these studies, differential effect on surrogate and clinical endpoints, less time duration of the study.

There is a paucity of data about the role of miR-21 in breast cancer and whether it can be considered as a marker for successful treatment? This study attempted to answer this question in resource limited setting. Such data is either very sparse or completely lacking for patients from India hence it was worthwhile to do this study. Non-significant results obtained for the relationship between miR-21 and clinical score may be true non-significance due to less sample size or short duration of the observation as the observations in this study were limited to 3rd cycle of the chemotherapy that is 42 days. In this study, plasma miR-21 level was analyzed but tissue miR-21 was not considered because of the feasibility reason. In the previous studies it was observed that plasma as well as tissue miR-21 behave similarly. In a study by Yan et al. miR-21 expression was seen by extracting the RNA from breast tissue samples of IDC before starting any therapy and it was concluded that upregulated miR-21 was correlated with more tumor involvement (54).

miRNAs have been seen to play role in drug resistance in recent years by targeting drug resistance related genes. The use of anti-miRNA in addition with chemotherapy to achieve better therapeutic effects has been promising (39). Chemoresistance in breast cancer occur due to disruption in the miRNA pathway. miRNA oligonucleotides have the capacity to increase the sensitivity of some drugs by increasing some miRNAs which were taken during BC. They also help in uplifting the efficiency of treatment by repressing expression of some miRNAs. The oligonucleotide of miR-21 has the potential to bind to HER2 which upgrades the sensitivity of chemo drug trastuzumab and terminate breast cancer cells (34). Further talking about expression of miR-21, Mei M et al. enlightened on the role of miRNAs in the resistance of chemotherapy of breast cancer. Chemotherapy drug Taxol was combined with miR-21 inhibitor in treatment to see the effects of combination in repression of breast cancer cells. It demonstrated significant decrease in the inhibitory concentration (IC50) of taxol, increase apoptosis and decreased invasiveness. This concludes that miR-21 might have valuable role in chemoresistance and its anti-miRNA oligonucleotide (AMO) can be used to increase the sensitivity of drugs along with chemotherapy for improvement in chemoresistance in future (100).

Altogether, the data has significant implications for future but prevention of metastasis is the main aim to decrease the mortality due to breast cancer in future. Even though there is cure for early detection and diagnosis of breast tumors, they are disseminated prior to diagnosis leading to recurrence in future. Next, primary tumors are used as substitute for taking

decisions related to systemic chemotherapy regimen to treat metastasis. Therefore, biomarkers could help identify molecular changes in metastasis and further early diagnosis and prevention of metastasis. This study is one of the early studies, particularly for Indian patients, showing the effect of chemotherapy on miR-21 and is a step in the direction of establishing miR-21 as prognostic biomarker in the breast cancer. Using miRNA profiles as a prognostic tool for breast cancer has always been challenging due to factors such as sampling and isolation timings of miRNAs, crude assessments of response rates to therapy and quantification of isolated RNA. The sampling and processing techniques in estimation of miR-21 by the method used in this study has some inherent technical issues which includes variable quantification ratio of RNA (260/280=2) due to less quantity of blood sample collection, contamination of nucleic acid due to phenol during RNA extraction. Further, handling errors including pipetting error and presence of contaminants arising from the puncture site which could be resolved by removing first few ml of blood before processing or presence of clots or hemolysis in serum or plasma further leading to miRNAs variations. But this is the most feasible and frequently used method as observed in previous studies.

Hence, there is a need for further studies considering the relationship between chemoresistance and miR-21, which could not be explored in this study. Future studies are required to be planned for these hurdles by development of better technologies, larger sample size, long duration of observation and using multiple miRNAs from plasma as well as tissues to validate the results obtained in this study.

CONCLUSION

Based on this study, it can be concluded that the fold change expression of miR-21 was upregulated after neoadjuvant chemotherapy comprising of Adriamycin- Cyclophosphamide (AC) regimen which was found to be significant. Also, when patients were analyzed for their clinical response using RECIST 1.1 scoring before and after chemotherapy it was seen that there was significant decrease in the tumor size overall after neoadjuvant chemotherapy treatment as compared to baseline proving the efficacy of this neoadjuvant chemotherapy regimen. However, the correlation between change in individual expression of miR-21 and clinical scores using RECIST 1.1 was not statistically significant. When other clinical parameters were analyzed including complete hemogram, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin and creatinine, it was found that there was significant decrease in mean of post-chemotherapy protein, albumin and hemoglobin when compared with pre-chemotherapy mean although they were within the normal range. The relation of miR-21 expression with clinical improvement is yet not fully understood. This need to be explored further by using larger sample size and using long duration of the observation.

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ANNEXURES

ANNEXURE-I

Institutional Ethics Committee Certificate

अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

No. AIIMS/IEC/2020/2059 Date: 01/01/2020

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2019-20/961**

Project title: **"Circulating miRNA-21 levels in breast cancer patients before and after chemotherapy and its association with clinical improvement"**

Nature of Project:	**Research Project**
Submitted as:	**M.D. Dissertation**
Student Name:	**Dr.Sanchi Sukhija**
Guide:	**Dr. Jaykaran Charan**
Co-Guide:	**Dr.Sneha Ambwani, Dr.Praveen Sharma, Dr.Poonam Abhay Elhence, Dr.Purvi Purohit, Dr.Puneet Pareek, Dr.Jeewan Ram Vishnoi & Dr.Shoban Babu Varthya**

This is to inform that members of Institutional Ethics Committee (Annexure attached) met on **23-12-2019** and after through consideration accorded its approval on above project. Further, should any other methodology be used, would require separate authorization.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Praveen Sharma
Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur

Enclose:
1. Annexure 1

Page 1 of 2

Basni Phase-2, Jodhpur, Rajasthan-342005, **Website:** www.aiimsjodhpur.edu.in, **Phone:** 0291-2740741 Extn. 3109
Email: ethicscommittee@aiimsjodhpur.edu.in

ANNEXURE-II

Patient information sheet (English)

1. Risks to the patients: There's no risk of death or any disability resulting directly due to imaging.

2. Confidentiality: Your participation will be kept confidential. Your medical records will be treated with confidentiality and will be revealed only to doctors/ scientists involved in this study. The results of this study may be published in a scientific journal, but you will not be identified by name.

3. Provision of free treatment for research related injury. Not applicable.

4. Compensation of subjects for disability or death resulting from such injury. Not Applicable

5. Freedom of individual to participate and to withdraw from research at any time without penalty or loss of benefits to which the subject would otherwise be entitled.

6. You have complete freedom to participate and to withdraw from research at any time without penalty or loss of benefits to which you would otherwise be entitled.

7. Your participation in the study is optional and voluntary.

8. The copy of the results of the investigations performed will be provided to you for your record.

9. You can withdraw from the project at any time, and this will not affect your subsequent medical treatment or relationship with the treating physician.

10. Any additional expense for the project, other than your regular expenses, will not be charged from you.

Patient :

_____

Signature

Name: _____

Place : _____

ANNEXURE-III

Patient information sheet (Hindi)

रोगी सूचना पत्रक

1. रोगियों के लिए जोखिम: इमेजिंग के दौरान सीधे तौर पर कोई विकिरण के कोई खतरे नहीं है। कोई हस्तक्षेपीय जीवन-धमनी की प्रक्रिया नहीं की जाएगी।

2.गोपनीयता: आपकी भागीदारी को गोपनीय रखा जाएगा। आपके मेडिकल रिकॉर्ड को गोपनीयता के साथ इलाज किया जाएगा और केवल इस अध्ययन में शामिल डॉक्टरों / वैज्ञानिकों को पता चलेगा। इस अध्ययन के परिणाम एक वैज्ञानिक पत्रिका में प्रकाशित हो सकते हैं, लेकिन आपको नाम से पहचाना नहीं जाएगा।

1. अनुसंधान संबंधी चोट के लिए नि: शुल्क उपचार की व्यवस्था किया गया नहीं।
2. ऐसी चोट से उत्पन्न विकलांगता या मृत्यु के लिए विषयों के मुआवजे का नहीं है

5.किसी भी समय दंड या लाभों के नुकसान के बिना किसी भी समय भाग लेने के लिए व्यक्ति को स्वतंत्रता होगी और अनुसंधान से वापस लेने के लिए स्वतंत्र, जिसके अनुरोध विषय अन्यथा हकदार होगा।

6.आपको जुर्माना या लाभ के नुकसान के बिना किसी भी समय भाग लेने और अनुसंधान से वापस लेने की पूरी आजादी है, जिस पर आप अन्यथा हकदार होंगे।

7. अध्ययन में आपकी भागीदारी वैकल्पिक और स्वैच्छिक है।

8. प्रदर्शन की जांच की परिणामों की प्रति आपके रिकॉर्ड के लिए आपको उपलब्ध कराई जाएगी।

9.आप किसी भी समय परियोजना से वापस ले सकते हैं, और यह आपके डॉक्टर के चिकित्सा उपचार या उपचार चिकित्सक के साथ संबंध को प्रभावित नहीं करेगा।

10. परियोजना के लिए कोई भी अतिरिक्त व्यय, आपके नियमित खर्चों के अलावा, आप से शुल्क नहीं लिया जाएगा।

मरीज़ :

हस्ताक्षर_____

नाम: _____

जगह: _____

ANNEXURE-IV

Informed Consent Form (English)

All India Institute of Medical Sciences, Jodhpur, Rajasthan

Title of the project : _____

Name of the Principal Investigator : _____Tel. No. _____

Patient/Volunteer Identification No. : _____

I, _____ S/o or D/o _____ R/o _____ give my full, free, voluntary consent to be a part of the study "A single clinical trial study to evaluate the correlation between miRNA 21 levels and response to AC-T therapy in locally advanced and metastatic breast cancer patients." the procedure and nature of which has been explained to me in my own language to my full satisfaction. I confirm that I have had the opportunity to ask questions.

I understand that my participation is voluntary and am aware of my right to opt out of the study at any time without giving any reason.

I understand that the information collected about me and any of my medical records may be looked at by responsible individual from AIIMS. I give permission for these individuals to have access to my records.

Date : _____ _____

Place : _____ Signature/Left thumb impression

This to certify that the above consent has been obtained in my presence.

Date : _____ _____

Place : _____ Signature of Principal Investigator

1. Witness 1

_____

Signature

Name: _____

Address : _____

2. Witness 2

_____

Signature

Name: _____

Address : _____

ANNEXURE-V

Informed Consent Form (Hindi)

- थीसिस / निबंध क□शीर्षक: _____
- पीजी छ□क□ज□म: _____ टल□न: _____
- रोगी / स्वयं सव□क पहच□न संख्य□: _____

मैं, _____ एस / ओय□ी /ओ_____

आर/ओ _____

अध्ययन "_____ MiRNA 21 स्तरों और स्थ□नीय रूप स□उन्नत और मर□ट□क स्तन कैंसर रोगियों में AC-T थ□पी की प्रतिक्रिय□ और□ीच सहसंबंध क□मूल्यां□न करन□क□लिए एक एकल नैद□नि□क परीक्षण अध्ययन _____" क□एक भ□ग बनन□क□ लिए मर□पूर्ण, स्वतंत्र, स्वैच्छिक सहमति दें, जिसकी प्रक्रिय□और प्रकृति मुझ□अपनी पूरी संतुष्टि क□लिए अपनी भ□ष□में समझ□ई गई है। मैं पुष्टि करत□हूं कि मुझ□प्रश्न पूछन□क□अवसर मिल□है।

मैं समझत□हूं कि मर□भ□गीदर□ी स्वैच्छिक है और मुझ□किसी भी कर□ण दिए बिन□किसी भी समय अध्ययन स□ब□हर निकलन□क□मर□अधिकर□की जन□कर□ी है।

मैं समझत□हूं कि मर□और मर□□ड□कल रिकॉर्ड क□□कत्रित की गई जन□कर□ी को _____ (कंपनीन□म) य□विनियम□क प्र□धिकरणों स□□म्मद□ि व्यक्ति द्व□र□देख□ज□सकत□है।मैं इन व्यक्तियों को अपन□अभिलेखों तक पहुंच क□लिए अनुमति दत□हूं

त□रीख : _____

जगह: _____ हस्त□क्षर / ब□ए अंगूठ□क□छ□प _____

यह प्रम□णित करन□क□लिए कि मर□उपस्थिति में उपरोक्त सहमति प्र□ की गई है

त□रीख : _____

जगह: _____ सिद्ध□ित अन्वष□क क□हस्त□क्षर_____

गवाह1: : _____

हस्ताक्षर: _____

तारीख : _____

गवाह2: : _____

हस्ताक्षर: _____

तारीख : _____

ANNEXURE-VI

Case Record Form

Title of the study- Circulating miR-21 levels in breast cancer patients before and after chemotherapy and its association with clinical improvement.

OPD/IPD Registration No : AIIMS/JDH/____/_____

Name: Date of enrolment :

Address: Age/Sex:

Occupation: Contact number:

PAST HISTORY:

FAMILY HISTORY:

PERSONAL HISTORY: Smoker/ Alcoholic/ Veg/ Non-veg

COMORBIDITIES:

TREATMENT HISTORY:

HISTOPATHOLOGIC TYPE OF CANCER:

HORMONE RECEPTOR STATUS:

ER : POSITIVE/NEGATIVE

PR : POSITIVE/NEGATIVE

HER2/neu : POSITIVE/NEGATIVE

Baseline Parameters

Anthropometric parameters	**Pre-chemotherapy**	**Post-chemotherapy**
Height (cm)		
Weight (kg)		
BMI (kg/m^2)		
Biochemical parameters		
Haemoglobin %		
Total Leukocyte count (TLC)		
Neutrophil count		
Platelet count		
AST		
ALT		
Indirect Bilirubin		
Direct Bilirubin		
Total Protein		
Albumin		
Creatinine		
RNU 6 (control)		
Delta Ct miR-21		

Clinical Response Assessment	**Pre-chemotherapy**	**Post-chemotherapy**
T. Diameter		
N. Diameter		
M. Diameter		
Target lesions		
Non-Target lesions		
New lesions		
SLD (sum of longest diameters of target lesions)		
Percentage change		
RECIST 1.1 group		
Survival		