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Pilot study demonstrating metabolic and anti-proliferative effects of in vivo anti-oxidant supplementation with N-Acetylcysteine in Breast Cancer

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ABSTRACT

Background: High oxidative stress as defined by hydroxyl and peroxyl activity is often found in the stroma of human breast cancers. Oxidative stress induces stromal catabolism, which promotes cancer aggressiveness. Stromal cells exposed to oxidative stress release catabolites such as lactate, which are uptaken by cancer cells to support mitochondrial oxidative phosphorylation. The transfer of catabolites between stromal and cancer cells leads to metabolic heterogeneity between these cells and increased cancer cell proliferation and reduced apoptosis in preclinical models. N-Acetylcysteine (NAC) is an antioxidant that reduces oxidative stress and reverses stromal catabolism and stromal-carcinoma cell metabolic heterogeneity, resulting in reduced proliferation and increased apoptosis of cancer cells in experimental models of breast cancer. The purpose of this clinical trial was to determine if NAC could reduce markers of stromal-cancer metabolic heterogeneity and markers of cancer cell aggressiveness in human breast cancer.

Methods: Subjects with newly diagnosed stage 0 and I breast cancer who were not going to receive neoadjuvant therapy prior to surgical resection were treated with NAC before definitive surgery to assess intra-tumoral metabolic markers. NAC was administered once a week intravenously at a dose of 150 mg/kg and 600 mg twice daily orally on the days not receiving intravenous NAC. Histochemistry for the stromal metabolic markers monocarboxylate transporter 4 (MCT4) and caveolin-1 (CAV1) and the Ki67 proliferation assay and TUNEL apoptosis assay in carcinoma cells were performed in pre- and post-NAC specimens.

Results: The range of days on NAC was 14–27 and the mean was 19 days. Post-treatment biopsies showed significant decrease in stromal MCT4 and reduced Ki67 in carcinoma cells. NAC did not significantly change stromal CAV1 and carcinoma TUNEL staining. NAC was well tolerated.

Conclusions: NAC as a single agent reduces MCT4 stromal expression, which is a marker of glycolysis in breast cancer with reduced carcinoma cell proliferation. This study suggests that modulating metabolism in the tumor microenvironment has the potential to impact breast cancer proliferation.

1. Introduction

Breast cancer is the most common type of cancer in women [1] and is the fourth leading cause of cancer death in women in the United States [1]. Moreover, current breast treatment strategies have significant side effects. There is a need for novel treatments in breast cancer, especially those with low morbidity.

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Epidemiological studies suggest that antioxidants such as *N-Acetylcysteine* (NAC) may reduce breast cancer mortality by reducing progression [2]. Antioxidants, which are well tolerated, offer the potential to fill this need.

NAC is one of the best-characterized antioxidants and it is approved by the US Food and Drug Administration (FDA) for intravenous or oral treatment of acetaminophen overdose and is also FDA-approved by inhalation as a mucolytic agent [3]. Because of its antioxidant properties, NAC is also commonly administered prophylactically for contrast-induced nephropathy and is being investigated in many diseases, including chemotherapy-induced toxicity such as doxorubicin cardiotoxicity, ischemia–reperfusion cardiac injury, acute respiratory distress syndrome, bronchitis, heavy metal toxicity, neurologic and psychiatric disorders, interstitial lung diseases, hepatitis, influenza, and HIV [4].

NAC is a precursor for reduced glutathione (GSH), which is the main intracellular antioxidant [5]. GSH is a water-soluble molecule found in millimolar concentration in cells. It is a low-molecular-weight peptide containing a thiol group, which provides its antioxidant activity [5]. The physiologic roles of GSH include being a scavenger of free radical and reactive oxygen species, forming conjugates with metabolites and xenobiotics, being a thiol buffer for many cellular proteins such as metallothioneins and thioredoxins, altering protein structures by reducing disulfide bonds, being an essential cofactor for many enzymes, and allowing the regeneration of other antioxidants such as tocopherols and ascorbate [4,6]. GSH is composed of three amino acids: cysteine, glycine, and glutamate [7]. Cysteine is the rate-limiting amino acid in the generation of GSH, and NAC has been developed as a drug instead of cysteine because of its greater stability [4]. NAC was investigated in the current breast cancer clinical trial because of its ability to reduce oxidative stress by increasing GSH levels.

Dysfunctional GSH homeostasis causes a reduced antioxidant response, activation of redox-regulated signal transduction with increased glycolysis, immune impairment, reduced ability to detoxify electrophilic xenobiotics, and increased cellular proliferation [8]. GSH is implicated in the etiology of several diseases, including cancer, aging, neurodegenerative diseases, pulmonary diseases, liver diseases, immune disorders, and cardiovascular diseases [6]. GSH is rapidly degraded extracellularly by γ-glutamyl transpeptidase (GGT) [4]. NAC, orally and intravenously in human subjects, enhances GSH intracellular production [9]. NAC is also an antioxidant directly without needing to be metabolized to GSH [4]. Hence, most studies investigating targeting dysfunctional GSH and redox homeostasis have used NAC instead of GSH itself [4]. No clinical trials have been conducted with NAC in breast cancer; NAC was investigated in the current trial because redox and GSH homeostasis is altered in this disease.

Oxidative stress induces a glycolytic and catabolic state in tumor stromal cells with the release of catabolites such as lactate [10]. These catabolites drive metabolic heterogeneity with transfer of catabolites from stromal cells to carcinoma cells to support mitochondrial metabolism [11,12]. Metabolic heterogeneity increases cancer cell proliferation, reduces apoptosis, and induces larger tumors with more frequent metastasis and shorter overall survival [11–18].

Metabolic heterogeneity exists in breast cancer [10]. High stromal monocarboxylate transporter 4 (MCT4) and low caveolin-1 (CAV1) expression are markers of glycolytic stromal cells in metabolically heterogeneous tumors [10,12,19–21]. NAC preferentially targets cells with altered glycolysis and, hence, stromal cells with high MCT4 and low CAV1 would presumably be more susceptible to NAC.

High stromal staining for MCT4 and low CAV1 occurs in the majority of breast cancers, suggesting transport of catabolites from cancer-associated stroma to highly proliferative cancer cells [22]. MCT4 is an exporter of glycolytic byproducts such as pyruvate and lactate [19]. Oxidative stress induces the expression of MCT4 in stromal cells and NAC can reduce MCT4 expression in preclinical models [10]. Also, loss of CAV1 in cancer-associated stroma induces glycolysis and the upregulation of MCT4 and stromal CAV1 expression can be rescued with NAC [18]. The purpose of this clinical trial was to determine if NAC could reduce markers of stromal-cancer metabolic heterogeneity and markers of cancer cell proliferation and apoptosis in human breast cancer.

NAC, which reduces oxidative stress, has been extensively studied as an anticancer agent in vitro and in vivo and has been shown to reduce cancer aggressiveness with reduced proliferation and increased apoptosis of cancer cells [10,11,23–27]. NAC's ability to limit tumor growth in some in vivo models is dependent on its antioxidant properties [28]. NAC also reduces catabolism, glycolysis, mitochondrial dysfunction, and inflammatory mediators by reducing oxidative stress [5,7,24,25]. However, NAC has not been investigated systematically in breast cancer. Also, no clinical trials have been performed to assess the effect of drugs on markers of the metabolic profile of human tumors as a primary end-point.

In sum, oxidative stress drives metabolic heterogeneity between tumor stromal cells and cancer cells and metabolic heterogeneity induces aggressive behavior in cancer. NAC preferentially targets tumors with increased stromal glycolysis, such as breast cancer [29]. Because of its antioxidant effect, NAC can reverse stromal-cancer metabolic heterogeneity, which drives cancer aggressiveness [10]. Hence NAC may be a drug with anticancer activity in human breast cancer. We hypothesized that, because of the metabolic effects of NAC in the tumor microenvironment, it can reduce cancer cell proliferation and increase apoptosis rates in subjects with breast cancer.

2. Materials and methods

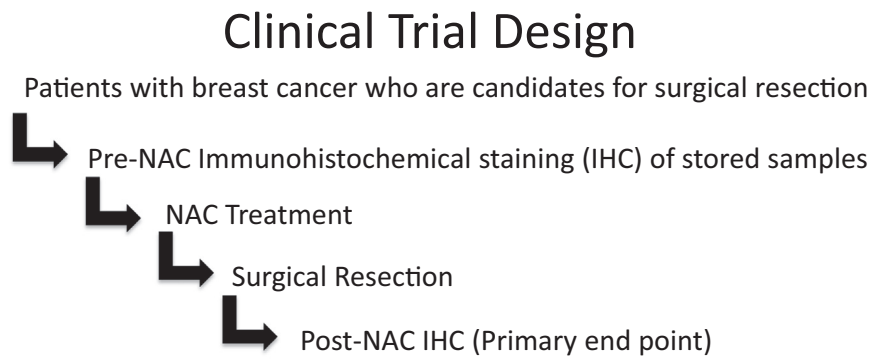
2.1. Trial design

The Institutional Review Board and Cancer Review Committee at Thomas Jefferson University (Philadelphia, PA) approved this clinical trial.

The clinical trial design is outlined in Figure 1. Eligible patients were those with a biopsy demonstrating breast cancer who were planned to undergo surgical resection without neoadjuvant therapy prior to surgery. Patients were treated with NAC for a minimum of 2 weeks in the period between biopsy and definitive resection. NAC was administered intravenously at a dose of 150 mg/kg weekly and orally at a dose of 600 mg twice daily on days not receiving intravenous drug. NAC was administered in this schedule because it has been shown to have anticancer activity in experimental models at this dose and is within the dose range used in clinical practice [6,9,23]. NAC treatment ceased no less than 48 hours prior to scheduled date of surgery. Immunohistochemistry was performed in pre- and post-NAC samples.

Inclusion criteria included stage 0/I breast cancer, ECOG performance status of 0 or 1, serum creatinine ≤2.0 mg/dL, serum bilirubin ≤2.0 × ULN, and a serum hemoglobin ≥8.0 mg/dL. Subjects were excluded if they had a history of bronchospasm or severe asthma.

The primary end point was change in the tumor microenvironment as marked by immunohistochemistry staining for MCT4 and CAV1 in the tumor stroma from pre- to post-NAC treatment specimens. Secondary end points were changes in the Ki-67 proliferation index and changes in carcinoma cell apoptosis by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay.



Day	Procedure
Rx week #0	Subject recruitment, screening, consent, and breast biopsy.
Rx week #1*	Confirm all inclusion criteria and positive biopsy for stage 0/1 breast cancer.
Rx week #2	Receive 1st intravenous dose of NAC. Begin oral NAC 600mg twice per day.
Rx week #3	Receive 2nd intravenous dose of NAC. Begin oral NAC 600mg twice per day
Rx week #4	Receive 3nd intravenous dose of NAC.
Rx week #5	Patient undergoes surgery (lumpectomy or mastectomy). This completes participation in the study.

*This timeline may be modified for initiation of NAC depending on patient recovery from initial biopsy and may be extended up to 5 weeks of treatment depending on when the patient is scheduled for surgery.

Fig. 1. NAC window of opportunity clinical trial design. Subjects with stage 0 or 1 breast cancer who were scheduled to undergo primary surgical resection without receiving neoadjuvant therapy were eligible to enroll. Subjects were administered NAC and subsequently underwent surgical resection. Immunohistochemistry was performed on pre- and post-NAC paired breast cancer samples.

2.2. Immunohistochemistry

Two tissue samples were prepared for each patient; one sample from a pre-NAC biopsy, and one sample from a post-NAC resection. Tissue samples were fixed in neutral buffered formalin and then embedded in paraffin. Formalin fixed paraffin embedded (FFPE) samples were sectioned at 4-μm thickness, then dewaxed and rehydrated through graded ethanols.

Antigen retrieval was performed in 10 mmol/L citrate buffer, pH 6.0, for 10 minutes using a pressure cooker. Sections were cooled, blocked for endogenous peroxidase with 3% H_2O_2, and blocked for endogenous biotin using the DakoCytomation Biotin Blocking System (Dako, Carpinteria, CA). Sections were next incubated at room temperature with 10% goat serum for 30 minutes and then incubated at 4°C with primary antibodies for CAV1 and MCT4 (Santa Cruz Biotechnologies, Santa Cruz, CA) and Ki-67 (Abcam, Cambridge, MA).

Primary antibody binding was detected by biotinylated species-specific secondary antibody (Vector Labs, Burlingame, CA), followed by a streptavidin-horseradish peroxidase conjugate (Dako). Immunoreactivity was revealed with 3,3'diaminobenzidine (Dako). All sections were counterstained with hematoxylin.

Apoptotic cells were identified using the TUNEL-based ApopTag Peroxidase In Situ Apoptosis Detection Kit (MilliporeSigma, Burlington, MA) as previously described [30].

Histochemical staining was graded by two breast pathologists who were blinded to the nature of whether specimens were pre- or post-treatment with NAC. Stromal MCT4 and CAV1 staining were scored semiquantitatively. The scoring was as follows: 0, 0% strong immuno-reactive stromal cells; 1, $<$ 5% strong immuno-reactive stromal cells; 2, 5%–30% strong immuno-reactive stromal cells; and 3, $>$ 30% strong immuno-reactive stromal cells.

Quantitative analysis of MCT4 and Ki67 was performed using digital pathology with Aperio software (Leica Biosystems, Buffalo Grove, IL) as previously described [31]. Briefly, tissue sections were scanned on a ScanScopeXT with an average scan time of 120 seconds (compression quality 70). Images were analyzed using the Color Deconvolution and the Colocalization Aperio Image Analysis tool. Areas of staining were color separated from hematoxylin counter-stained sections and the intensity of the staining was measured on a continuous scale. For each case, three representative areas of the tumor were analyzed.

2.3. Statistical analysis

Strength-of-staining scores were compared between pre- and post-NAC samples by a paired, two-tailed Student's *t*-test. Significant *P* values were considered $<$ .05.

3. Results

3.1. Patients

A total of 12 female patients with stage 0 and 1 breast cancer were enrolled and pre- and post-NAC samples were obtained. Average age was 53 years (range, 43–62 years). Most common types of disease were invasive ductal carcinoma (six of 12) and ductal carcinoma in situ (five of 12) and there was one subject with papillary breast cancer (one of 12). No patients had known metastatic disease at the time of diagnosis and treatment. Toxicity was assessed using CTCAE v4.0 (Common Terminology Criteria for Adverse Events).

Patients received NAC an average of 19 days (range, 14–27 days) between enrollment in the study and definitive surgical resection.

3.2. Immunohistochemistry

Stromal expression of MCT4 was reduced after treatment with NAC (Fig. 2A). Average staining intensity of stromal MCT4

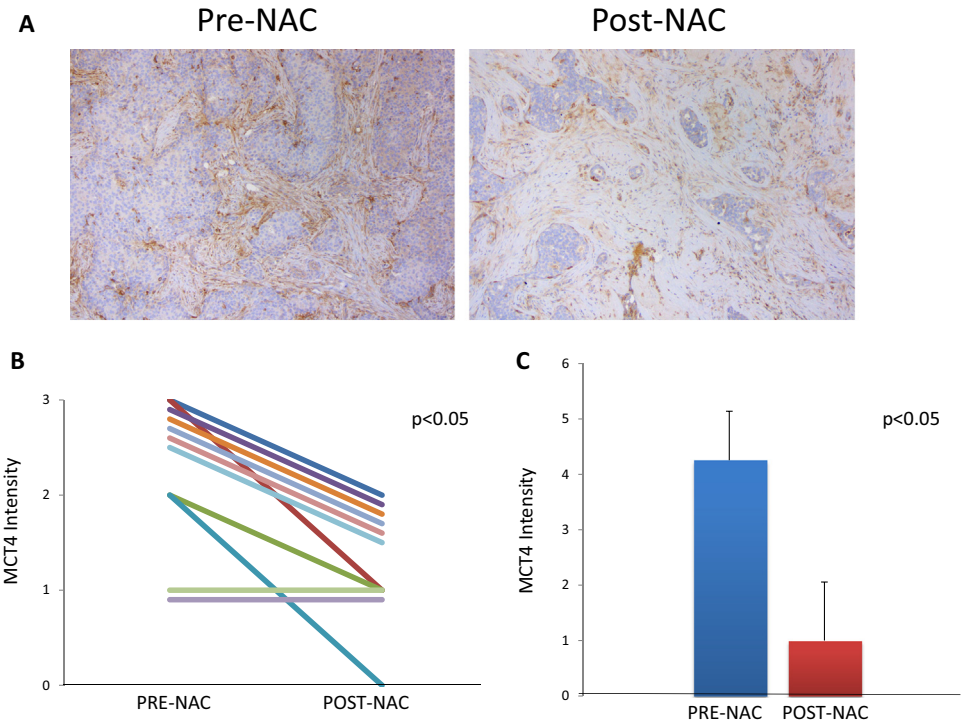


Fig. 2. Stromal MCT4 expression in pre- and post-NAC breast cancer samples. MCT4 immunostaining was performed on paired pre- and post-NAC breast cancer samples and a representative example is shown (A). Note that post-NAC there is reduction of MCT4 stromal staining. Original magnification: 20×. MCT4 immunostaining in the stroma was scored on paired pre- and post-NAC breast cancer samples by two pathologists independently. Note that post-NAC there is reduction of MCT4 stromal staining ($P < .05$) (B). MCT4 staining was quantified digitally using the Aperio software and arbitrary intensity units (a.i.) were obtained. Note that MCT4 expression is reduced in post-NAC samples ($P < .05$) (C).

as scored by two independent pathologists was reduced from 2.3 to 1.4 after treatment with NAC ($P < .001$) (Fig. 2B). Digital quantification of MCT4 stromal staining was also reduced from 4.3 to 1 arbitrary intensity units ($P < .001$) using Aperio digital pathology quantification (Fig. 2C).

There was a trend toward increased CAV1 stromal staining post-NAC (Fig. 3), but it did not reach statistical significance. The two left side panels of Figure 3A are from a subject who had increased CAV1 expression after NAC, while the two right-sided panels are from a subject who did not have a change in CAV expression. The average staining intensity of stromal CAV1 was 2.2 on pre-treatment specimens, and increased to 2.3 on post-treatment specimens ($P > .05$; Fig. 3).

Carcinoma cells had lower proliferation rates after NAC (Fig. 4). The pre-treatment specimens had an average of 5.9% proliferating cells as measured by the Ki-67 proliferation assay, and this decreased to 4.2% on post-treatment specimens ($P < .05$; Fig. 4).

There was a trend toward increased TUNEL staining post-NAC (Fig. 5), but it did not reach statistical significance. The average number of TUNEL-positive carcinoma cells per high power field was 6.9 on pre-treatment specimens, and was 7.4 on post-treatment specimens ($P > .05$; Fig. 5).

4. Discussion

We demonstrate in a clinical trial that NAC reduces carcinoma cell proliferation rates in patients with stage 0 and I breast cancer. NAC also alters a stromal marker of metabolism with reduced MCT4 expression. Previous epidemiologic studies have shown that antioxidant use during breast cancer treatment is associated with reduced mortality [2]. On the other hand, a previous clinical trial that assessed the effects of a 2-year supplementation with NAC on recurrence or survival in head and neck squamous cell carcinoma and lung cancer did not show a benefit [32]. NAC supplementation had never been evaluated in patients with breast cancer and this is the first clinical trial that demonstrates that NAC reduces carcinoma cell proliferation.

Anticancer drugs aim to directly reduce cancer cell proliferation and thus to improve outcomes. Multidrug anticancer regimens are based on the premise that different but often overlapping pathways such as DNA damage and signal transduction are engaged, leading to reduced proliferation. NAC induces metabolic stress and it has been described to synergize with drugs that cause DNA damage [10]. Future clinical trials will determine if the reduced rates of proliferation demonstrated in this study translate into a clinically meaningful impact on patient survival either alone or in combination with other drugs, such as those that target DNA damage. Indeed, a reduction in the rate of tumor cell proliferation may be predictive of drug sensitivity and prognosis and may prove to be a useful biomarker.

Proliferation is a hallmark of cancer cells and it is a core change in the conversion of a normal to malignant cell [33]. Support from stromal cells is critically required for cancer cell proliferation and most studies have focused on pro-survival growth factors and cytokines generated by the tumor microenvironment [33]. However, the metabolic environment may be an additional driver of proliferation in breast cancer that can be targeted with NAC and will need to be investigated further.

The mechanism by which NAC targets tumor growth in breast cancer may be because of reduced glycolysis and catabolism in stromal cells because MCT4 expression was also reduced in our study. In this pilot clinical trial of breast cancer, we assessed the effect of NAC on stromal-epithelial metabolic coupling by quantifying the expression of MCT4 in tumor stromal cells. MCT4 is the main transporter of lactate out of cells and, hence, a marker of glycolysis because lactate is the end-product of glycolysis [11].

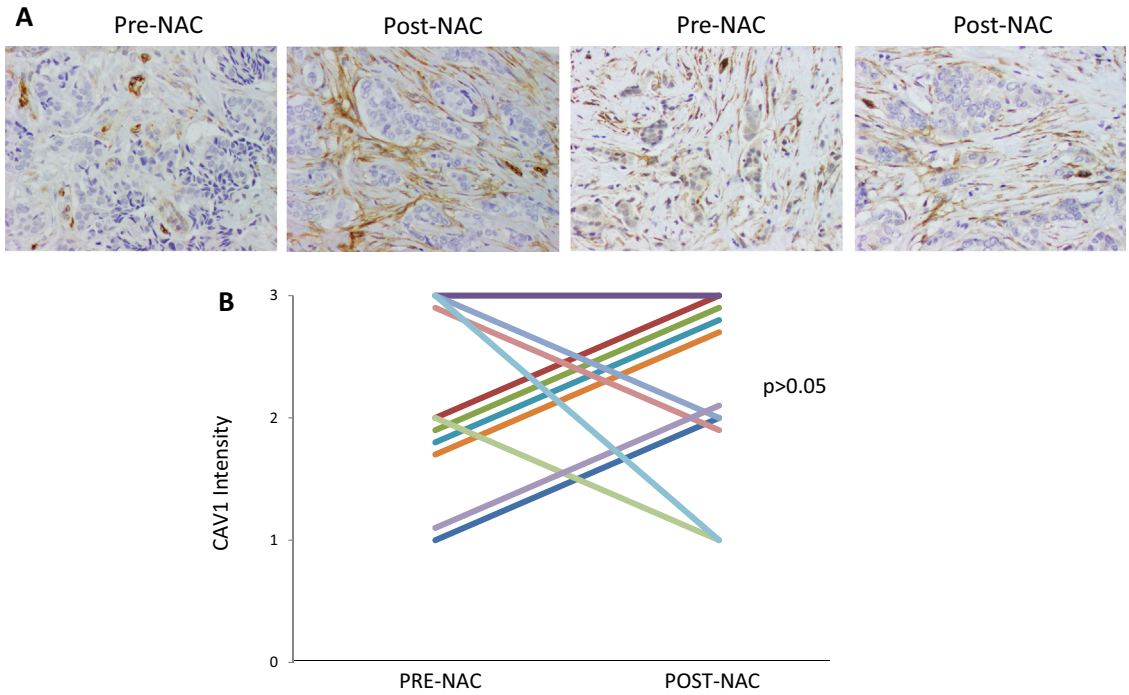


Fig. 3. Effect of NAC on stromal CAV1 expression in breast cancer. CAV1 immunostaining was performed on paired pre- and post-NAC breast cancer samples and a representative example is shown. Original magnification: 20× (A). CAV1 immunostaining in the stroma was scored by two pathologists independently on paired pre- and post-NAC breast cancer samples. Note that post-NAC there is no significant increase of CAV1 stromal staining ($P > .05$) (B).

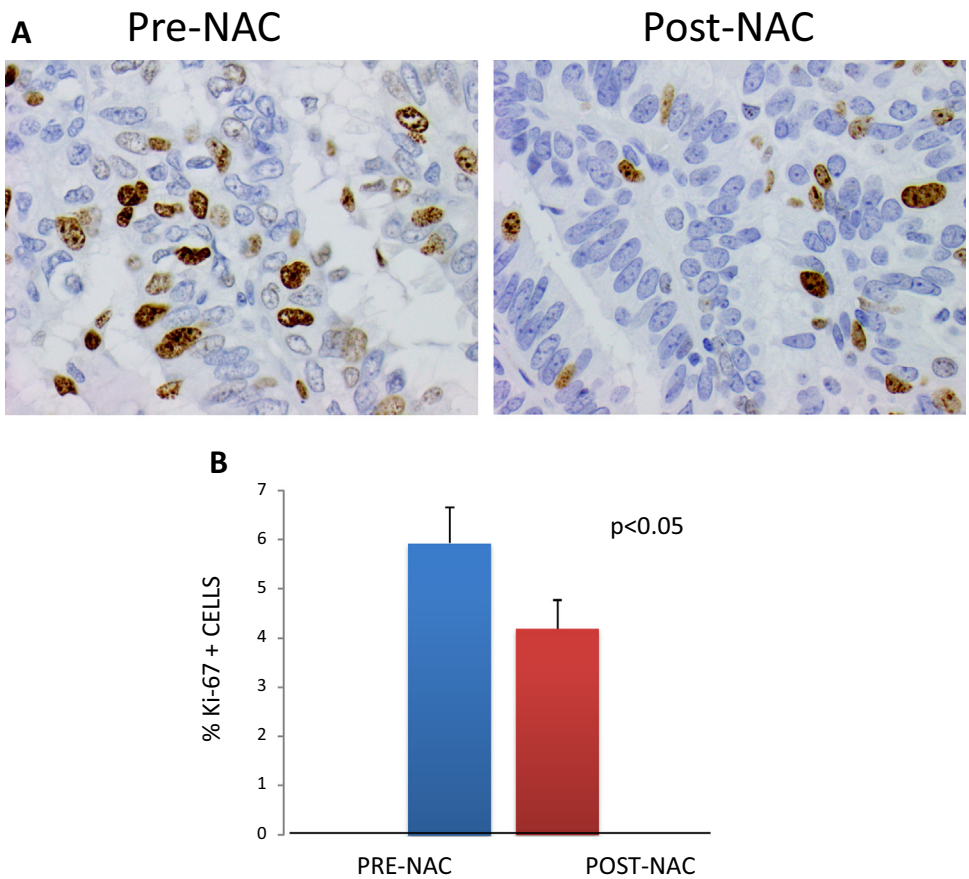


Fig. 4. Carcinoma cell Ki-67 in breast cancer pre- and post-NAC. Ki-67 immunostaining was performed on paired pre- and post-NAC breast cancer samples and a representative example is shown. Note that post-NAC there is reduction of Ki-67 carcinoma staining. Original magnification: 60× (A). Ki-67 staining of carcinoma cells was quantified digitally using the Aperio software and percentage of positive cells was obtained. Note that Ki-67 is reduced in post-NAC samples ($P < .05$) (B).

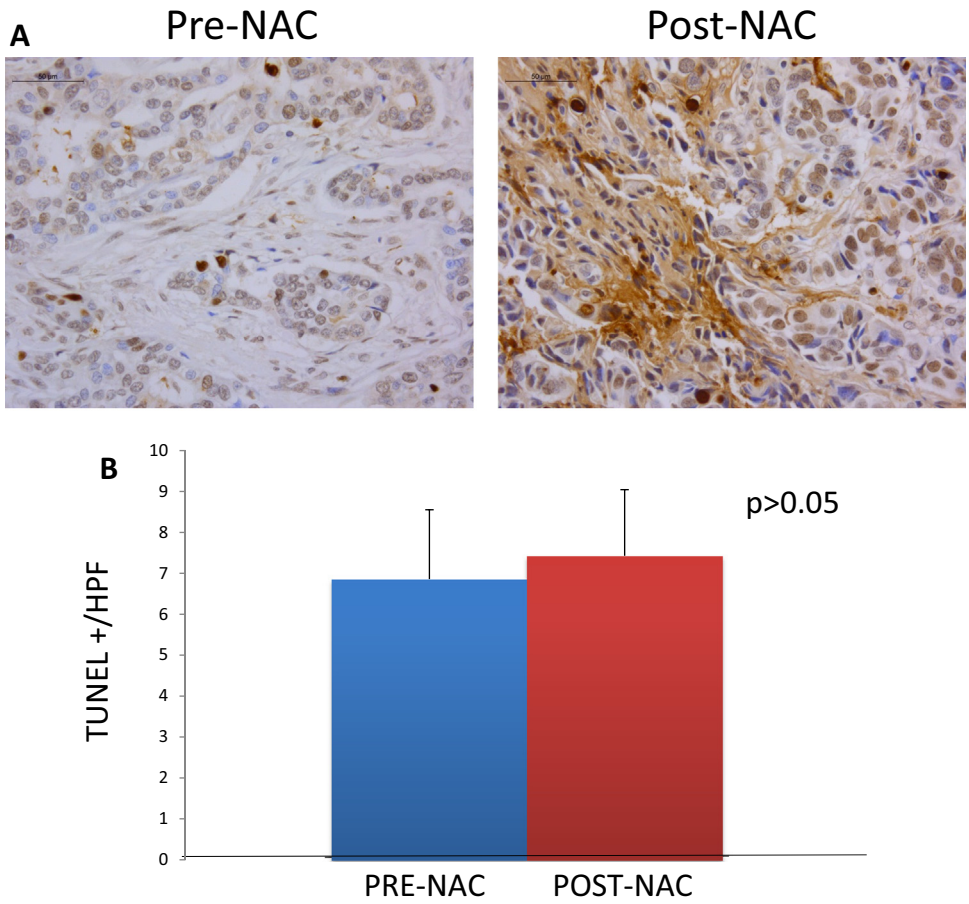


Fig. 5. Carcinoma cell TUNEL staining in breast cancer pre- and post-NAC. TUNEL staining to evaluate apoptosis was performed on paired pre- and post-NAC breast cancer samples and a representative example is shown. Original magnification: 40× (A). TUNEL staining in the stroma was scored on paired pre- and post-NAC breast cancer samples independently by two pathologists. Note that post-NAC there is no significant increase of TUNEL staining ($P > .05$) (B).

High expression of MCT4 in tumor stromal cells induces cancer aggressiveness with increased glycolysis and catabolism and NAC can reduce MCT4 expression [19,21]. Increased stromal MCT4 is a marker of metabolic coupling between cancer and stroma [19]. NAC should be an effective agent in tumors with tumor metabolic coupling [34] because NAC reduces MCT4 expression in experimental models of tumor stromal cells [18,35]. We have now demonstrated that NAC can reduce MCT4 expression in the tumor stroma of patients with breast cancer.

NAC is directly an antioxidant and is also a precursor of reduced GSH, which is the main intracellular antioxidant [5]. NAC reduces disulfide bonds in proteins, which alters their structures and disrupts their ligand bonding, competing with larger reducing molecules in sterically less accessible spaces and ultimately altering signal transduction. The antioxidant activity of NAC is attributed in experimental models to its fast reactions with free radicals that have an unpaired electron in their bonding structures and include the hydroxyl radical, ($^{\bullet}OH$), nitrogen dioxide radical ($^{\bullet}NO_2$), carbonate radical (CO_3^{2-}) and thiol radicals [4]. NAC also reacts with superoxide, hydrogen peroxide, and peroxynitrite, although more slowly than with the first group of free radicals. NAC and GSH have overlapping although not the same cellular effects and it is unclear if the effects of NAC are because of one or both. It is thought that the physiologic functions and therapeutic effects of NAC are largely associated with maintaining the levels of intracellular GSH but future studies will need to determine if the anticancer activity of NAC in breast cancer is because of its direct effects or its effects on increasing GSH.

The current clinical trial demonstrates that NAC reduced carcinoma cell proliferation. NAC also altered the metabolism marker MCT4 in the tumor stroma of subjects with breast cancer in this trial. These results are important because there is controversy as to whether NAC has anticancer properties because several studies have shown cancer progression [36–38]. Also, there is controversy as to what dose of NAC should be used in oncology trials because some advocate using higher doses because of its pharmacokinetics [39]. The terminal half-life of NAC is 5.6 hours after a single intravenous administration and 30% of the drug is cleared by renal excretion [40]. The bioavailability of NAC is less than 5%, which is thought to be because of its N-deacetylation in the intestinal mucosa and first pass metabolism in the liver [40]. The dose of NAC in this clinical trial is a dose commonly used in experimental cancer models and in clinical practice, although the current dose is lower than the highest recommended dose [6,9,23]. We show in this study that NAC has pharmacodynamic effects on breast carcinoma cells in patients with breast cancer despite its pharmacokinetic profile. Breast cancer cells may be particularly sensitive to NAC and the current dose can be used in phase II clinical trials in patients with breast cancer. Higher doses may be more efficacious and will also need to be evaluated.

New pharmacodynamic effects of NAC in human cancers have been discovered in this clinical trial because it has been demonstrated that NAC reduces MCT4, which is a marker of catabolism in stromal cells and reduces carcinoma cell proliferation. One of the major obstacles to successful clinical translation of promising preventive agents is a lack of pharmacodynamic biomarkers to provide an early read out of biological activity and for optimizing

doses to take into large-scale randomized clinical trials. MCT4 may be a suitable pharmacodynamic biomarker that could accelerate the developmental pipeline for drugs targeting glycolytic metabolism.

NAC was safe and well tolerated in this clinical trial. This is consistent with previous clinical trials and the pharmaco-vigilance data, which has detected only mild side effects and little toxicity [32]. Nausea, vomiting, rhinorrhea, rash, urticaria, pruritus, bronchospasm, and tachycardia have been described [9]. NAC administration in clinical trials in breast cancer assessing efficacy are expected to show low toxicity and side effects based on the current trial.

In sum, this pilot clinical trial of NAC shows that it is safe and has biological activity in breast cancer. This trial demonstrates that conventional doses of NAC reduce proliferation of carcinoma cells and reverts stromal MCT4, which is a marker of catabolism. Future studies need to be conducted to determine whether stromal metabolic changes are the driver of NAC's anticancer effects. Clinical trials will need to be performed to test if NAC and other antioxidants and metabolic modulators are effective in breast cancer.

Acknowledgments

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