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Anti-angiogenic potential of CoenzymeQ$_{10}$, riboflavin and niacin in breast cancer patients undergoing tamoxifen therapy

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ABSTRACT

Tumour angiogenesis is a complex mechanism consisting of multi-step events including secretion or activation of angiogenic factors by tumour cells, activation of proteolytic enzymes, proliferation, migration and differentiation of endothelial cells. Both primary and metastatic tumours in the breast are dependent on angiogenesis. In the present study, 84 breast cancer patients were randomized to receive a daily supplement of CoQ$_{10}$ 100 mg, riboflavin 10 mg and niacin 50 mg (CoRN), one dosage per day along with tamoxifen (TAM) 10 mg twice a day. Serum pro-angiogenic levels were elevated in untreated breast cancer patients (Group II) and their levels were found to be reduced in breast cancer patients undergoing TAM therapy for more than 1 year (Group III). When these group III breast cancer patients were supplemented with CoRN for 45 days (Group IV) and 90 days (Group V) along with TAM, a further significant reduction in pro-angiogenic marker levels were observed. Supplementing CoRN to breast cancer patients has found to decrease the levels of pro-angiogenic factors and increase the levels of anti-angiogenic factors. A reduction in pro-angiogenic marker levels attributes to reduction in tumour burden and may suggest good prognosis and efficacy of the treatment, and might even offer protection from cancer metastases and recurrence.

1. Introduction

Breast cancer development is a complex process associated with an accumulation of genetic and epigenetic changes that run through the steps of initiation, promotion and progression. Although localized breast cancer can be cured by surgery, it has a high mortality rate primarily due to frequent metastasis while the primary tumour is undetected. Angiogenesis is the formation of new capillaries from preexisting blood vessels, not only it is important in physiological processes but also contributes in a variety of pathological processes and various inflammatory disorders (Folkman, 1995). Both primary and metastasis tumours in the breast are dependent on angiogenesis and primary malignant breast tumours are among those human neoplasms that exhibit the greatest angiogenic activity. Recently, the significance of tumour angiogenesis as a prognostic indicator has been documented in various kinds of human tumours (Weidner et al., 1992; Toi et al., 1993).

Tamoxifen (TAM) is a non-steroidal anti-oestrogen drug, which has led to an increase in both disease-free and overall survival of breast cancer patients after primary surgery (Rutqvist et al., 1995). A complicating factor is the relapse in breast cancer patients during TAM therapy and in this subset of patients, treatment is only palliative and the recurrent breast cancer is incurable (van Dalen et al., 1996). CoQ$_{10}$ plays an important role in electron transport chain (Crane, 1993), functions as an antioxidant and prevent lipid peroxidation (Nohl et al., 1999), increases phagocytic activity in tumour induced mice (Folkers et al., 1982). Several clinical trials administrating CoQ$_{10}$ in cancer patients have indicated a tumour suppressive effect (Folkers et al., 1993, 1997; Lockwood et al., 1994, 1995). In breast cancer patients, administering 90 mg/day CoQ$_{10}$ showed complete regression. An increased dose of 390 mg/day of CoQ$_{10}$ to breast cancer patients and follow up study for five years showed complete regression of metastases in these patients (Lockwood et al., 1994). CoQ$_{10}$ was found to increase the levels of serum IgG in patients and this increase is attributed to a transcriptional increase in mRNA and a translational increase in the level of the apoenzymes for the CoQ$_{10}$ enzymes (Folkers et al., 1982). Riboflavin influences epithelial integrity, tissue flavin concentrations, rate of prostaglandin biosynthesis and glutathione metabolism (Siassi and Ghadirian, 2005). Riboflavin administration was found to decrease the risk of developing cancer by increasing the levels of total flavins, which has the capacity to capture reactive metabolites thereby, decreases the carcinogen binding to cellular macromolecules like DNA (Pangrekar et al., 1993). Riboflavin deficiency enhances the risk of esophagal cancer development at initiation and promotion stages (Siassi and Ghadirian, 2005).

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Table 1.1
Characteristic of breast cancer patient's in the study group

Characteristics	*n*	%
Age – year		
Median – 57 years		
Range – 43–70 years		
Tumour size (T)		
T1	4	5
T2	33	39
T3	26	31
T4	21	25
Nodal status (N)		
N0	26	30.9
N1	44	52.4
N2	13	15.5
N3	1	1.2
N4	0	0
Metastasis (M)		
M0	84	100
M1	0	0
Histology		
Ductal invasive	82	97.6
Lobular invasive	2	2.4
Surgery		
Conservative	2	2.4
Mastectomy		
Simple	11	13
Radical	2	2.4
Modified radical	56	66.6
Patey's	10	12
Modified Patey's	1	1.2
No surgery	2	2.4
Family history of cancer		
Yes	9	10.7
No	75	89.3
Diet		
Mixed	80	95.2
Vegetarian	4	4.8
Treatment		
Tamoxifen alone	8	9.5
Chemotherapy + Tamoxifen	40	47.6
Radiation + Tamoxifen	26	31
Chemotherapy + Radiation + Tamoxifen	10	11.9

Patient characteristics of group III, IV and V breast cancer women.
n = number of patients, % = percentage of patients.

In addition to redox functions in energy metabolism, niacin, in the form of NAD^+ participates in a variety of ADP-ribosylation reactions, which is responsible for the majority of polymer synthesis and plays an important role in DNA damage response, including repair, maintenance of genomic stability and signaling events for apoptosis (Virag and Szabo, 2002; Weitberg and Corvese, 1990).

Earlier studies in our laboratory with co-enzyme Q_{10} (CoQ_{10}), riboflavin and niacin (CoRN) supplementation in rat mammary carcinoma was found to enhance antitumour activity by increasing the expression of the tumour suppressor gene MnSOD (manganese superoxide dismutase), thereby preventing cancer cell proliferation. It has also been found to restore lipid peroxide levels and activities of the enzymatic and nonenzymatic antioxidants to near normal, thus demonstrating its mitochondrial antioxidant activity (Perumal et al., 2005b). CoRN supplementation was found to prevent cancer cachexia by inhibiting host energy loss by increasing the gluconeogenesis pathway by the cofactors of CoRN, which participate in oxidation–reduction reactions, numerous metabolic pathways and thereby enhance glycolysis, Kreb's cycle and adenosine triphosphate (ATP) production via the electron transport chain in the host tissue (Perumal et al., 2005a). CoRN supplementation to breast cancer patients has been found to reduce the tumour marker levels of CEA and CA 15-3 (Premkumar et al., 2007b) and decrease the levels of serum cytokines IL-1β, IL-6, IL-8, TNF-α and VEGF (Premkumar et al., 2007a).

Antiangiogenic therapy is a highly promising new strategy because of the dependence of growing and metastasize capacity of a tumour on the formation of new blood vessels (Miller et al., 2001). Many antiangiogenic agents have been developed that are directed against the endothelial cells of the tumour vasculature. Targeting the endothelium of the tumour blood vessels is favoured over targeting the tumour itself, as somatic mutations frequently occur in tumour cells, making the tumour resistant to apoptotic stimuli. One of the advantages of antiangiogenic therapy is believed to be the lack of induction of resistance to angiogenic inhibitors, which is explained by the fact that endothelial cells are genetically stable cells that are considered not to mutate into drug-resistant variants. Further evidence for the antiangiogenic activity of conventional as well as experimental cancer therapies comes from a growing number of studies that have shown that damage of blood vessels precede or accompanies tumour regression after radiation therapy or administration of a variety of biological response modifiers such as interferon, TNF, interleukins or endotoxin (Oehler and Bicknell, 2000).

2. Materials and methods

Patients were recruited from the Medical Oncology Department of the Government Royapettah Hospital, Chennai, India, via their physicians according to the process approved by the Institutional Human Ethical Review Board. Informed consent was obtained from all patients with due explanation of the study. They were aged 43 to 70 years with histopathology-confirmed breast cancer. Patients with diabetes mellitus, renal and hepatic diseases were excluded from this study. Patients' characteristics are given in Table 1.1.

2.1. Study design

Three different groups of patient were recruited in the Groups I, II and III: Group I: 42 socio-economically and age-matched disease-free, healthy controls; Group II: 84 untreated breast cancer patients; Group III: 84 breast cancer patients treated for more than 1 year with TAM Group IV and V: Group III patients followed up for 45 days (Group IV) and 90 days (Group V) after supplementation with C_oQ_{10} (100 mg), riboflavin (10 mg) and niacin (50 mg) one dosage per day along with TAM (10 mg twice a day).

Patients were advised to take one capsule of C_oQ_{10} (100 mg Kaneka®Q10, Kaneka Corporation, Osaka, Japan), one tablet of riboflavin and niacin (10 mg riboflavin and 50 mg niacin, Madras Pharmaceuticals, Chennai, India) and two tablets of TAM (10 mg Nolvadex®, AstraZeneca, Bangalore, India) per day. All tablets were taken after breakfast, with a second tablet of TAM after dinner. The patients were asked not to take any vitamin supplement during the study period. Compliance was checked by counting the number of tablets handed out to the patients and recollected at the end of the study.

2.2. ELISA

Angiogenic marker levels of PGE_2 (PGE_2 Assay, Parameter™ R&D Systems, Minneapolis, MN, USA), TGF-β1 (Quantikine®, Human TGF-β1

Table 1.2
Primer sequences for PCR reaction for each target gene

Gene	Primer
MMP 2	Sense: 5′-GTCCTGACCAAGGATATAGCC-3′ Anti-sense: 5′-AGACCCAGTACTCATTCCCTG-3′
MMP 9	Sense: 5′-AGTTTGGTGTCGCGGAGCAC-3′ Anti-sense: 5′-TACATGAGCGCTTCCGGCAC-3′
TIMP-1	Sense: 5′-ATCCTGTTGTTGCTGTGGCTGATAG-3′ Anti-sense: 5′-TGCTGGGTGGTAACCTCTTTATTTCA-3′
TIMP 2	Sense: 5′-GTTTTGCAATGCAGACGTAG-3′ Anti-sense: 5′-ATGTCAAGAAACTCCTGCTT-3′
RPL-19	Sense: 5′-CTGAAGGTCAAAGGGAATGTG-3′ Anti-sense: 5′-GGACAGAGTCTTGATGATCTC-3′

Table 1.3
PCR amplification conditions and product length for each target gene

Gene	Amplification conditions	Product length (bp)
MMP 2	Denaturation at 94 °C for 45 s. Annealing at 58 °C for 1 min and extension at 72 °C for 1 min for 34 cycles	465
MMP 9	Denaturation at 94 °C for 1 min 20 s. Annealing at 60 °C for 1 min 20 s and extension at 72 °C for 2 min for 34 cycles	753
TIMP-1	Denaturation at 94 °C for 30 s. Annealing at 58 °C for 1 min and extension at 72 °C for 1 min 50 s for 27 cycles	667
TIMP 2	Denaturation at 94 °C for 1 min 20 s. Annealing at 60 °C for 1 min 20 s and extension at 72 °C for 2 min for 34 cycles	539
RPL-19	Changed according to different target gene	194

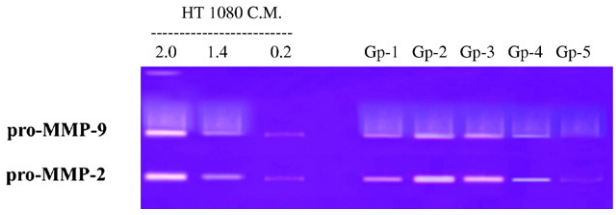


Plate 1. Gelatin zymography of the study subjects. Gelatin zymography of patient's serum in different groups. Serum samples were normalized for protein concentration and analyzed. HT-1080 conditioned medium with different concentration used as standard reference. Gp-1: Group I, control subjects; Gp-2: Group II, untreated patients; Gp-3: Group III, TAM alone treated; Gp-4: Group IV, TAM+CoRN treated (45d); Gp-5: Group V, TAM+CoRN treated (90d).

Immunoassay, R&D Systems, Minneapolis, MN, USA), Epidermal Growth Factor Receptor (EGFr ELISA, Siemens Medical Solutions Diagnostics Tarrytown, NY, USA), basic Fibroblast Growth Factor (Quantikine®, Human FGF basic Immunoassay, R&D Systems, Minneapolis, MN, USA), Serum Hepatocyte Growth Factor (HGF-ELISA kit, Institute of Immunology, Tokyo, Japan), Human Thrombospondin (ChemiKine™ Human TSP-1, Chemicon International, CA, USA), Endostatin (ChemiKine™ Human Endostatin kit, Chemicon International, CA, USA), Human TIMP-1 ELISA (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA), Human TIMP-2 Immunoassay (R&D Systems, Minneapolis, MN, USA), u-PA (Chromolixe™ u-PA, Biopool, Umea, Sweden) test measures single chain u-PA and two chain u-PA, but not u-PA in complex with PAI. t-PA (Imulyse™ t-PA, Biopool, Umea, Sweden) measures free t-PA as well as t-PA in complex with PAI-1. PAI-1 (Imulyse™ PAI-1, Biopool, Umea, Sweden) detects the active and latent forms of PAI-1, as well as PAI-1 in complex with PAs. PAI-2 (Imulyse™ PAI-2, Biopool, Umea, Sweden) detects free PAI-2 and about 60% of PAI-2 in complex with PAs. The IMUBIND® Total suPAR ELISA kit (American diagnostics Inc., Stamford, CT) was used to measure human soluble urokinase-type plasminogen activator receptor in plasma, Soluble E-selectin (Human E-Selectin Immunoassay, R&D Systems, Minneapolis, MN, USA), soluble ICAM-1 (Human soluble ICAM-1/CD54 Immunoassay, R&D Systems, Minneapolis, MN, USA) and soluble VCAM-1 (Human sVCAM-1 Immunoassay, R&D Systems, Minneapolis, MN, USA) were determined using the enzyme-linked immunosorbent assay (ELISA) kits according to the instructions provided by the manufacturer. The standard curves were prepared by using a group of serially diluted standards.

Quantification of soluble elastin peptides were performed by a competitive ELISA as described by Wei et al. (1993) with some modifications. Wells of microtiter plates were coated with 2 µg/ml of elastin peptides (CB573; Elastin Products Company Inc., Owensville, MO). Simultaneously, in a separate plate (precoated with 0.5% bovine serum albumin), samples were mixed 1:1 (v/v) with a 1:500 dilution of rabbit anti-bovine neck elastin antibody (Elastin Products Company Inc.). After overnight incubation of both plates at 4 °C, contents of each sample-antibody mixture well were transferred to corresponding wells in the elastin peptide coated plate and allowed to react for 30 min at 37 °C, followed by secondary antibody (anti-rabbit IgG peroxidase conjugate, diluted 1:2000, Bangalore Genei, India). After 60 min at 37 °C, peroxidase activity was detected using *o*-phenylene diamine hydrochloride as a substrate at 490 nm. All washes between steps and dilutions of samples and antibodies were performed in phosphate-buffered saline containing 0.05% Tween 20 and 0.5% bovine serum albumin. Extracts obtained from unimplanted elastin served as controls. Elastin peptide values of the samples were quantified comparing with the standard curve. Values are expressed as µg/mg protein.

BSP and OPN levels were measured according to the method of Fedarko et al. (2001). Briefly, the serum sample were processed in a anion exchange column chromatography, 100 µl of serum samples were diluted 1:10 in a 50% formamide-40-mM phosphate buffer (pH 7.4) and were reduced with 2 mM DTT at 100 °C for 5 min to disrupt the binding of BSP or OPN by complement factor H in serum. Residual reducing agent and formamide were removed by strong anion exchange column chromatography using 300 µl of resin in a chromatography column. After loading the 1 ml sample, the column was washed with 6 × column volumes of TBS solution [0.05 M Tris–HCl (pH 7.5), 0.15 M NaCl] containing 0.05% Tween 20 (TBS-T). BSP and OPN (antibody gift from Larry W. Fisher, National Institute of Dental and Craniofacial, Research, NIH, Bethesda, Maryland 20892) were eluted with TBS-T containing 1 M NaCl. A competitive ELISA was done using 100 µl of processed sample. Microtiter plates were coated with 20 ng/well BSP or OPN overnight in 50 mM carbonate buffer (pH 8.0). The standard curves for BSP and OPN were obtained by using standard protein at concentrations. Hundred µl of samples and standards were incubated for 2 h with shaking at room temperature with 100 µl of 1:200,000 dilution of LF-100 antibody (for BSP) or a 1:100,000 of

Table 2
Levels of angiogenic growth factors in study subjects

Parameters	Group I	Group II	Group III	Group IV	Group V
	Control, normal age-matched, postmenopausal women (42)	Pretreatment untreated breast cancer women (84)	Treatment with tamoxifen (84)	45 days after treatment with CoQ_{10}, riboflavin and niacin along with tamoxifen (84)	90 days after treatment with CoQ_{10}, riboflavin and niacin along with tamoxifen (84)
Endostatin (ng/ml)	224.7±56.9	$308.5\pm74.8^{a^{**}}$	$350\pm82.7^{b^{***},cNS}$	$652.2\pm105.4^{d^{***}}$	$687\pm162.7^{e^{***},f^{***}}$
bFGF (pg/ml)	0.2±0.2	$10.8\pm2.9^{a^{***}}$	$7.8\pm2.0^{b^{***},cNS}$	$5.4\pm1.4^{d^{**}}$	$5.0\pm1.3^{e^{**},f^{***}}$
HGF (ng/ml)	0.5±0.4	$3.6\pm0.5^{a^{***}}$	$2.9\pm0.4^{b^{***},cNS}$	$1.5\pm0.3^{d^{**}}$	$1.1\pm0.3^{e^{***},f^{*}}$
EGFR (ng/ml)	72.82±18.3	$186.2\pm41.8^{a^{***}}$	$169.4\pm32.7^{b^{***},cNS}$	$118.2\pm28.3^{d^{**}}$	$93.7\pm24.9^{e^{**},fNS}$
TGF-β1 (ng/ml)	28.2±6.3	$67.8\pm19.2^{a^{***}}$	$51.2\pm16.8^{b^{***},c^{**}}$	$39.4\pm8.6^{d^{**}}$	$32.3\pm8.1^{e^{**},fNS}$
dThdPase (ng/ml)	5.8±1.6	$23.7\pm8.4^{a^{***}}$	$21.6\pm5.7^{b^{***},cNS}$	$15.3\pm4.1^{d^{**}}$	$11.8\pm3.2^{e^{**},f^{***}}$
PGE_2 (pg/ml)	83.5±17.2	$227.6\pm63.32^{a^{***}}$	$147.2\pm33.7^{b^{**},c^{*}}$	$108.2\pm26.2^{d^{*}}$	$97.5\pm22.3^{e^{*},fNS}$
TSP (ng/ml)	282±72.8	$1320.23\pm283.43^{a^{***}}$	$818.4\pm221.62\ b^{***,c^{**}}$	$592.26\pm161.2^{d^{**}}$	$436.76\pm111.36^{e^{**},f^{**}}$

Values are expressed as mean±SD. Number of subjects are indicated in parentheses.
Comparisons were made between: a – Group I and Group II; b – Group I and III; c – Group II and III; d – Group III and IV; e – Group III and V; f – Group I and V. Statistical significance expressed as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – Non Significant.

Table 3
Levels of Matrix degrading proteinase in the study subjects

Parameters (ng/ml)	Group I	Group II	Group III	Group IV	Group V
	Control, normal age-matched, postmenopausal women (42)	Pretreatment untreated breast cancer women (84)	Treatment with tamoxifen (84)	45 days after treatment with CoQ_{10}, riboflavin and niacin along with tamoxifen (84)	90 days after treatment with CoQ_{10}, riboflavin and niacin along with tamoxifen (84)
Pro-MMP2	90.46±23.38	$540.2\pm108.92^{a***}$	$393.6\pm92.94^{b***,c**}$	$280.67\pm61.3^{d**}$	$255.4\pm68.21^{e**,f***}$
Pro-MMP9	1022.7±270.31	$1388.89\pm202.9^{a***}$	$1272.29\pm200.6^{b**,c*}$	$1050.3\pm152.54^{d**}$	$952.68\pm120.89^{e**,fNS}$
TIMP 1	620.61±125.9	$920.4\pm125.12^{a**}$	$1200.64\pm280.1^{b***,c*}$	$1580.69\pm425.28^{d**}$	$1620.7\pm475.3^{e***,f***}$
TIMP 2	92.49±23.9	$232.7\pm60.3^{a***}$	$298.32\pm72.4^{b***,c*}$	$572.8\pm112.7^{d**}$	$608.31\pm138.4^{e***,f***}$

Values are expressed as mean±SD. Number of subjects are indicated in parentheses.
Comparisons were made between: a – Group I and Group II; b – Group I and III; c – Group II and III; d – Group III and IV; e – Group III and V; f – Group I and V. Statistical significance expressed as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – Non Significant.

LF-124 antibody (for OPN) in TBS-T+5% skimmed milk powder in polypropylene 96-well plates. After suitable incubation and washing with TBS-T buffer, secondary antibody of goat antirabbit peroxidase-labeled antibody conjugate, human serum adsorbed (Bangalore Genei, Chennai, India) at 1:2000 was then added, and the plates were incubated for 1 h. After three washes with TBS-T, TMB substrate (Bangalore Genei, Chennai, India) was added and after final 20-min incubation, the colour reaction was stopped by the addition of 25 µl of 1 N H_2SO_4. Absorbance was read at 450 nm and the data were analyzed using the program AssayZap (BioSoft, Cambridge, United Kingdom). The values are expressed as ng/ml. Thymidine phosphorylase (dThdPase) was quantified in serum by using the method of Katayanagi et al. (2003).

2.3. Gelatin zymography

MMP-2 and MMP-9 quantification was performed by gelatin zymography. SDS-substrate gels were prepared by a modification of standard SDS-PAGE electrophoresis (Laemmli, 1970). Type I gelatin was added to the standard acrylamide polymerization mixture at a final concentration of 1 mg/ml. The protein concentrations in the supernatant were estimated by the method of Lowry et al. (1951). Equal protein (20 µg) from each serum sample were mixed (3:1) with sample buffer (10% SDS, 40% glycerol, 0.25 M Tris–HCl, pH 6.8, and 0.1% bromophenol blue) and loaded into the wells of a 4% acrylamide stacking gel and 10% acrylamide resolving gel. HT1080 standard control, containing known concentrations of MMP-2 and MMP-9 was included and serial dilutions of the sample were performed to determine gelatinolytic activity. Gels were run at 4°C and later were soaked in 2.5% Triton X-100 with gentle shaking for 30 min at ambient temperature with one change of detergent solution. The gels were rinsed with water for 20 min and incubated overnight at 37°C in substrate buffer (50 mM Tris–HCl buffer pH 8.0, 5 mM $CaCl_2$ and 0.02% Tween 20). After incubation, the gels were stained for 1 h in 0.5% Coomassie blue R-250 in acetic acid: methanol: water (1:3:6), and de-stained in acetic acid: methanol: water (1:4:6). Proteolytic areas appeared as clear bands against a blue background after intensive destaining. The bands measured had a molecular weight of 72 kDa for pro-MMP-2 and of 92 kDa for pro-MMP-9. The gels were quantified with a Biorad image analysis software system.

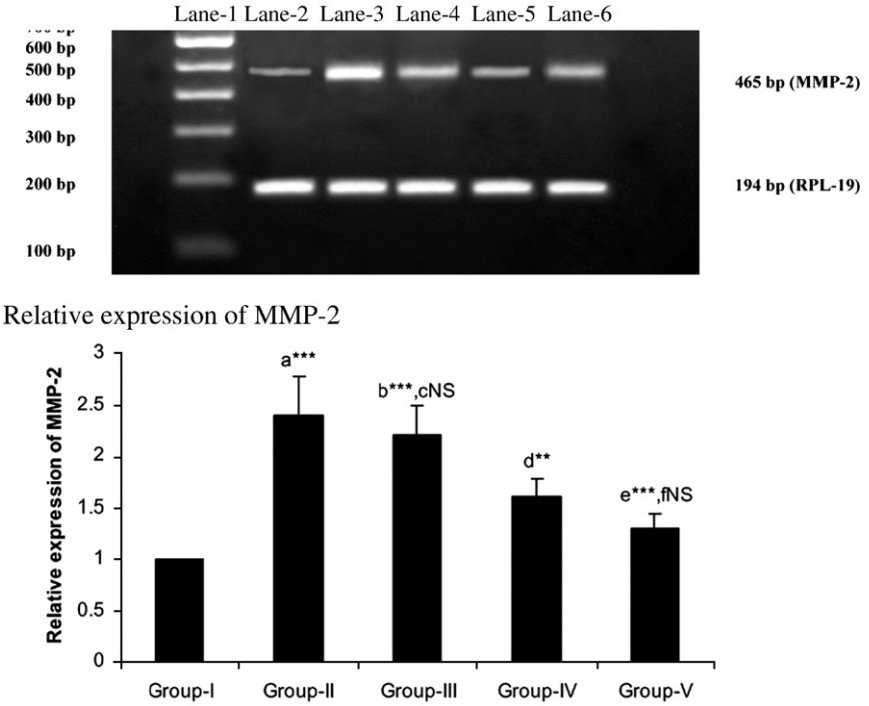


Plate 2. Representative MMP-2 expression in study subjects. Lane-1: 100 bp DNA ladder; Lane-2: Gp-I control subjects; Lane-3: Gp-II untreated patients; Lane-4: Gp-III TAM alone treated; Lane-5: Gp-IV TAM+CoRN treated (45d); Lane-6: Gp-V TAM+CoRN treated (90d). Relative expression of MMP-2. Values are expressed as Mean±S.D. Comparisons were made between a: group I and II; b: group I and III; c: group II and III; d: group III and IV; e: group II and V; f: group I and V. Statistical significance expressed as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – Non significant.

2.4. Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

RT-PCR for MMP-2, -9, TIMP-1, and 2 mRNA expressions in lymphocytes were done according to instructions in the kit (Qiagen One Step RT-PCR mix). Briefly, the reaction mixture contained 10 μl of 5× Qiagen One step RT-PCR Buffer containing final concentration of 2.5 mM $MgCl_2$, 2 μl of dNTP Mix (0.4 mM of each dNTP as final concentration), 5 μl of each sense and antisense primers of MMP-2, -9, TIMP-1, and -2, 5 μl of sense and antisense primers of housekeeping RPL-19 (each of 0.6 μM final concentration), 1.0 μg of template RNA, 2 μl of Qiagen One step RT-PCR enzyme mix was made up to 50 μl with RNase free water. RPL 19 was used as an internal control. The specific sets of primers and the target gene amplification conditions are shown in Tables 1.2 and 1.3.

To compare the amount of steady state mRNA, 5 μl of each PCR product was resolved onto 2% agarose gel using TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). After electrophoresis, the gels were viewed under UV light and digital images were captured on Biorad gel documentation system. The expression of each target gene was standardized with internal control gene expression and represented as a ratio.

2.5. Analysis of uPA by immunoblotting

The protein concentration of the serum was estimated by the method of Lowry et al. (1951). The samples (equal amount of protein; 50 μg) were boiled with sample solubilising buffer (SSB) for 5 min and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). The gel was transferred onto a nitrocellulose membrane (Hybond C+, Amersham life sciences) at 30 V for 3 h. Membrane was then washed thrice with PBS (Phosphate buffered saline, 0.01 M, pH 7.2) and blocking was done with TBST buffer (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% non-fat dry milk. Then, the membrane was incubated with primary antibody 1: 1000 (rabbit monoclonal anti-uPA, Callbiochem International, CA, USA) in TBST buffer containing 1% non-fat dry milk and agitated gently at room temperature for 3 h. After incubation with the primary antibody, the blots were washed thrice for 5 min with TBST buffer and incubated for 75 min at room temperature with goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (1:500 dilutions) in phosphate-free TBST buffer containing 5% non-fat dried milk. The bands were detected using Diaminobenzidine reagent chromogen system (6 mg of 3,3' diaminobenzidine dihydrochloride and 30 mg of nickel chloride in 10 ml of 50 mM Tris–HCl, pH 7.5 containing 10 μl of H_2O_2).

2.6. Data analysis

All test values were expressed as mean±standard deviation (SD). The results were computed statistically by Social Science Computer Package version 11.0 (SPSS 11.0, Chicago, IL, U.S.A.) using student unpaired and paired *t*-test. Analysis within Group III, IV and V were done using students paired *t*-test, while the other group comparisons were done using unpaired *t*-test. *p* value of 0.05 was considered significant.

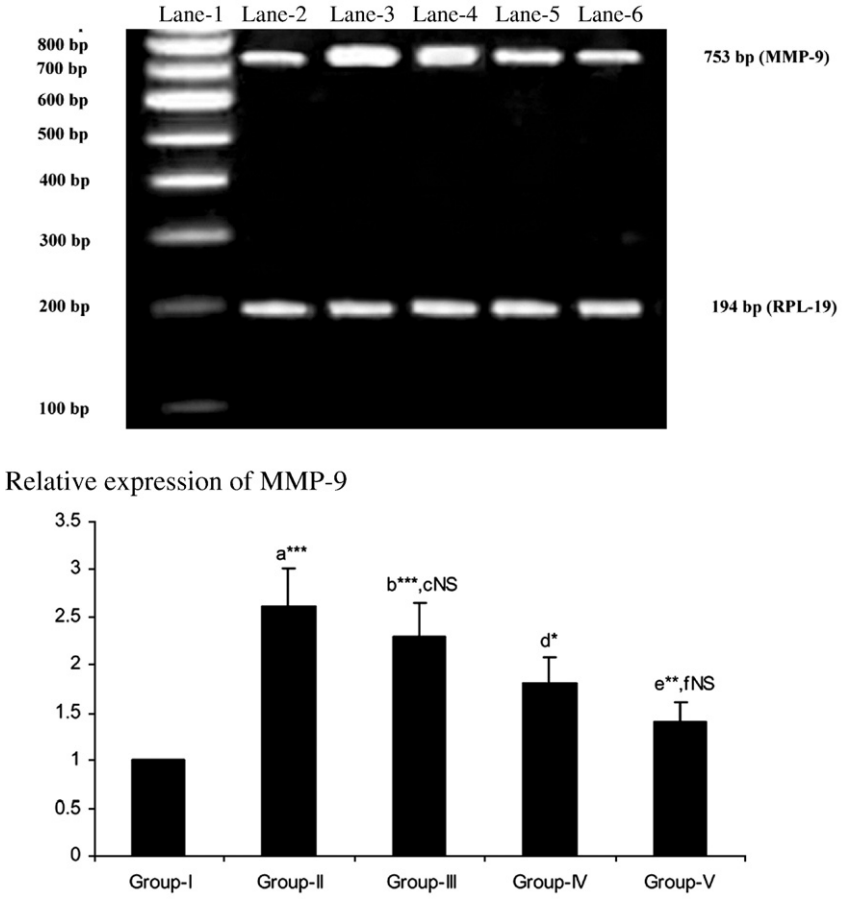


Plate 3. Representative MMP-9 expression in study subjects. Lane-1: 100 bp DNA ladder; Lane-2: Gp-I control subjects; Lane-3: Gp-II untreated patients; Lane-4: Gp-III TAM alone treated; Lane-5: Gp-IV TAM+CoRN treated (45d); Lane-6: Gp-V TAM+CoRN treated (90d). Relative expression of MMP-9. Values are expressed as Mean±S.D. Comparisons were made between a: group I and II; b: group I and III; c: group II and III; d: group III and IV; e: group II and V; f: group I and V. Statistical significance expressed as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – Non significant.

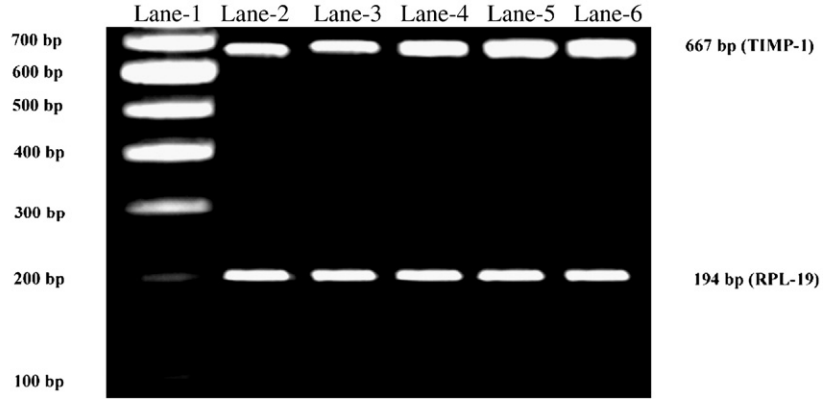
3. Results

In Table 2, the angiogenic marker levels of bFGF, HGF, EGFR, TGF-β1, dThdPase, PGE_2 and TSP levels were found to be significantly ($p<0.001$) increased in Group II compared to control Group I subjects. The levels were found to be significantly decreased ($p<0.001$) in group III when compared to group II. When group III patients were supplemented with CoRN for 45 and 90 days the level of angiogenic markers reduced significantly (levels of significant difference expressed in Table 2) in Group IV and Group V patients. The levels of Endostatin significantly increased in Group IV and V patients compared to Group II and Group III patients.

In our study we detected pro-MMP-9 (92 kDa) and pro-MMP-2 (72 kDa) by gelatin zymography (Plate 1) and the concentration of the test samples were determined by using a standard HT1080 medium (Table 3). pro-MMPs level were found to be significantly increased ($p<0.001$) in group II cancer patients compared to control Group I. pro-MMP2 and pro-MMP9 levels in group III patients were significantly reduced compared to Group II patients. In Group IV and V patients supplemented with CoRN along with TAM there was a significant reduction ($p<0.01$) in pro-MMP2 and pro-MMP9 compared to Group III patients. The serum levels of TIMP-1 and TIMP-2 were found to be significantly increased ($p<0.01$) in Group IV and V patients compared to Group III patients. The mRNA expression of MMPs and TIMPs from RNA isolated from blood were showed in Plates 2–5. In Plate 2 we found significant decrease ($p<0.01$) in MMP-2 mRNA expression of Group IV patients compared to Group III patients and a significant decrease of ($p<0.001$) was found in Group V patients compared to Group III patients. In Plate 3 MMP-9 mRNA expression in Group IV patients were ($p<0.05$) and Group V patients were ($p<0.01$) compared to Group III TAM treated patients. In Plate 4 TIMP-1 mRNA expression were found to be significantly increased ($p<0.01$) in Group IV and Group V compared to Group III patients. In Plate 5 TIMP-2 mRNA expression were found to be significantly increased ($p<0.01$) in Group IV and ($p<0.001$) in Group V compared to Group III patients.

In Table 4 group II patients had a significant increase ($p<0.001$) in the levels of uPA, tPA and suPAR compared to controls. No significant change in levels of uPA, tPA, suPAR and PAIs were found in Group III patients treated with TAM compared to group II. But, in Group IV and V patients there was significant decrease ($p<0.001$) in levels of uPA, tPA and suPAR and significant increase ($p<0.001$) in levels of PAI-1 and PAI-2 compared to Group III patients. The protein expression of secreted uPA (55 kDa) were determined by immunoblot in the serum (Plate 6) and we found a non-significant change in the protein expression in Group III patients compared to Group II. There was a significant decrease ($p<0.01$) in protein expression in Group IV and Group V patients compared to Group III patients.

In Table 5, we found the levels of Adhesion molecules in Group II were significantly higher ($p<0.001$) than control subjects and there was a significant reduction ($p<0.05$) in group III patients compared to group II patients. There was a significant change ($p<0.01$) in Group IV and Group V patients treated with CoRN when compared with Group III patients. Levels of elastin peptides in group II patients were found to be significantly increased ($p<0.001$) and group III patients had a significant reduction ($p<0.05$) in the levels compared to group II patients, significant reduction ($p<0.001$) were found in group IV and V patients compared to group III patients after treatment with CoRN for 45 and 90 days respectively. The BSP and OPN levels were tabulated in Table 5 and the study results showed a significant increase ($p<0.001$) in BSP and OPN levels in Group II patients compared to Group I controls



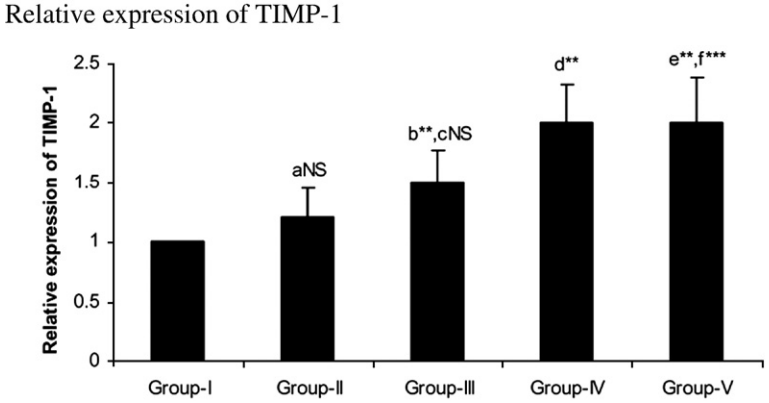


Plate 4. Representative TIMP-1 expression in study subjects. Lane-1: 100 bp DNA ladder; Lane-2: Gp-I control subjects; Lane-3: Gp-II untreated patients; Lane-4: Gp-III TAM alone treated; Lane-5: Gp-IV TAM + CoRN treated (45d); Lane-6: Gp-V TAM + CoRN treated (90d). Relative expression of TIMP-1. Values are expressed as Mean ± S.D. Comparisons were made between a: group I and II; b: group I and III; c: group II and III; d: group III and IV; e: group II and V; f: group I and V. Statistical significance expressed as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – Non significant.

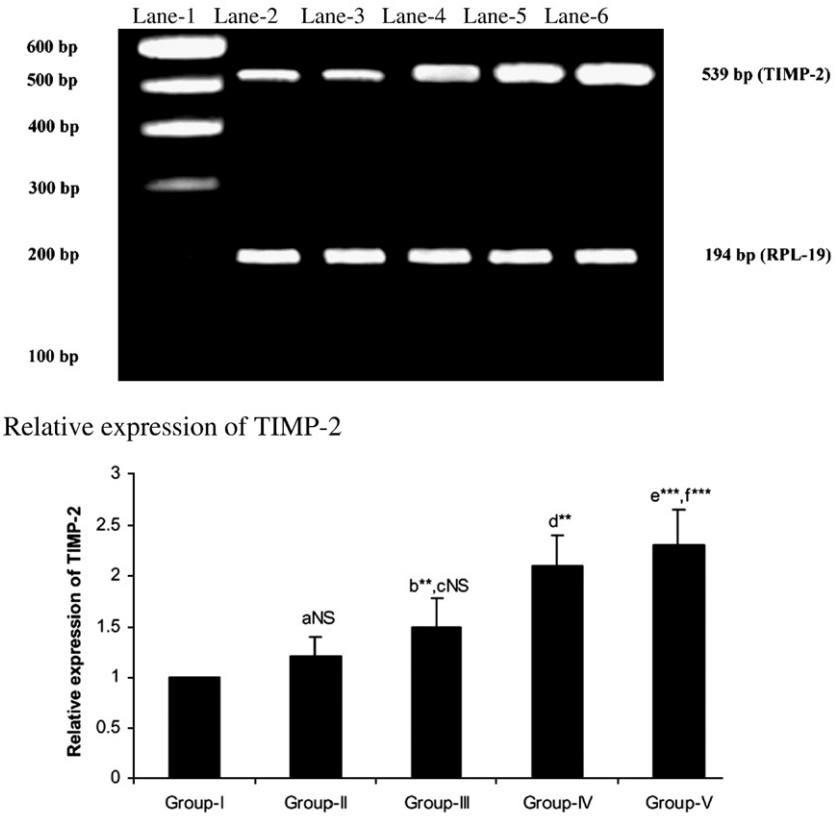


Plate 5. Representative TIMP-2 expression in study subjects. Lane-1: 100 bp DNA ladder; Lane-2: Gp-I control subjects; Lane-3: Gp-II untreated patients; Lane-4: Gp-III TAM alone treated; Lane-5: Gp-IV TAM + CoRN treated (45d); Lane-6: Gp-V TAM + CoRN treated (90d). Relative expression of TIMP-2. Values are expressed as Mean ± S.D. Comparisons were made between a: group I and II; b: group I and III; c: group II and III; d: group III and IV; e: group II and V; f: group I and V. Statistical significance expressed as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – Non significant.

and there was a significant decrease ($p<0.01$) in Group III patients treated with TAM compared to Group II untreated patients. When the Group III patients were treated with CoRN for 45 days and 90 days there was a significant change ($p<0.05$ for 45 days and $p<0.01$ for 90 days) in serum levels of BSP and OPN.

4. Discussion

The formation of new blood vessels, "angiogenesis" is a necessity for growth of both primary and metastatic tumours, since tumour growth beyond 1–2 mm^3 requires development of an adequate blood supply due to the oxygen diffusion limit between capillary and cells (Folkman, 1990). Angiogenesis is a highly regulated process involving sequential activation of a series of receptors by various ligands, in order to initiate degradation of the basement membrane, cell migration, endothelial cell proliferation and tube formation (Ferrara et al., 2003; Scott et al., 1998).

Angiogenic factors such as Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor (bFGF), Hepatocyte growth factor (HGF), angiogenin, Transforming Growth Factor (TGF)-beta, Tumour Necrosis Factor (TNF)-alpha, Matrix metalloproteinases (MMPs) and urokinase Plasminogen Activators (uPA) are positive regulation of angiogenesis (Gasparini, 2001). On the other hand, antiangiogenic factors, such as Trombospondin-1 (TSP-1), angiostatin, endostatin, IL-12, Plasminogen Activator Inhibitor (PAI) and Tissue Metalloproteinases Inhibitors (TIMPs) are negative regulators of angiogenesis and they counteract the action of angiogenic factors, in normal situations leading to a balance between angiogenesis and anti-angiogenesis (Folkman, 1995). Balance between angiogenesis and anti-angiogenesis can shift towards angiogenesis after tumour growth

Table 4
Levels of plasminogen activators and inhibitors in the study subjects

Parameters	Group I	Group II	Group III	Group IV	Group V
	Control, normal age-matched, postmenopausal women (42)	Pretreatment untreated breast cancer women (84)	Treatment with tamoxifen (84)	45 days after treatment with CoQ_{10}, riboflavin and niacin along with tamoxifen (84)	90 days after treatment with CoQ_{10}, riboflavin and niacin along with tamoxifen (84)
uPA (μg/l)	0.38 ± 0.08	$1.82 \pm 0.09^{a***}$	$1.2 \pm 0.07^{b***,cNS}$	$0.52 \pm 0.07^{d***}$	$0.51 \pm 0.09^{e***,fNS}$
tPA (μg/l)	2.2 ± 0.73	$6.4 \pm 1.2^{a***}$	$5.7 \pm 1.42^{b***,cNS}$	$3.2 \pm 1.31^{d***}$	$2.9 \pm 1.11^{e***,fNS}$
PAI-1 (μg/l)	3.6 ± 1.2	$5.8 \pm 1.72^{a***}$	$6.9 \pm 1.97^{b***,cNS}$	$13.8 \pm 3.6^{d***}$	$15.3 \pm 4.7^{e***,f***}$
PAI-2 (μg/l)	2.23 ± 0.58	$16.2 \pm 2.6^{a***}$	$18.3 \pm 5.5^{b***,cNS}$	$25.8 \pm 6.1^{d***}$	$29.3 \pm 8.2^{e***,f***}$
SuPAR (ng/ml)	2.3 ± 0.5	$7.2 \pm 1.83^{a***}$	$5.8 \pm 1.24^{b***,cNS}$	$3.6 \pm 1^{d***}$	$3.2 \pm 0.8^{e***,f**}$

Values are expressed as mean ± SD. Number of subjects are indicated in parentheses.
Comparisons were made between: a – Group I and Group II; b – Group I and III; c – Group II and III; d – Group III and IV; e – Group III and V; f – Group I and V. Statistical significance expressed as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – Non Significant.

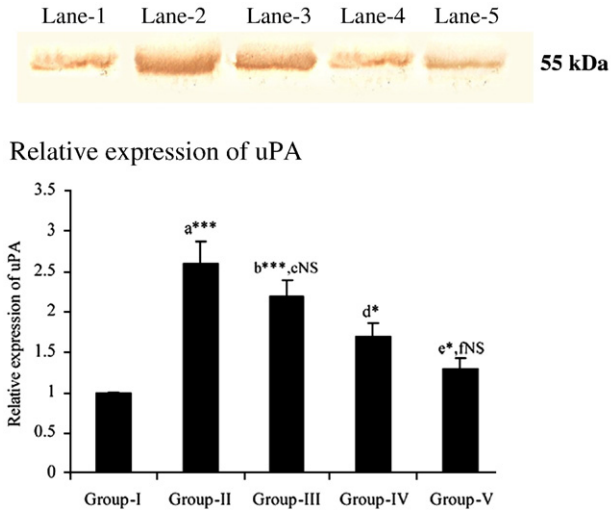


Plate 6. Protein expression of uPA in study subjects. Lane-1: Gp-I control subjects; Lane-2: Gp-II untreated patients; Lane-3: Gp-III TAM alone treated; Lane-4: Gp-IV TAM + CoRN treated (45d); Lane-5: Gp-V TAM + CoRN treated (90d). Relative expression of uPA. Values are expressed as Mean ± S.D. Comparisons were made between a: group I and II; b: group I and III; c: group II and III; d: group III and IV; e: group II and V; f: group I and V. Statistical significance expressed as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – Non significant.

(Potgens et al., 1995). Angiogenic switch is induced by release of specific growth factors produced by tumour or stromal cells (Gasparini, 2001). Only some clones of a primary tumour become angiogenic. In fact, angiogenic activity of primary solid tumours presents intra and inter-individual heterogeneity (Gasparini, 1995).

In an earlier study, we have reported that supplementing CoRN to breast cancer patients reduced the serum tumour marker levels of CEA and CA 15-3 (Premkumar et al., 2007b) and regulated the level of inflammatory cytokine levels of IL-1β, IL-6, IL-8, TNF-α and VEGF (Premkumar et al., 2007a). In this study, supplementing CoRN to breast cancer patients has found to decrease the levels of pro-angiogenic factors and increase the levels of anti-angiogenic factors. A reduction in pro-angiogenic levels attributes to reduction in tumour burden (Kozlowski et al., 2003). CoRN supplementation downregulates the serum levels of interleukins IL-1β, IL-6, IL-8, TNF-α and VEGF (Premkumar et al., 2007a), which could possibly downregulate the expression and secretion of pro-angiogenic markers in breast cancer patients supplemented with CoRN. To substantiate this hypothesis, we measured the levels of both pro-angiogenic and anti-angiogenic markers in breast cancer patients supplemented with CoRN for 45 and 90 days along with TAM.

CoRN supplementation downregulates the levels of IL-1β which in turn downregulates the expression of a wide number of angiogenic markers bFGF (Rivera et al., 1994), EGFR (Wan et al., 2001), TGFβ1 (Villiger et al., 1993) including angiogenic cytokine IL-8 (Pantschenko et al., 2003). Takahashi et al. (1999) reported that IL-1 upregulated the expression of COX-1, COX-2, iNOS, EGF, bFGF, HGF and TGFβ1 in gastric ulcer rats and when an IL-1 receptor antagonist (IL-1RA) was used to block the expression of IL-1, there was a significant reduction in mRNA levels of COX-2, iNOS, CINC-1, HGF, and bFGF.

Basic Fibroblast Growth Factor (bFGF) has been shown to stimulate the proliferation of a variety of cells, suggesting a key role of bFGF in wound healing, embryonic development, neuronal regeneration and angiogenesis (Baird and Klagsbrun, 1991; Sliutz et al., 1995). Soluble fragment of EGFR protein (sEGFR) can be detected in the serum of patients with breast cancer and other solid tumours since a 110 kDa portion is shed from the full length 170 kDa protein (Baron et al., 2003). There was a non-significant reduction in EGFR levels in group II patients, which may predict poor prognosis. Investigators have reported that EGFR over-expression may predict poor prognosis and resistance to hormone therapy (Fontanini et al., 1995; Mukaida et al., 1991; Neal et al., 1985), which requires an effective combination therapy to reduce the levels of pro-angiogenic EGFR. There was a significant reduction in EGFR levels in group IV and V patients. Our finding is in line with a study of Gregorc and coworkers, who found a correlation of a higher response rate associated with decrease in sEGFR levels during treatment in patients with cancer (Gregorc et al., 2004). The development of EGFR antagonists represents a promising novel anticancer therapeutic approach.

HGF can promote cell motility, invasiveness and metastatic phenotype in a variety of tumour cells. In fact, the removal of the primary tumour clearly decreased the serum HGF level in primary breast cancer patients (Nakamura et al., 1984). Taniguchi et al. (1995) reported from their study in 134 breast cancer patients, that more than one third of primary breast cancer patients and more than 80% of those with recurrent disease had a significant increase in the circulating level of HGF. The increase in the serum HGF level was significantly associated with markers representing the aggressiveness of the primary tumour. Furthermore, the serum HGF level was frequently increased in breast cancer patients with recurrent disease. This also strongly suggests a close correlation between the appearance of HGF in sera and tumour progression and the significant increase in group III patients, which may be associated with occurrence of relapse. The significant suppression of HGF levels in group IV and V patients are indicative of good prognosis. Several growth factors or cytokines including TNF-α, IL-1, and TGF-β are known to be responsible for the production of HGF in stromal cells (Tamura et al., 1993). In addition, recent studies underlined the importance of release and activation mechanism of HGF from its extracellular matrix-bound forms (Naldini et al., 1992; Masumoto and Yamamoto, 1993).

Increased *COX-2* transcription results in enhanced production of Prostaglandins (PGs), including PGE_2 (Dannenberg et al., 2005). PGE_2 is found to activate the expression of multiple angiogenic mediators like EGFR, VEGF and bFGF expression (Sakai et al., 2001; Joyce and Meklir,

Table 5
Levels of adhesion molecules, elastin peptides, BSP and OPN in the study subjects

Parameters (ng/ml)	Group I	Group II	Group III	Group IV	Group V
	Control, normal age-matched, postmenopausal women (42)	Pretreatment untreated breast cancer women (84)	Treatment with tamoxifen (84)	45 days after treatment with CoQ_{10}, riboflavin and niacin along with tamoxifen (84)	90 days after treatment with CoQ_{10}, riboflavin and niacin along with tamoxifen (84)
E-Selectin (ng/ml)	20.2 ± 5.8	$88.3 \pm 20.8^{a***}$	$74.2 \pm 17.4^{b***,c*}$	$39.6 \pm 10.3^{d**}$	$35.3 \pm 8.6^{e**,fNS}$
ICAM-1 (ng/ml)	142.3 ± 32.7	$486.7 \pm 123.3^{a***}$	$348.8 \pm 74.3^{b***,c*}$	$208.8 \pm 56.7^{d**}$	$182.3 \pm 49.2^{e**,f**}$
VCAM-1 (ng/ml)	470.2 ± 111.3	$988.6 \pm 210.6^{a***}$	$782.3 \pm 188.6^{b**,c*}$	$593.7 \pm 162.9^{d**}$	$557.8 \pm 148.6^{e**,fNS}$
Elastin peptide (U/L)	4.8 ± 1.1	$19.6 \pm 5.3^{a***}$	$14.2 \pm 3.4^{b***,c*}$	$8.4 \pm 2.1^{d***}$	$6.3 \pm 1.7^{e***,fNS}$
BSP (ng/ml)	128.5 ± 22.6	$613.2 \pm 88.8^{a***}$	$416.6 \pm 99.2^{b***,c**}$	$322.7 \pm 87.3^{d*}$	$271.3 \pm 81.3^{e**,f***}$
OPN (ng/ml)	342.3 ± 78.2	$972.8 \pm 179.3^{a***}$	$632.7 \pm 111.9^{b***,c**}$	$480.1 \pm 101.7^{d*}$	$454.3 \pm 97.5^{e**,f*}$

Values are expressed as mean ± SD. Number of subjects are indicated in parentheses.
Comparisons were made between: a – Group I and Group II; b – Group I and III; c – Group II and III; d – Group III and IV; e – Group III and V; f – Group I and V. Statistical significance expressed as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; NS – Non Significant.

1994). Significant reduction ($p<0.05$) in PGE_2 levels were found in Group IV and V patients. Hence, blocking PGE_2 expression would potentiate the anti-angiogenic effect in cancer patients. dThdpase levels in group III patients had no significant change compared to Group II patients, which shows that TAM administration does not alter dThdpase levels, the results are in line with earlier reports which have shown that TAM administration upregulates the dThdpase expression. Though, the upregulation of dThdpase in breast cancer cell lines can enhance the chemotherapeutic anti-cancer drug activity of doxifluridine, capecitabine, and/or methotrexate (Shintani et al., 2004), TAM also enhances the expression of dThdpase in endometrium increasing the risk of developing endometrial hyperplasia, polyps and carcinoma (Nishida et al., 1996). In Group IV and V patients supplemented with CoRN for 45 days and 90 days respectively there was a significant decrease ($p<0.01$) in dThdpase levels compared to Group III patients. Several types of cytokines such as IL-1, TNF-α and IFN-γ also upregulated the expression of dThdpase in both malignant and nonmalignant cells (Eda et al., 1993).

Endostatin has been shown to upregulate thrombospondin, another major endogenous angiogenesis inhibitor known to be suppressed during the angiogenic switch (Abdollahi et al., 2004). The ability of endostatin to inhibit tumour growth and angiogenesis *in vivo* is demonstrated by extensive studies using animal models (Li and Olsen, 2004). The levels of serum endostatin were tabulated in Table 2, in Group III patients treated with TAM there was no significant change in levels of serum endostatin levels compared to Group II patients. The result has been found to be contrary to an earlier study report by Kuroi et al. (2001), who reported, that administration of TAM to cancer patients has been found to increase the levels of endostatin in plasma. Levels of Endostatin in group IV and V patients were significantly increased. Earlier reports have shown that an increase of circulating endostatin of about one-third of the normal serum levels may represent an effective therapeutic dose to inhibit many solid tumours (Zorick et al., 2001).

The serum concentration and mRNA expression levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 in TAM treated group III patients were not reduced and the results are in line with earlier reports of Nilsson et al. (2007), who reported that TAM exposure increased both intracellular and extracellular protein levels of MMP-9 compared to untreated controls and the levels of TIMP-1 and TIMP-2 remained unchanged after TAM therapy. The levels of MMP-2 and MMP-9 were decreased and TIMP-1 and TIMP-2 increased on CoRN supplementation, which shows the beneficial effect of CoRN supplementation to TAM therapy. It has also been suggested that patient outcome may depend on the balance between MMPs and their tissue inhibitors, for instance between MMP-2 and TIMP-2 in breast cancer (Nakopoulou et al., 2003). MMPs regulate cancer-cell growth, differentiation, apoptosis, tumour angiogenesis and immune surveillance, suggesting that MMPs may also affect earlier stages of tumour progression. MMPs have been found to promote angiogenesis by degrading the ECM, allowing endothelial cells to invade the tumour stroma. However, several MMPs have been shown to indirectly regulate angiogenesis by releasing membrane-sequestered pro-angiogenic factors including VEGF, bFGF, and TGF-β (Kalluri, 2003). MMPs expression and secretion is regulated by cytokines and if there are ways by which the downregulation of cytokines could lead to suppression of MMPs production, this would have biological consequences that could benefit the host. Hagemann et al. (2004) reported that MMP induction was dependent on upregulation of TNF-α, which was produced by the macrophages in response to co-cultivation with tumour cells. Addition of a neutralizing TNF-α antibody downregulated increased invasiveness of the co-cultivated tumour cells as well as the expression of MMP-2 and -9 mRNA in the macrophages. TNF-α release was reduced as a consequence of MMP inhibition. IL-1β upregulated the expression of MMP-2 in cardiac microvascular endothelial cells (Mountain et al., 2007) and MMP-9 in cultured mesangial cells (Yokoo and Kitamura, 1996). Xue et al. (2003) reported that administration of anti-IL-1β decreased significantly the levels of MMP-2 and MMP-9.

The levels of uPA, tPA and suPAR were found to be increased in group II and III patients suggesting that plasminogen activators has reached the blood stream. uPA can reach the blood stream through release from activated macrophages and tPA is released by the tumour vasculature. Peripheral blood levels of uPA have been reported to be increased in patients with carcinomas of the breast, uterine, cervix and endometricum. High serum levels of suPAR were associated with shorter survival rates in breast cancer patients (Grondahl-Hansen et al., 1995). The levels of PAs and suPAR were decreased and the levels of PAI's were increased after CoRN supplementation, which proves the beneficial effect of CoRN supplementation. The beneficial effect may be due to CoRN mediated downregulation of IL-1β. IL-1β has been reported to regulate the expression and secretion of plasminogen activator system during embryo implantation (Chung et al., 2001).

The levels of E-Selectin, ICAM and VCAM were found to be elevated in cancer conditions and in patients with poor prognosis to cancer (Gearing et al., 1992; Zhang and Adachi, 1999; Hamazaki et al., 1996). There was a significant decrease ($p<0.01$) in Group IV and Group V patients treated with CoRN when compared with Group III patients. A reduction in Adhesion molecules is indicative of beneficial prognostic effect of the drug. The effect of CoRN on downregulation of adhesion molecules may be due to the effect of inflammatory cytokines IL-1, TNF-α and IFN-γ which downregulates the expression of these adhesion molecules (O'Hanlon et al., 2002). The levels of elastin peptides were found to be significantly decreased in CoRN supplemented patients, which may be due to downregulation of MMP production in these patients. MMP-mediated elastin degradation is the initial step in elastin calcification in the rat subdermal implantation model and that inhibition of MMPs leads to significant reduction in calcification (Vyavahare et al., 2000; Bailey et al., 2003). MMP-9 and MMP-2 bind and degrade insoluble elastin to generate soluble peptides (Mecham et al., 1997). These elastin peptides can interact with a 67-kd transmembrane protein, the elastin laminin receptor (ELR), (Hinek et al., 1988) which is present on the surface of most cells. Activation of ELR by elastin peptides triggers diverse biological activities in various cell types including synthesis and release of elastase, liberation of free radicals, increased Ca^{2+} influx in endothelial cells, NO-dependent vasorelaxation, proliferation of arterial smooth muscle cells, chemotaxis of monocytes and fibroblasts, and apoptosis. (Faury et al., 1998; Hinek, 1996; Mochizuki et al., 2002). BSP and OPN are secreted proteins which are associated with poor survival in breast cancer and with bone metastases development. Decrease in serum BSP and OPN levels is indicative of good treatment prognosis, as both these markers are indicative of metastases and bone invasion, the risk of developing metastases in Group IV and V patients treated with CoRN is reduced.

5. Conclusion

Significant increase in pro-angiogenic levels found in untreated breast cancer patients were found to be reduced in TAM administered patients, but a significant decrease in pro-angiogenic marker and increase in anti-angiogenic marker levels were found in TAM co-administered with $CoenzymeQ_{10}$, riboflavin and niacin patients. Study proves the beneficial effect of CoRN supplementation to breast cancer patients undergoing TAM therapy, as a reduction in pro-angiogenic levels attributes to reduction in tumour burden and may suggest good prognosis and efficacy of the treatment, and might even offer protection from metastases and recurrence of cancer.

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