



Genetic Predisposition of PON1 L55M (T172A) and Oxidative Stress in Breast Carcinogenesis among Pakistani Population

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ABSTRACT

Breast cancer has become a global health issue across the world including Pakistan. In 2020, an estimated five-year prevalence of breast cancer is 30.1% depicting the major cause of socio-economic burden in our country. Owing to the fact that oxidative stress plays a crucial role in breast cancer progression, this study aimed to inspect the role of exonic region variant PON1 L55M and the effect of PON1 enzyme activity in accelerating the disease among Pakistani females. In this population-based case-control study n=222 females were included. PON1 L55M (T172A) genetic variation was determined by means of tetra-ARMS PCR. PON1 paraoxonase and arylesterase enzyme activities and levels of lipid peroxidation marker were assessed to figure out the level of oxidative stress. The odds ratio was calculated using MedCalc® software and gene counting. SPSS® 16.0 was used to perform Chi-square test, independent t-test and Pearson correlation. In our results, we found that genetic variant PON1 55M (172A) was significantly ($p<0.001$) correlated with an increased risk of breast cancer. PON1 activities were found to be decreased ($p<0.001$) with increased MDA levels ($p<0.001$) in the diseased group. Pearson correlation indicated an inverse correlation ($r = -0.1215$, $p<0.01$) between PON1 enzyme activity and MDA levels. In breast cancer patients with 55M allele, activity of PON1 was found to be decreased ($p<0.001$). It is concluded that increased levels of MDA with a concomitant decrease in PON1 activity could be responsible for disease acceleration via increased oxidative burden. Reduced PON1 activity in breast cancer patients with PON1 L55M variant might be a contributing factor to the disease progression.

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Authors' Contribution

SAZJ performed the study and collected data. TA contributed to data analysis. MZ contributed to sample preparation. GH provided samples and responsible for diagnosis of breast cancer patients. ZH is a guarantor, conceived the idea and finalized the draft.

Key words

Breast cancer, Paraoxonase1, Oxidative stress, Gene polymorphism, Lipid peroxidation, Tetra-ARMS PCR

INTRODUCTION

Breast cancer is the utmost prevailing cancer and more frequent cause of mortality in females around the world. An estimated rate of breast cancer incidence and mortality in 2020 were 24.5% and 15.5% around the globe respectively (Ferlay *et al.*, 2021). Pakistan has reported the highest breast carcinoma cases in Asian population (Noreen *et al.*, 2015). As reported by global cancer observatory, the expected five-year prevalence of breast malignancy in Pakistan is 30.1 % (Globocan, 2020).

Majority of the females are diagnosed with this deadly disease at later stage with poor prognosis (Wang *et al.*, 2019). Risk factors contributing to the disease are mainly age, genealogy, menstruation before time, hormonal therapies, drinking alcohol, physical inactivity, socioeconomic burden, poor knowledge of the disease and oxidative stress (Asif *et al.*, 2014; Jelic *et al.*, 2021).

Oxidative stress is one of the etiological factors that have been associated with greater risk of breast carcinoma (Taler-Verčič *et al.*, 2020). Overproduction of oxidants which damage biomolecules and reducing antioxidant capacity needed to sustain normal physiological functions are the main causative agents that cause oxidative stress (Voronkova *et al.*, 2018). Antioxidants are scavengers that encounter free radicals to balance the oxidation-reduction status of the cell (Gupta *et al.*, 2012). There are several antioxidant enzymes present in a living system including glutathione peroxidase, glutathione reductase, glutathione S-transferase, superoxide dismutase, catalase, paraoxonase etc. (Abid *et al.*, 2022; Asaduzzaman *et al.*, 2010). Paraoxonase (PON) has three members including

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PON1, PON2 and PON3. The enzyme PON1 distributed comprehensively in liver, kidney, intestine and blood circulation. Subsequently, PON2 is absent in serum while PON3 is present in a very min quantity (Baig *et al.*, 2016). It has been identified that PON1 has a potential role in detoxifying carcinogenic free radicals (Pan *et al.*, 2019).

PON1 gene is localized on chromosome 7 (7q21.3) and comprised of 9 exons which codes information for PON1 protein synthesis (Taler-Verčič *et al.*, 2020). Several single-nucleotide polymorphisms (SNPs) were recognized in *PON1* gene; some of them are responsible for its functionality. The most frequently studied SNPs in coding region are *Q192R* and *L55M*, whereas SNP *C108T* found in promoter region of the gene (Mackness and Mackness, 2015). Transmutation of single nucleotide resulting in swapping of amino acid from glutamine to arginine positioned 192 and leucine with methionine positioned 55. These *Q192R* and *L55M* polymorphic sites are appeared to have a critical role in gene regulation and effect on the phenotypic expression among distinctive groups (Pan *et al.*, 2019). PON1 enzyme activity has been found to be decreased in multiple cancers including breast cancer (Balci *et al.*, 2012).

It has been reported that PON1 has a significant effect in reducing oxidative stress in various clinical conditions (Kotur-Stevuljević *et al.*, 2020). Reactive oxygen species (ROS) are produced persistently during increased oxidative burden that causes oxidation of cellular material including lipids and hence cause elevated levels of lipid peroxidation (Phaniendra *et al.*, 2015). Lipid peroxidation is an indicator of oxidative stress and a free radical-mediated cellular injury that results in formation of lipid peroxides that are derivatives of polyunsaturated fatty acids. These lipid peroxides are less stable and readily decomposes to build reactive carbonyl compounds such as malondialdehyde (MDA). Therefore, MDA is employed as an oxidative stress marker (De Leon and Borges, 2020; Gianazza *et al.*, 2021). Owing to the fact that being HDL (high density lipoprotein) associated enzyme, PON1 has potential to detoxify lipid peroxides which plays defensive role against lipid peroxidation (Jeelani *et al.*, 2020). Hence, evaluation of PON1 is crucial to understand its association in susceptibility and progression of breast cancer (Pan *et al.*, 2019).

The goal of current research is to evaluate the role of *PON1 L55M* polymorphism and oxidative stress with increased risk of breast cancer. Present case-control study investigates PON1 activity and MDA levels simultaneously to measure redox imbalance. This study also determines the relationship between genetic polymorphism of *PON1 L55M* with the activity of PON1 antioxidant among Pakistani population.

MATERIALS AND METHODS

Study subjects

All participants were female aged between forty to sixty-five years. Blood samples from breast cancer patients (n= 111) and age matched healthy controls (n= 111) were collected from a local hospital of Karachi. Participants were not included in this study if they had prior history of tumor or experienced any comorbidity.

Sample collection

Whole blood sample was collected through venipuncture. Total 5 mL blood sample was drawn in K₂ EDTA coated tube and gel vacutainer for DNA extraction and serum separation, respectively. On centrifugation, serum was separated at 3500 rpm for 5 min.

PON1 L55M (T172A) polymorphism

Genomic DNA was extracted by the protocol described earlier (Kleines *et al.*, 2003). Briefly, RBCs were ruptured from whole blood using red cell lysis buffer containing 0.31M sucrose, 0.01M Tris (pH 7.6), 0.002M MgCl₂ and 0.003M sodium azide. Lysed cells were centrifuged at 10,000 rpm for 1 min followed by resuspension of pellet in cell lysis buffer. Protein was precipitated using 6.4M ammonium acetate solution and precipitated proteins were centrifuged at 14,000 rpm for 5 min followed by supernatant collection. Isopropanol was added into the supernatant and mixed gently till DNA thread appeared. The mixture was then centrifuged at 12,000 rpm for 1 min to pellet down DNA and stored at -70°C till further analysis.

Tetra Primer-Amplification Refractory Mutation System (Tetra-ARMS) PCR was performed to identify *PON1 L55M (T172A)* genetic variation. Two external non-allele specific and two internal allele specific primers were included in the assay. PCR reaction mixture included 1X PCR buffer (2M KCl, 1M Tris pH 8.3, 1M MgCl₂ and 10mM dNTPs), 1U taq polymerase, 10μM each primer and DNA template. Total 35 cycles were executed at annealing temperature 59°C and amplicons were scrutinized using 2% agarose gel. Primer sequences are given in Table I.

Table I. Sequences of primers used for determination of *PON1 L55M (T172A)*.

Primers	For position <i>L55M</i>
FO (Forward outer)	5'-GGCTTTTGTACGTTTGTG-3'
RO (Reverse outer)	5'-CCGAAGAACACAAATATGCA-3'
FI (Forward inner)	5'-CAGAAACTGGCTCTGAAGTCA-3'
RI (Reverse inner)	5'-TCCATTAGGCAGTATCTCGAA-3'

Enzyme activity

PON1 paraoxonase and arylesterase activities were restrained by previously described procedure (Gan *et al.*, 1991). Serum was incubated with reaction mixture containing paraoxon (1.0 mmol/L), CaCl_2 (1.0 mmol/L) and glycine/NaOH buffer (50mmol/L, pH 10.5). Rate of paraoxon substrate hydrolysis and formation of 4-nitrophenol was monitored at 412nm. Activity of enzyme was calculated via molar extinction coefficient $18,290 \text{ M}^{-1}\text{cm}^{-1}$ and results were indicated as U/mL. Activity of PON1 arylesterase was measured by using phenylacetate as substrate. Control and diseased samples were added into the reaction mix containing 20mM TrisHCl pH 8.0, 1mM phenylacetate and 1mM CaCl_2 . Rate of substrate hydrolysis was monitored at 270nm. Molar extinction coefficient for the assay was 1310 /M.cm and enzyme activity was expressed as U/L.

Measurement of lipid peroxidation

To quantify levels of lipid peroxidation, MDA-TBA (thiobarbituric acid) adduct was measured as described earlier (Hashim *et al.*, 2009; Ohkawa *et al.*, 1979). Assay mixture included 20% TCA (trichloroacetic acid), 0.8% TBA, 8.1% SDS and serum sample. This reaction mixture was kept for one hour in boiling water bath, then chilled on ice and n-butanol was subsequently added. Centrifuged at 3500 rpm for 10min. Absorbance of obtained organic layer was taken at 532nm. Levels of MDA was expressed as $\mu\text{M}/\mu\text{L}$ and 1, 1, 3, 3-tetramethoxypropane was employed as standard.

Statistical analysis

Data was analyzed using SPSS® software version 16.0. Chi-square test was employed to assess genotypic and allelic frequencies of PON1 L55M variation among healthy and diseased group. Odds ratio was calculated through gene counting method and MedCalc® software for genotypic analysis. Independent T-test was performed to calculate mean and standard deviation for PON1 enzyme activities and MDA. Pearson correlation (r) was performed to determine relationship between PON1 paraoxonase enzyme activity and MDA levels. Statistical data was considered significant with p-value less than or equals to 0.01.

RESULTS

Figure 1 signifies the results of amplified products of tetra-ARMS PCR for the identification of *PON1* L55M single nucleotide polymorphism in control and breast cancer patients. Genotypic and allelic frequencies for observational groups are illustrated in Figure 2.

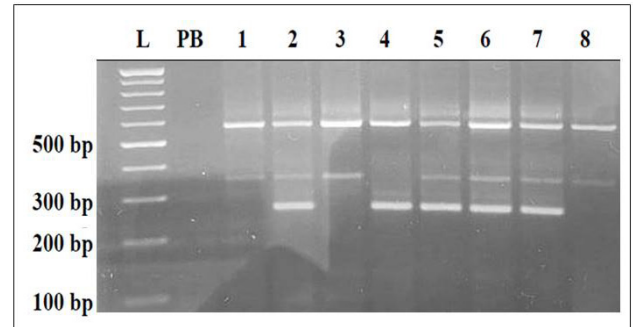


Fig. 1. Agarose gel indicating tetra-ARMS PCR products for *PON1* L55M polymorphism. L: ladder; PB: PCR blank; Lane 1, 3 and 8: homozygous LL (TT); Lane 2, 5, 6 and 7: heterozygous LM (AT); Lane 4: homozygous mutant MM (AA).

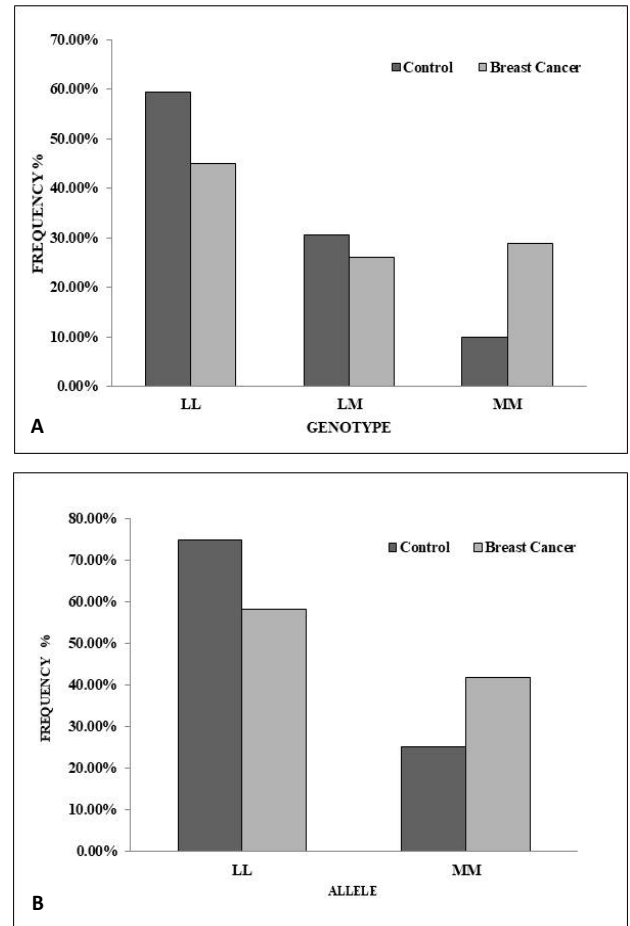


Fig. 2. Frequency of *PON1* L55M genotypes (A) and alleles (B). Bars in A represent LL, LM and MM genotypes in control (n=111) and diseased group (n=111). Bars in B presented L and M alleles in healthy females (n=111) and breast cancer patients (n=111).

Table II. *PON1* L55M (T172A) genotype and allele frequency distribution in control and breast cancer patients (n=111).

55 T----> A	Control	Breast cancer	OR	95% CI	Chi square
Genotypes					
TT (LL)	66 (59.46%)	50 (45.04%)	0.558	0.3282-0.9517	$\chi^2 = 202.92^{***}$
AT (LM)	34 (30.63%)	29 (26.13%)	0.800	0.4462-1.4376	
AA (MM)	11 (9.91%)	32 (28.83%)	3.682	1.7466-7.7635	
Alleles					
TT (LL)	166 (74.77%)	129 (58.11%)	0.467	0.3126-0.7005	
AA (MM)	56 (25.23%)	93 (41.89%)	2.137	1.4275-3.1993	

OR, odds ratio; CI, confidence Interval; *, statistically significant (**p < 0.001).

Genotypic frequency of LL (TT) homozygote was 66/111 (59.46%) and 50/111 (45.04%) in control and disease participants, respectively. Percent frequency of LM (AT) heterozygote was 34/111 (30.63%) and 29/111 (26.13%). Whereas, mutant homozygote MM (AA) for above mentioned groups were 11/111 (9.91%) and 32 (28.83%), respectively.

The frequency of mutant M (A) allele was found to be significantly higher ($p < 0.001$) in diseased group (41.89%) as compared to control group (25.23%). With respect to risk of breast cancer development, wild type 55L (T) allele was taken as standard. The obtained data represents that 55M (A) allele was significantly correlated with an elevated risk of disease (OR= 2.137, 95% CI= 1.4275-3.1993, $p < 0.001$). Genotype and allele frequency distribution of PON1 L55M polymorphism are presented in Table II.

PON1 paraoxonase and arylesterase activities and levels of MDA are presented in Figure 3. PON1 activity towards paraoxon was found significantly lower ($p < 0.001$) in serum samples of breast carcinoma patients versus healthy subjects. Similar trend of result was obtained towards substrate phenylacetate. However, in comparison with control group, significantly higher levels of MDA were found in diseased samples ($p < 0.001$).

In disease and control group, decreased PON1 enzyme activity was measured with concomitant increased in MDA levels significantly ($p < 0.01$). Pearson correlation determined that there was an inverse correlation ($r^2=0.243$, $r=-0.1215$) between PON1 enzyme activity and MDA levels in both study subjects as shown in Figure 4.

Activity of Paraoxonase was found to be significantly ($p < 0.001$) decreased in breast carcinoma patients with increased risk allele (M) and in contrast to healthy group with decreased M allele. Similarly significant relationship ($p < 0.001$) was obtained between L allele and increased enzyme activity in control group as compared to breast cancer patients (Fig. 5).

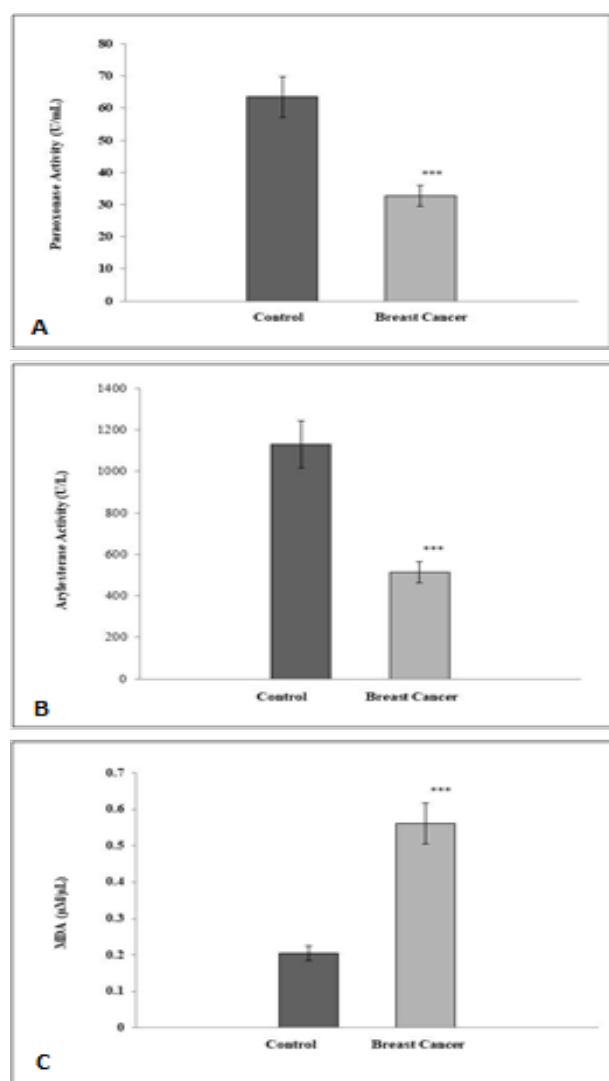


Fig. 3. Paraoxonase (A) and arylesterase (B) activities, and MDA levels (C) in healthy and diseased subjects. Bars representing mean $\pm$ SD of experimental groups (n=111). Statistical significance ***p < 0.001.

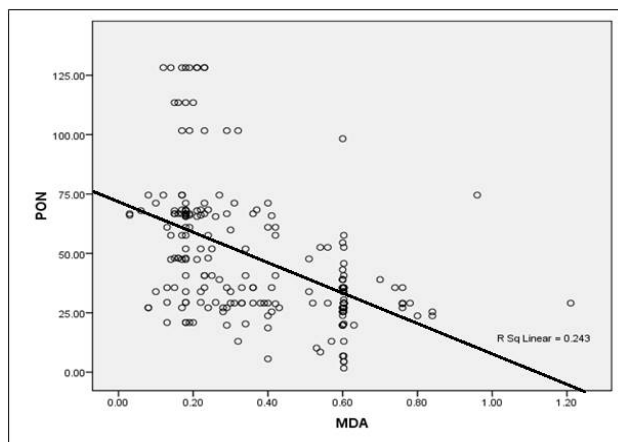


Fig. 4. Inverse correlation between PON1 enzyme activity and MDA levels ($r=-0.121$ and $**p < 0.01$) was considered statistically significant.

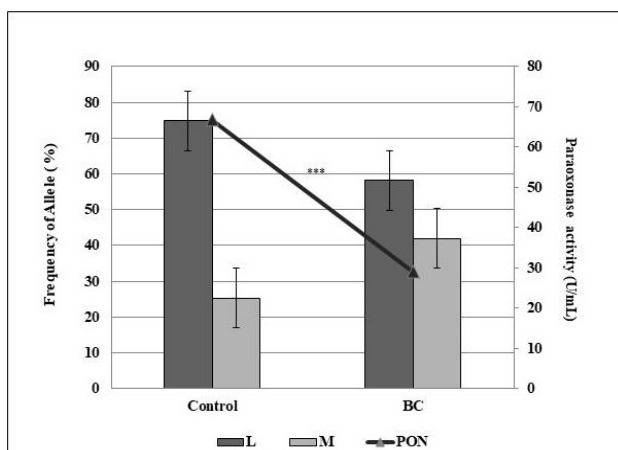


Fig. 5. Interrelation between allelic frequencies and PON1 activity in diseased and normal groups. X-axis represents study groups. Primary Y-axis represents L and M allele frequencies (%). Whereas, Secondary Y-axis represents antioxidant activity (U/mL). Statistical significance= $***p < 0.001$.

DISCUSSION

Breast cancer is the most common malignancy in women worldwide as the aetiopathogenesis of disease is not fully understood. Oxidative stress seems to appear as a major risk factor involved in the disease progression and its advancements (Hecht *et al.*, 2016; Wawruszak *et al.*, 2021). In current study, we determined the relationship of *PON1* L55M polymorphism with elevated risk of breast malignancy. Our results showed significant association ($p < 0.001$) between MM genotype, mutant M allele and breast cancer risk. Current investigations also determine

that PON1 enzyme activities are significantly ($p < 0.001$) reduced in cancerous females in contrast with healthy females. These results intimate the key role of PON1 in breast illness. It has also been demonstrated that the role of genetic variant 55M in breast cancer patients and control group among Iranian women. Their findings proposed that 55M variant is a genetic risk factor for onset of the disease and due to variation in the exonic region it could also affect PON1 enzyme structure and function (Farmohammadi *et al.*, 2020). It has been reported previously that women with MM genotype and M allele are more at risk of developing breast cancer (Hu *et al.*, 2016; Pan *et al.*, 2019). Moreover, it was also suggested that individuals who had only one M allele have an elevated risk of getting malignant breast signifying that even a single alteration is important that can change PON1 enzyme activity (Naidu *et al.*, 2010; Tolooi, 2021; Wu *et al.*, 2017).

Paraoxonase-1, a lipolactonase antioxidant is known to play a role in scavenging carcinogenic free radicals to maintain oxidative burden incriminated in the advancement of multiple cancers (Arenas *et al.*, 2018; Kaya *et al.*, 2016). Researchers demonstrated that reduced PON1 activity is a consequence of excessive production of ROS causing increased oxidative stress involved in carcinogenesis (Arenas *et al.*, 2018; Balci *et al.*, 2012). In addition, suppressed ability of antioxidant detoxification is might be due to alteration in the coding region of PON1 gene (Saadat, 2012; Wen *et al.*, 2015). Furthermore, we have determined relationship between allelic frequencies and PON1 activity. It was found that there is a significant ($p < 0.001$) decrease in PON1 activity in patients with 55M allele. Similar trend of results were observed by Kaya *et al.* suggesting, genetic vulnerability in PON1 gene might be correlated with PON1 activity among Turkish women (Kaya *et al.*, 2016). Eraldemir and co-investigators suggested that lower PON1 activity accompanied by MM genotype may increase susceptibility of breast cancer to genetic deterioration by reduction in capability to counteract pro-oxidants and nutritive carcinogens (Eraldemir *et al.*, 2019).

Under normal physiological conditions ROS are produced at a basal level persistently while, increased production might have deleterious effects. Excessive ROS not only interact and cause damage to biological material but also affect redox status of the cell. Oxidative imbalance begins due to higher ROS production with low antioxidant defense capacity (Georgieva *et al.*, 2017). PON1, being HDL linked antioxidant has a crucial effect on lipid peroxidation. In current study we measured MDA as lipid peroxidation marker in serum samples from breast cancer patients and healthy controls. We found significant increase ($p < 0.001$) in MDA levels in patients group set against

controls. These results are validated by lower Paraoxonase and arylesterase activities in breast cancer patients. Previous studies also provide evidences that MDA levels are increased in disease conditions due to excessive ROS production ultimately causing higher lipid peroxidation hence reducing antioxidant capacity (Hashim *et al.*, 2009; Kangari *et al.*, 2018). We also found significant ($p < 0.01$) inverse correlation between MDA and PON1 activity in both groups. It has been reported that reduction in PON1 enzyme concentrations are might be due to insufficient HDL extent in patient's serum as opposed to age and sex matched healthy controls (Mogarekar *et al.*, 2019).

CONCLUSION

In conclusion, the current study demonstrated a strong relationship between *PON1 L55M* genetic variations with its reduced antioxidant defense capability. Furthermore, decreased PON1 activity with concomitant increased in MDA levels indicating involvement of oxidative stress in breast cancer progression. Our results provide preliminary information which if further investigated may shed some light to understand the functions of antioxidants in breast carcinogenesis.

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IRB approval and ethical approval

Prior to study for the sample collection, ethical approval was taken from Institutional Bioethics Committee (IBC), University of Karachi (IBC# KU-56/2018) and informed consent forms were filled by each participant. All procedures were carried out according to Helsinki declaration 1964 and later amendments.

Statement of conflict of interest

The authors have declared no conflict of interest.

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