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Involvement of Total Antioxidant Activity and *eNOS* Gene rs1799983/rs2070744 Polymorphisms in Breast Carcinogenesis

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Abstract

Globally, breast cancer is the common malignancy affecting women and understanding its associated molecular events could help in disease prevention and management strategies. The present study was set to investigate an association between total antioxidant capacity (TAC) and endothelial nitric oxide synthase (*eNOS*) polymorphisms with breast cancer. For this purpose, 100 subjects were participated in this work, including 50 female patients diagnosed with breast cancer recruited from Oncology hospital, Baghdad - Iraq and 50 healthy women as a control group. The concentration of antioxidants was measured in the serums collected from blood samples of breast cancer patients and healthy controls. While *eNOS* SNPs (rs1799983, G894T and rs2070744, T 786C) were assessed using TaqMan SNP genotyping and utilising genomic DNA extracted from the participants. The results showed that the antioxidants levels were significantly ($P < 0.0001$) reduced in blood samples of breast cancer patients in comparison to that of that healthy controls (0.144 ± 0.097 and 0.587 ± 0.239 respectively). Additionally, the homozygous GG genotype G894T (rs1799983) could retain beneficial impact for the protection from breast cancer potential. While SNP genotyping results showed that both of the homozygous CC and heterozygous TC genotypes (rs2070744 T > C SNP) seem to contribute to the susceptibility of breast cancer development in the investigated set of patients. Overall, the present study findings suggest an association between reduced antioxidant capacity and *eNOS* gene polymorphisms in breast carcinogenesis.

Keywords: Antioxidants, *eNOS*, SNPs, Breast cancer.

اشترك فاعلية مضادات الأكسدة الكلية و ظاهرة تعدد الاشكال لجين *eNOS* (rs2070744 , rs1799983) في تسرطن الثدي

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الخلاصة

على الصعيد العالمي ، يعد سرطان الثدي هو الورم الأكثر شيوعًا الذي يصيب النساء ، ويمكن أن يساعد فهم الأحداث الجزيئية المرتبطة به في الوقاية من المرض واستراتيجيات إدارته. وعلى هذا الأساس ، فقد تم إعداد

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الدراسة الحالية للتحقيق في العلاقة بين السعة الكلية لمضادات الأكسدة TAC وتعدد الأشكال لجين *eNOS* مع سرطان الثدي. تضمنت الدراسة مشاركة 100 امرأة، بما في ذلك 50 مريضة تم تشخيصهن بالسرطان في مستشفى الأورام، بغداد - العراق و 50 امرأة من الأصحاء ظاهرياً كمجموعة سيطرة. تم قياس تركيز مضادات الأكسدة في الدم عن طريق الكشف عن مستوى إجمالي مضادات الأكسدة في العينات التي تم جمعها لمريضات سرطان الثدي ومجموعة السيطرة. بينما تم تقييم تعدد الأشكال ((rs1799983 *eNOS* SNPs و G894T و rs2070744، T 786C باستخدام الترميز الجيني TaqMan SNP. أظهرت النتائج أن مستويات مضادات الأكسدة انخفضت معنوياً ($P < 0.0001$) في عينات دم مريضات سرطان الثدي مقارنة بتلك الموجودة في مجموعة السيطرة (0.097 ± 0.144 و 0.239 ± 0.587 على التوالي). بالإضافة إلى ذلك، بينت النتائج أن النمط الجيني (rs1799983 G894T) متماثل الزيجوت يمكن ان يكون له تأثير مفيد للحماية من احتمالية الإصابة بسرطان الثدي. بينما يبدو أن كلا من الأنماط الجينية CC متماثلة الزيجوت و TC متغايرة الزيجوت (rs2070744 T > C SNP) تساهم في قابلية تطور سرطان الثدي في مجموعة المرضى التي تم فحصها. بشكل عام، تشير نتائج الدراسة الحالية إلى وجود ارتباط بين انخفاض قدرة مضادات الأكسدة وتعدد الأشكال الجينية لـ *eNOS* في سرطان الثدي.

Introduction

Breast cancer is a life threatening malignancy that affects one in every eight women worldwide as stated by recent epidemiological statistics [1]. The high predicted global cancer statistics (12.5%) has put breast cancer as the second leading cause of cancer mortality in women with approximately 700,000 deaths in 2020 [2]. This emphasizes that much more efforts need to be allocated to understand the molecular events associated with breast cancer initiation and progression. The identified disease-associated biological changes could be assessed further for their potential to improve current patients' risk stratification, disease management and the development of novel therapeutics targets [3, 4].

Total antioxidant capacity (TAC) is an indicator of the quantity of free radicals scavenged by a biological sample. It is well-established that reactive oxygen, which are a subset of free radicals, and nitrogen species are responsible for DNA damage that contribute to genomic instability which is a hallmark of carcinogenesis [5]. Serum TAC is an oxidative DNA damage biomarker evaluated in different neoplastic conditions. Several lines of evidence have suggested that TAC levels are negatively associated with different types of cancers [6-8].

Endothelial nitric oxide synthase (*eNOS*) is an enzyme encoded by *NOS3* gene that maps to the 7q35-7q36 region on chromosome 7 and has more than 168 polymorphisms. A number of *eNOS* polymorphisms have been linked to the development of several solid malignancies, however, the reported findings have been broadly inconsistent [9-11]. *eNOS* catalyses the production of nitric oxide (NO) from L-arginine and molecular oxygen. *eNOS* has been recognized as a key regulator molecule for maintaining endothelial homeostasis and other biological processes [12] and has antioxidant potential and is believed to modulate cancer-related events such as inflammation, apoptosis, cell cycle, invasion and metastasis [13-15]. It is also linked to tumor angiogenesis that supplies a tumor with a supportive microenvironment rich with oxygen and nutrients to sustain its growth [16]. However, its oncogenic impact on endothelial cell proliferation is less clear and studies have even reported its dual role in tumorigenesis represented by either antiapoptotic or proapoptotic influence of the targeted cells [17-19]. This could be due to the nature of NO where its effects seem to be cell type-specific and concentration-dependent. The importance of NO concentration is illustrated by the observation that S-nitroso-N-acetylpencillamine (a NO donor) stimulates proliferation of myoblast cells at low concentrations (1–10 μM), but inhibits the proliferation of the same cell

line at higher concentrations (50 μM) [20]. In this regard, a number of studies have reported that the release of lower *eNOS* levels can promote cancer cells cycle progression and proliferation [21-23]. On the other hand, a plethora of recent evidence has linked *eNOS* up regulation to carcinogenesis [18, 24, 25]. Similarly, studies have shown that direct inducible nitric oxide synthase (*iNOS*) transduction could be cytotoxic and thus leads to inhibit the growth of tumour cells [26]. This finding was further supported by evidence that showed induced tumorigenesis in *iNOS* knockout mice model (*iNOS* $^{-/-}$) [27] indicating that the expression of NO can suppress tumorigenesis. Depending on the cellular milieu, release of low concentrations of NO can stimulate angiogenesis, inhibit apoptosis, or stimulate proliferation and invasion.

Locally in Iraq, breast cancer tops the list of women-effected malignancies and accounts for 22.2% of the diagnosed cases [28]. However, no previous study has investigated its association with *eNOS*. Exploring the potential molecular biomarkers based on abnormal variation in gene expression to prognosticate survival of cancer patients has recently been our field of interest [3, 29-32]. This study was therefore set to investigate *eNOS* genetic polymorphism at the loci of 894G>T (rs1799983) and 786T>C (rs2070744) and to evaluate the contribution of different haplotypes variations on estimated serum levels of TAC of recruited individuals since genetic variants of *eNOS* gene may influence its role in breast carcinogenesis.

Materials and Methods

Subjects and Sampling

A total of hundred peripheral blood samples were collected from 50 breast cancer patients and 50 from apparently healthy women as a control during the period of January 3rd to March 23rd, 2022. The recruited breast cancer patients and their apparently healthy counterparts aged between 30-70 years. Breast cancer patients had been diagnosed by specialist physicians at Baghdad Teaching Hospital and Oncology Hospital, Baghdad, Iraq. Participants' demographic information and clinicopathological data were obtained from patients' hospital records and a questionnaire was prepared for the healthy controls. Informed consent was obtained from all participants enrolled in this study and the Biomedical Research Ethics Committee of the National Cancer Research Center, University of Baghdad approved this study (Reference no. NCRCEC/01/002).

Total Antioxidants Capacity (TAC) Assay

To determine the total antioxidant capacity (TAC) levels in the blood serum of breast cancer patients and their healthy counterparts, serum was separated first by refrigerated centrifugation of the collected blood samples at 3000 rpm for 10 min. TAC was then estimated utilizing Band-Williams modified protocol [33]. This included the mixing of 30 μl of each serum sample with 3000 μl of Diphenyl-1-picrylhydrazyl (DPPH). The mixture was then left to stand in dark place for 30 min. Ascorbic acid (100mg/100ML) was used for standard calibration curve. Ultimately, samples' absorbance was read at 517nm wavelength and TAC concentrations were calculated according to the following equation:

$$TAC (mg/100ML) = (A * Slope)$$

$$A = \text{absorbance}$$

$$Slope = (y_2 - y_1) / (x_2 - x_1)$$

DNA Extraction and *eNOS* SNPs Polymorphisms Genotyping Using TaqMan SNP Genotyping

Genomic DNA extraction from the whole fresh blood samples was directly performed using blood DNA extraction kit, Cat. 46300-Norgen®, Canada, according to the manufacturing protocol. While the sequence of *eNOS* gene SNPs of interest (rs1799983, G894T located to exon 8, and rs2070744, T 786C maps to gene promoter) were amplified using TaqMan fluorescent oligonucleotide primers and probes. The primers and props sequences used in the present study are given in Table 1.

Table 1: Primers and probes sequences used for the detection of *eNOS* gene SNPs (rs1799983 and rs2070744)

SNP	Primers	Sequence	Probes	Sequence
*rs1799983	Forward	CAGGAGACAGTGGATGGAGG	FAM	AGATGATCCCCCAGAACTCTTC
	Reverse	CCCACCCAGTCAATCCCTTT	Vic	ATGAACCCCCAGAACTCTTCC
**rs2070744	Forward	TTTCTCCAGCCCCTCAGATG	FAM	TTCCCTGGCCGGCTG
	Reverse	GCAGAGAGACTAGGGCTGAG	Vic	CCCTGGCTGGCTGACC

*chr7:150998867-150999114, ** Chr7:150992991-150690079

For this assay, DNA samples both for breast cancer patients and healthy control, were genotyped for the above mentioned *eNOS* gene two SNPs. The RT-PCR reaction mixture total volume was 25 µl composed 12.5µl of TaqMan master (PROBE), 0.5µl of each fluorescence probes, 0.5µl from each forward and reverse primer (10µM), 5µl of template DNA, and 5.5µl nuclease-free water. RT-PCR amplification reaction was performed using a programmed thermocycler with one hold cycle of 95°C for 15 min, 45 cycles of 95°C for 5 sec, 60°C for 20 sec and 72°C for 15 sec, followed by one cycle of 72°C for 5 min.

Statistical Analysis

Data analysis was performed using IBM® SPSS® statistical software (Version 27.0; IBM SPSS, Armonk, NY, USA). Spearman's rank was used to assess the correlation between the investigated parameters. While Mann-Whitney-U test was used to compare significance between means. Whereas Chi-square test was used to compare the observed results of the variables in this study. All measures were obtained from three replicates and presented as a mean of values, from which the standard error (SE) of the mean was calculated. Statistical significance was set at $p < 0.05$.

Results and Discussion:

1- Serum total antioxidants capacity levels in breast cancer patients

Breast cancer patients showed to have much lower mean level of serum total antioxidants capacity (0.144 ± 0.097) than that of their healthy counterparts (0.587 ± 0.239). This four folds reduction in the total antioxidants capacity mean level in breast cancer patients in comparison the healthy controls resulted in highly significant difference ($P < 0.0001$) (Figure 1). Such findings suggest an involvement of the lowering levels of total antioxidants capacity in the breast cancer pathogenicity.

Additionally, breast cancer patients with lower histopathological stages (PT1) showed to have comparable TAC mean level to that of patients with higher histopathological stages (PT2) (0.144 ± 0.087 vs. 146 ± 0.087 respectively, $P > 0.05$).

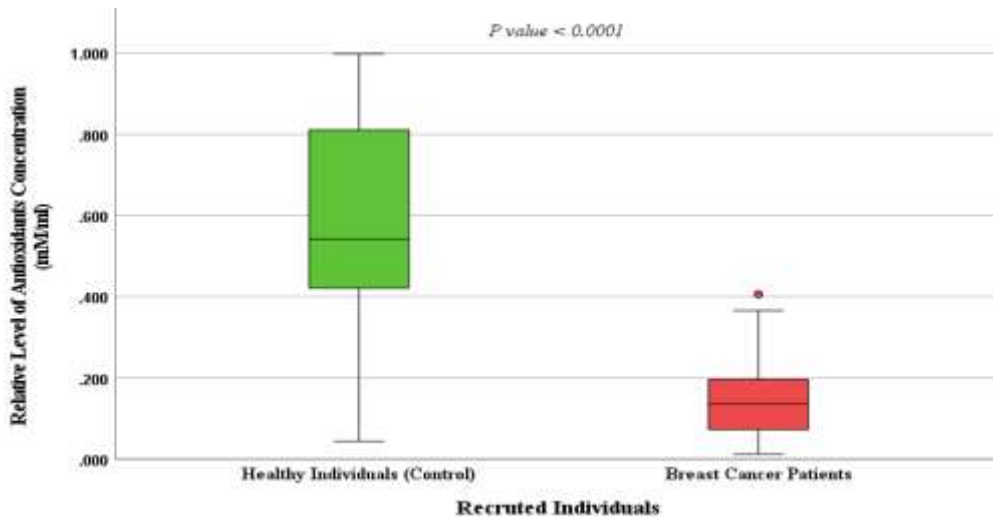


Figure 1: The relative levels of the antioxidants concentrations (assessed by measuring the total antioxidant capacity levels mM/ml) in breast cancer patients and apparently healthy individuals, where the mean of antioxidants levels showed to be significantly reduced in blood samples of patients with breast cancer in comparison to that of that healthy controls (0.144 ± 0.097 and 0.587 ± 0.239 respectively). The p value is derived from Mann-Whitney test.

2- Molecular analysis

rs1799983 G894T Polymorphism:

G894T (rs1799983) *eNOS* genotype distribution and allele frequency analysis were performed to assess the genetic variations in this genomic region in the investigated breast cancer patients and healthy controls. G894T (rs1799983) *eNOS* genotyping analysis showed significantly ($p < 0.01$) lower frequency of the homozygous GG genotype in the assessed breast cancer patients than healthy controls (2% versus 40% respectively, $\chi^2 = 17.19$, $p < 0.01$, Table 2). The present study results suggest that homozygous GG genotype may retain protective value from breast cancer potential.

By contrast, the frequency of the TT genotype in breast cancer patients appeared to be much higher than in the healthy controls (34% versus 4% respectively). This resulted in significant differences between the compared groups in the frequency of this homozygous genotype ($\chi^2 = 11.84$, $p < 0.01$, OR = 2.67, CI = 1.16-4.09, Table 2). Based on this analysis and the obtained OR value, our study results suggest that homozygous TT may retain a potential risk factor for breast cancer genetic predisposition in the investigated set of Iraqi patients.

Furthermore, a relatively higher frequency to the heterozygous GT genotype was detected in the investigated breast cancer patients than in the healthy control subjects (64% versus 56% respectively). Chi-square test on the other hand did not show significant differences between the two groups ($\chi^2 = 0.266$ NS, OR = 0.502, CI = 0.27 – 0.92, Table 2).

Table 2: The frequency of genotype and alleles at rs1799983 SNP of the studied breast cancer and healthy controls

Genotype	Control	Patients	χ^2	OR	CI
GG	20 (40%)	1 (2%)	17.19 **	Ref.	--
GT	28 (56%)	32 (64%)	0.266 NS	0.502	0.27 – 0.92
TT	2 (4%)	17 (34%)	11.84 **	2.67	1.16-4.09
Alleles frequencies					
G	0.68	0.34	--	--	--
T	0.32	0.66	--	--	--
** (P<0.01), NS: Non-Significant, GG: represents the wild-type genotype.					

Regarding to the alleles frequency in the rs1799983 G894T genomic location, G allele frequency was higher in healthy controls compared with breast cancer patients (0.68 and 0.34 respectively). This was not the case in respect to the T allele frequency where its frequency was higher (0.66) in breast cancer patients than that of the healthy control group (0.32, Table 2).

rs2070744 T > C Polymorphism

Genotypes distribution and alleles frequency analysis of rs2070744 T > C SNP maps to *eNOS* in the studied breast cancer patients and controls is presented in Table 3. The results showed that the frequency of homozygous TT wild type genotype was higher in healthy controls individuals in comparison to that of the breast cancer patients (6% versus 14% respectively, $\chi^2=1.60$, NS, Table 3). In contrast, the frequency of homozygous CC genotype was significantly ($P<0.01$) higher in breast cancer patients than healthy controls (58% versus 12% respectively, $\chi^2=15.11$, OR= 2.08, $P<0.01$, CI=0.91 -4.17, Table 3). While breast cancer patients exhibited significantly ($P<0.01$) lower frequency of heterozygous TC genotype than healthy controls (36% versus 74% respectively, $\chi^2=6.57$, OR=1.13, $P<0.01$, CI=0.79 – 1.84, Table 3). Based on the above presented results, both the homozygous CC and heterozygous TC genotypes seemed to contribute to the risk of breast cancer development in the investigated set of patients.

Table 3: The frequency of genotype and alleles at rs2070744 with breast cancer and healthy controls

Genotype	Control	Patients	χ^2	OR	CI
TT	7 (14%)	3(6%)	1.60 NS	Ref.	--
TC	37 (74%)	18(36%)	6.57 **	1.13	0.79 – 1.84
CC	6(12%)	29(58%)	15.11 **	2.08	0.91 -4.17
Alleles frequencies					
T	0.51	0.24	--	--	--
C	0.49	0.76	--	--	--
** ($P\leq 0.01$), NS: Non-Significant, TT : represents the wild-type genotype.					

3- Total antioxidants capacity levels according to the *eNOS* gene polymorphism status in breast cancer patients and healthy controls

Further analysis was performed to assess the potential association between TAC levels and the genetic variation based on the *eNOS* gene polymorphism (rs1799983 and rs2070744). The results showed that both heterozygous and homozygous genotypes (GT and TT) of rs1799983 SNP showed to be significantly ($p<0.01$) associated with decreased serum TAC levels in breast cancer patients compared with apparently healthy subjects (0.0879 and 0.0945 versus 0.5851 and 0.491mM/ml respectively, Table 4). Also, all rs2070744 SNP in *eNOS* genotypes, with different zygosity status, showed to be significantly ($p<0.01$) associated with decreased serum TAC in breast cancer patients compared with apparently healthy subjects (0.0755, 0.0955 and 0.0877 versus 0.631, 0.553 and 0.7165 mM/ml, for CC, CT and TT respectively, Table 5).

Table 4: Serum total antioxidant capacity levels according the investigated eNOS- SNP genotypes.

eNOS SNPs	Genotype	Total Antioxidants Capacity (mM/ml)		P- value
		Healthy Controls	Breast Cancer Patients	
rs1799983 G894T	GG	0.5618 ± 0.06	-	-
	GT	0.5851 ± 0.08	0.0879 ± 0.02	0.0005 **
	TT	0.491 ± 0.07	0.0945 ± 0.05	0.0071 **
	P- value	0.658 NS	0.672 NS	-
rs2070744 T786C	TT	0.631 ± 0.17	0.0755 ± 0.02	0.0002 **
	TC	0.553 ± 0.09	0.0955 ± 0.03	0.00045 **
	CC	0.7165 ± 0.12	0.0877 ± 0.02	0.0002 **
	p- value	0.419 NS	0.640 NS	-

** (P<0.01).

Additionally, T allele frequency, for rs2070744 T > C genomic location, was higher in the healthy control group compared to breast cancer patients (0.51 and 0.24 respectively). While the frequency of C allele was higher in the breast cancer patients group in comparison to the healthy controls (0.76 and 0.49 respectively). Further detailed analysis of the obtained results showed that the examined SNPs of eNOS gene (rs2070744 and rs1799983) in breast cancer patients and healthy controls were significantly correlated with each other in addition to their haplotype patterns (Table 4). Interestingly, the results also demonstrated that antioxidant concentrations in blood samples of recruiting individuals elucidate an inverse significant correlation of the SNPs status with their haplotype patterns.

Table 4: Spearman's rank correlations of the assessed SNPs of eNOS gene (rs2070744 and rs1799983) in breast cancer patients and healthy controls

		rs1799983 SNP	rs2070744 SNP	Haplotype	Antioxidant	
Spearman's Rank Correlations rho (ρ)	Individuals	Correlation Coefficient	.538**	.454**	.533**	-.800**
		Sig. (2-tailed)	<.001	<.001	<.001	<.001
	rs1799983 SNP Pattern	Correlation Coefficient		.600**	.736**	-.777**
		Sig. (2-tailed)		<.001	<.001	<.001
	rs2070744 SNP Pattern	Correlation Coefficient			.828**	-.787**
		Sig. (2-tailed)			<.001	<.001
	Haplotype	Correlation Coefficient				-.768**
		Sig. (2-tailed)				<.001
		N	100	100	100	100

** . Correlation is significant at the 0.01 level (2-tailed).

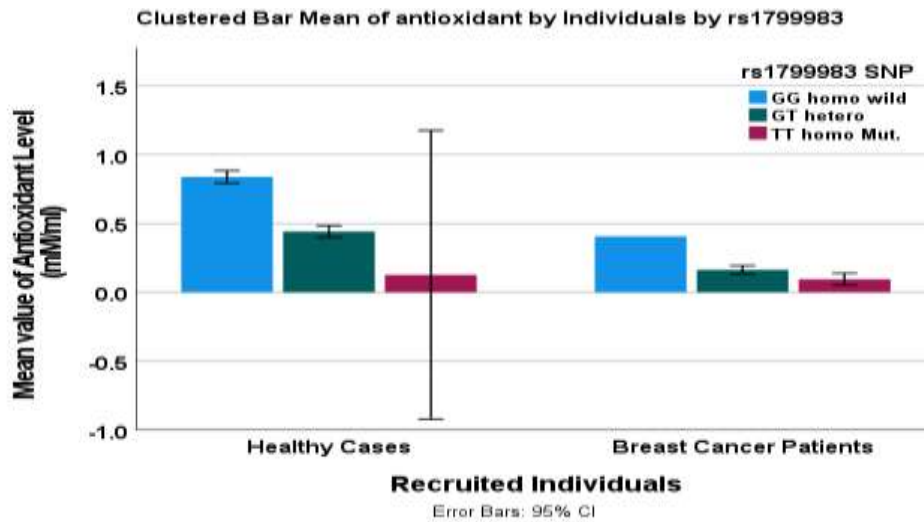


Figure 2: Clustered Bar-chart of the mean of total antioxidant capacity in the studied breast cancer patients and healthy controls based on the genotypic pattern of rs1799983-eNOS SNP. Bars represent mean values from three biological repeats and error bars represent 95% confidence intervals of the mean value .

Additionally, the results demonstrated a similar trend of increased range of total antioxidant levels in blood samples of both categories of recruited individuals “healthy cases and patients” that carry different patterns of rs1799983 SNP, illustrating higher levels in the wild type (GG) of rs1799983 SNP in comparison to those with hetero patterns (GT), and the latter was higher than the homo mutants (TT) (Figure 2). However, the overall antioxidants levels in each SNP category (homo wild type, hetero and homo mutants) of patients were significantly lower than their counterparts of healthy individuals.

Furthermore, individuals with wild type patterns (TT) of rs2070744 SNP showed higher levels compared to those with hetero patterns (TC) which is in turn was higher than the homo mutants (CC). Nevertheless, the overall antioxidants concentrations in each SNP category (homo wild type, hetero and homo mutants) of patients were significantly lower than their counterparts of healthy individuals (Figure 3).

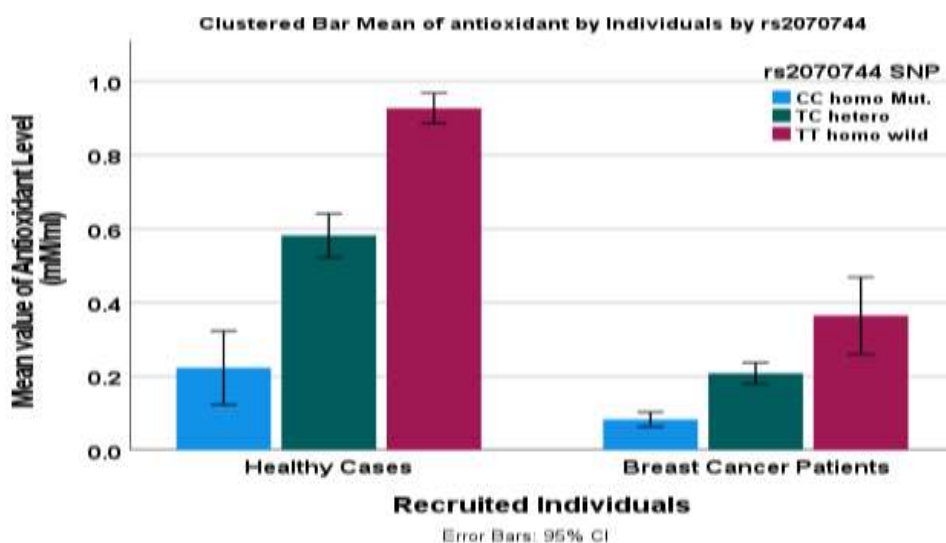


Figure 3: Clustered Bar-chart of the mean of total antioxidant capacity in the studied breast cancer patients and healthy controls based on the genotypic pattern of rs2070744-eNOS SNP. Bars represent mean values from three biological repeats and error bars represent 95% confidence intervals of the mean value.

Moreover, individuals with haplotype pattern (GT) showed to have increased levels of antioxidants which was more obvious in healthy cases (Figure 4). This observation seemed to be consistent with aforementioned results and suggested GT haplotype pattern as a key contributor to the increase in the antioxidant capacity level in healthy controls.

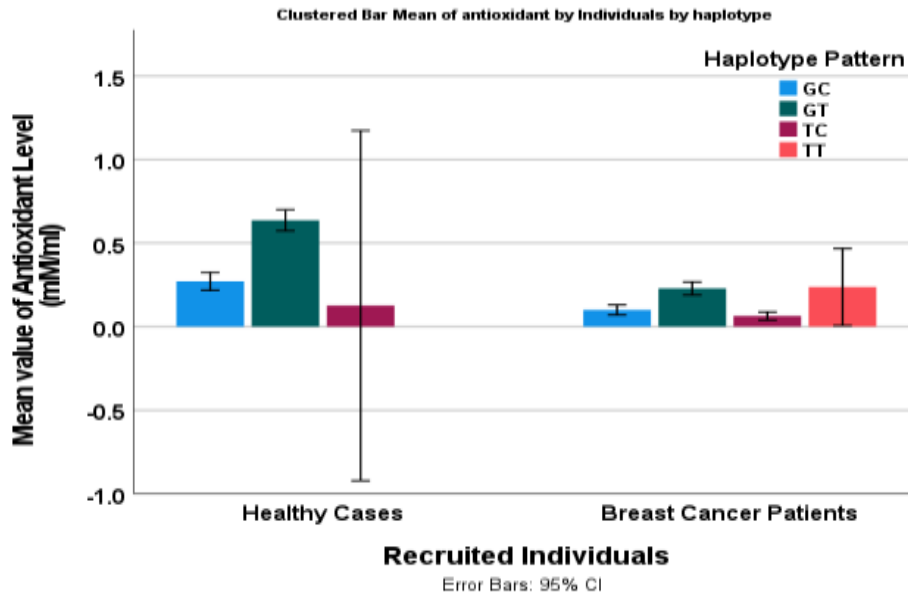


Figure 4: Total antioxidant capacity levels in blood samples of breast cancer patients alongside the healthy cases in relation to their haplotypes patterns of both studied SNPs “rs1799983 SNP and rs2070744 SNP” on the first allele for each. Bars represent mean values from three biological repeats and error bars represent 95% confidence intervals of the mean value.

Discussion

Antioxidant capacity is an integral part of the body mechanisms against damage caused by free radicals that play important roles in the development of a number of serious health issues including cancer. Within the context, the present study results have highlighted the potential involvement of reduced serum levels of total antioxidant capacity in breast cancer pathogenicity where the vast majority (80%) of the patients had very low levels of antioxidant capacity. This could be quite interesting in terms of the developing of the prevention strategies especially to those known to have high risk of cancer. Indeed, recent directions in cancer prevention have focused on the integration of dietary antioxidants can adjust the risk of developing cancer by approximately 40% [34-36].

As antioxidants contribute to maintain genomic integrity [37], it would be interesting to assess their level in cancer that is marked by its high levels of genomic instability [38, 39]. A number of studies have reported reduced total antioxidant capacity in different types of solid tumours [40], including those with HER2⁺ breast cancer [41]. Very recently, the oxidative damage has been investigated by malondialdehyde estimation in relation to miR-146a expression indicating their dual function to predict breast tumorigenesis [29]. The present findings seem to be consistent with other research which found reduced antioxidant capacity levels in cervical cancer patients [42]. Additionally, it is believed that antioxidant capacity contributes to treatment resistance which is one of the major obstacles in cancer management

[43]. This is quite interesting venue in cancer research that could be adapted for the development of novel therapeutic targets.

In respect to the G894T (rs1799983) *eNOS* genotyping analysis, our study results showed that homozygous GG genotype may be beneficial for the protection from breast cancer potential. While homozygous TT may confer a potential risk for breast cancer development in the investigated set of Iraqi patients. Additionally, the genotypes distribution and alleles frequency analysis of rs2070744 T >C SNP both the homozygous CC and heterozygous TC genotypes seemed to contribute to the risk of breast cancer development in the investigated set of patients. The statistical analysis showed that all of the assessed different *eNOS* haplotypes are negatively associated with the reduced levels of total antioxidant capacity in breast cancer. These findings further support the potential role of *eNOS* genetic variation in carcinogenesis that is highlighted by a few recent published studies [13, 14, 44]. Endothelial nitric oxide synthase (*eNOS*)-NO signaling axis functions to promote the growth of prostate cancer stem-like cells [24]. Additionally, studies have highlighted the involvement of endothelial nitric oxide synthase gene variants in the aggressiveness of uterine cervical and breast cancer [13, 45]. The identified significant association between the *eNOS* genetic polymorphisms and the reduced levels of antioxidants could be assessed further, in large cohort, to investigate its utility to improve the current breast cancer patients' risk stratification. Following diagnosis and pre-treatment, cancer patients' risk stratification is an integral part of personalised medicine with the aim of improving survival rate, minimise treatment-associated toxicity and enhancing patients' quality of life.

Conclusions

Overall, the present study highlighted the potential impact of both total antioxidant capacity and *eNOS* gene polymorphisms in breast cancer pathogenicity. This was manifested through the significantly reduced TAC in breast cancer patients and the risk association of TT (G 894T), TC and CC (T786C) of *eNOS*-SNPs.

"Conflict of Interest: The authors declare that they have no conflict of interest."

"All experiments were conducted in accordance with Helsinki Declaration of 1975, as revised in 2000. Informed consent for all human subjects included in the study was also obtained.

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