

Association of Genetic Polymorphism of ABCG2 Gene and the Occurrence of Oxaliplatin-Induced Peripheral Neuropathy in Patients with Colorectal Cancer

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ABSTRACT

Oxaliplatin use in gastrointestinal malignancies is limited by neurotoxicity. This study aims to identify single-nucleotide polymorphisms (SNPs) in the ABCG2 gene associated with oxaliplatin-induced peripheral neuropathy (OIPN) in Egyptian Colorectal Cancer (CRC) patients treated with oxaliplatin-based chemotherapy (CT). All eligible CRC patients between the ages of 18 and 80 participated in the study, and those with a neurological illness or condition limiting neurologic function were excluded. On the first day of every CT cycle, OIPN was assessed and scored using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE v.4). Blood was utilized to extract genomic DNA to detect SNPs in the ABCG2 gene (at rs2231137 and at rs3114018) using High Resolution Melting (HRM) technique followed by direct sequencing method for every melting cluster using 3500 genetic analyzers for the samples selected from each cluster. The occurrence of grade 2-3 OIPN was higher in patients carrying the G/G genotype of ABCG2 (rs2231137) than those with the G/A genotype at the same locus (rs2231137) (96.7% versus 82.0%; $P= 0.05$). While, grade 2-3 OIPN occurrence was not significantly different in patients carrying genotypes (C/C, A/C, and A/A) of ABCG2 at rs3114018 (92.9%, 82.1% and 80.0% respectively; $P= 0.309$). In conclusion, the occurrence of OIPN among Egyptian Colorectal Cancer (CRC) patients was more associated with the G/G allele genotype of ABCG2 (rs2231137). While, patients carrying different genotypes (C/C, A/C, and A/A) of ABCG2 at rs3114018 were similarly associated with OIPN in this patient population.

Keywords: Oxaliplatin-Induced Peripheral Neuropathy; ABCG2 gene; Colorectal Cancer; High-Resolution Melting Technique; 3500 Genetic Analyzer.

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1. Introduction

The third most prevalent diagnosis and the most common cause of cancer-related mortality is colorectal cancer (CRC). In CRC, both genetic

and epigenetic factors contribute to the growth of invasive cancer in the normal colonic mucosa [1-2]. The incidence of CRC is increased by some factors such as obesity, inactivity, and smoking, and the primary etiological hazards for it have

been reported, interactions between both environmental and genetic factors have been identified as the primary etiology of CRC [3-5].

Oxaliplatin, which consists of a 1, 2-diamino-cyclohexane ring, is the first cytotoxic platinum-based chemotherapeutic drug to be effective against CRC. It works by generating DNA crosslinks and adducts which suppress cell growth and promote apoptosis. Combining oxaliplatin with either capecitabine (CAPOX or XELOX) or 5-FU (FOLFOX) has been shown to improve overall survival (OS) and disease-free survival (DFS) in CRC patients whose tumors have undergone complete resection. When combined with other drugs like fluoropyrimidines, oxaliplatin has been shown to have a significantly better effect than when administered alone [6-7]. FOLFOX therapy against 5-FU/LV or 5-FU/irinotecan combinations in stage III patients has been reported to OS. Finally, these combination regimens have been considered to be the best option for treating individuals with stage III CRC, except for those for whom oxaliplatin is contraindicated [8]. Oxaliplatin cessation may result from a major dose-limiting clinical issue known as oxaliplatin-induced peripheral neurotoxicity. If treatment is discontinued, this neuropathy can be reversible [9]. As reported by the Food and Drug Administration (FDA), peripheral neuropathy affects more than 60% of oxaliplatin-treated patients [10]. The symptoms of acute and chronic OIPN include cold-sensitive sensory, neuropathic pain in limbs, dysphoric syndrome, ototoxicity, and autonomic nerve dysfunction [7].

ABCG2 (ATP-binding cassette subfamily G, member 2) is also referred to as breast cancer resistance protein (BCRP). These proteins are found across several organs, including the colon, liver, kidney, and cancerous cells [11]. The metabolism of various substances is influenced

by ABCG2, including anticancer drugs and xenobiotics with the potential to cause cancer.

Inhibition of ABCG2 has become an innovative therapeutic strategy for the treatment of cancer that aims to increase the bioavailability of chemotherapeutic medications and prevent drug resistance by preventing drug efflux [12-13]. The unusual distribution of carcinogenic xenobiotics has the potential to enhance the local carcinogen burden and promote carcinogenesis in particular cells and organelles [13-14].

Numerous studies in epidemiology have investigated the association between the ABCG2 polymorphism and the risk of chemotherapy-induced peripheral neuropathy, however, the real association between the ABCG2 polymorphism and chemotherapy-induced peripheral neuropathy (OIPN) using data collected from colorectal cancer patients treated with oxaliplatin regimens.

2. Patients and methods

2.1. Design: A prospective cohort study.

2.2. Setting: Ain Shams University Hospitals' oncology department, Cairo, Egypt

2.3. Patients: Eighty patients were evaluated for eligibility Based on the following criteria:

2.4. Inclusion criteria

- Patients with CRC receiving treatment involving oxaliplatin chemotherapy
- Patients aged between 18 to 80 years

2.5. Exclusion Criteria

- History of allergic responses to substances with equivalent chemical or biological compositions to Oxaliplatin.
- Neurologic illness or patients with

disease affecting neurologic function.

This investigation has been guided by the principles of the Helsinki Declaration which was revised in 2013. The study procedure was approved by Ain Shams University's ethics committee. The research procedure was explained to all patients, and only those who provided written informed permission were enrolled. Clinical trials.gov. registration number is NCT02428101.

Before the study started, all eligible individuals were submitted to a comprehensive neurologic examination, and those who showed baseline indications or symptoms of peripheral neuropathy were eliminated.

2.6. Clinical evaluation of neurotoxicity

On the first day of each CT cycle, OIPN was assessed and graded using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE), version 4.0.

In an incident of persistent (at least 14 days) paresthesias, transient painful paresthesias, or functional impairment, the oxaliplatin dose would be lowered to 75 mg per square meter. Oxaliplatin was terminated in cases of persistent painful paresthesias or functional impairment.

2.7. Baseline assessment

At baseline, the following procedures were carried out on all patients;

- Collecting patient's history and demographic data.
- Patient education about the signs and symptoms of neuropathy, as well as how to report them.

2.8. Follow-up evaluation:

Patient follow-up was done using a follow-up card used every cycle (every 2 weeks) for patients' self-evaluation and a daily record of the

occurrence of sensory/motor peripheral neuropathy. Burning, tingling, numbness, weakness, stabbing-like pain, loss of sensation, and responsiveness to mildly unpleasant stimuli were the predominant symptoms and signs indicated.

Patients were shown how to use the follow-up card and how to report all expected signs and symptoms.

All through the 12 cycles (Six Months), patients were assessed every 2 weeks according to:

- 1- Patients follow-up card evaluation and giving out new cards.
- 2- Weekly phone calls to ensure compliance to follow up
- 3- Assessment of severity of sensory and motor OIPN.

The standard terminology criteria for adverse events (CTCAE) version 4 (2010) was used to evaluate the frequency and severity of sensory and motor OIPN following each cycle.

2.9. End of study evaluation:

Patients were evaluated for the following after 12 cycles of the FOLFOX protocol:

- The development of peripheral neuropathy.
- The difference in grade between high (grades 2-3) and low (grade 1) motor and sensory OIPN.
- The highest grade of sensory/ motor peripheral neuropathy reached by patients in any particular cycle was recorded as the OIPN for that cycle.

2.10. Genomic DNA samples and ABCG2 gene polymorphisms genotyping

Using a DNA isolation kit (**Bio Basic Isolated, Canada**), genomic DNA was obtained

from a peripheral blood (lymphocyte) sample for Eighty CRC patients. Genotyping was carried out for the ABCG2 gene SNPs at rs2231137 and rs3114018 Utilizing the High-Resolution Melting (HRM) technique followed by a direct sequencing method for every melting cluster using 3500 genetic analyzers for the samples selected from each cluster.

2.11. Screening of ABCG2 by High-Resolution Melting (HRM) Method

The High-Resolution Melting technique was used to investigate genetic variation in the ABCG2 gene. The DNA melting profile is precisely measured using this approach. The length and nucleotide content of the PCR product depends on the melting temperature of dsDNA. Numerous varieties can be identified in this manner, as even a single base difference produces a different melting curve. **Table 1** displays the list of primers used for HRM scanning of particular regions. The single reaction mixture with a total volume of 10 μL /well was prepared using 5 μL of MeltDoctor[®] (Thermo Fisher, USA) 0.5 μM of forward primer, and 0.5 μM reverse primer (the stock concentration was 100 μM), 0.5 μL DNA (100 ng/ μL), and filled up to the final volume with nuclease-free water (3.5 μL). The reaction was performed in 384-micro well plates using a real-time PCR system (ViiA 7, APPLIED BIOSYSTEM) in duplicate samples. These were the reaction conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at the specific temperature depending on the primers used, for 1 min. The fluorescence was read after each cycle. Directly afterward, the melting curve was determined, the plate being incubated at 95 °C for 10 s, then annealing temperature for each primer for 1 min. this was followed by 95 °C for 10 sec with an increment of 0.025 °C/sec for 10 s with plate reading, then

60 °C for 15 sec. Based on HRM melting curve analysis, the direct sequencing approach was used to confirm genetic variation for each melting cluster for the samples selected from each cluster.

2.12. DNA sequencing

PCR was used to prepare selected samples for sequencing. The reaction mixture filled with water to a final volume of 25 μL per sample was composed using a 12.5 μL **Taq PCR master mix kit** (QIAGEN, Germany), 1 μL of 10 p moles/ μL primers mix, and DNA (100 ng total conc). These were the reaction conditions: initial denaturation at 95 °C for 5 min, 35 amplification cycles of denaturation at 95 °C for 30 s, annealing at a specific temperature depending on the primers used for 45 s, and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min (list of primers for sequencing are shown in **Table 1**). Then, PCR products were purified using QIAquick PCR Purification Kit[®] (Qiagen, Germany). Agarose electrophoresis of 2 μL of each sample was used to determine the DNA concentration and size of the PCR product. Following the manufacturer's instructions, samples were applied for a sequencing process using BigDye Terminator V3.1 (Applied Biosystems, USA) based on the band intensity. Using the BigDye X-Terminator kit and the manufacturer's protocol, the PCR-sequencing product was purified (Applied Biosystems, USA). Further, each purified sample was placed in 30 μL to 96 titration plate wells, and the 3500 Genetic Analyzer (Applied Biosystems, USA) was used for analysis.

2.13. Identification to Polymorphisms

The Novosnp program (Novosnp 3.0.1 - Windows - i686 version) was used to examine the ABCG2 Genomic DNA sequencing findings. The sequencing findings of selected samples were compared to HRM clusters. Based on this,

the genotype for each melting cluster was determined, and the genetic variance in each sample was verified.

Table 1. The primer list used in HRM scanning

Polymorphism	Primer Sequences	Annealing/ temp	Product size
ABCG2G34A At rs2231137	Forward 5'-TGC AAT CTC ATT TAT CTG GAC TA-3'	57 °C	163 bp
	Reverse 5'-AAT GCC TTC AGG TCA TTG GA-3'		
ABCG2 A/A At rs3114018	Forward 5'- TGATGTTTCCTTCAGCCACTG-3'	59 °C	124 bp
	Reverse 5'TTGTGGAAACCTCACAAAAGTG-3'		

2.14. Statistical analysis

IBM® SPSS® Statistics version 22 (IBM® Corp., Armonk, NY, USA) was used for statistical analysis. When applicable, the mean and standard deviation or the median and range were used to describe numerical data. Frequency and percentage were used to convey qualitative data. The relationship between the qualitative variables was investigated using the chi-square test or Fisher's exact test. Two successive measurements of non-normally distributed numerical variables were compared using the Wilcoxon-signed ranks test (non-parametric paired t-test). All tests were two-tailed. A p-value < 0.05 was considered significant.

3. Results

From January 2015 to March 2017, the study was carried out. Only 120 participants who met the inclusion criteria were enrolled in the trial from a total of 170 patients. Genotyping analysis was possible in 80 patients only and the follow-up was done for 6 months (12 cycles of treatment).

3.1. Occurrence of peripheral neuropathy, both sensory and motor:

Out of 80 patients in total, 70 patients (87.5%) developed different grades of sensory peripheral neuropathy; 15(18.8%) were grade I, 29 (36.3%) were grade II, and 26 (32.5%) patients were grade III. Motor neuropathy was observed in 62 patients (77.5%) as follows; 16 (25.8%) grade I, 43 (69.4%) grade II, and only 3 (4.8%) patients developed grade III. (**Table 2**).

3.2. SNPs associated with OIPN

During treatment, 87.5% of patients in the current study developed OIPN, which was associated with the ABCG2 SNPs rs3114018 and rs2231137. Peripheral neuropathy and different grades of sensory and motor peripheral neuropathy were included in the analysis. The 80 patients had two SNPs of ABCG2 at rs2231137 Wild-type (G/G allele) and Heterozygous (G/A allele). Patients with Wild-type showed an increased frequency of severe OIPN compared with heterozygous [96.7% G/G allele versus 82.0% G/A allele; odds ratio (OR) = 6.366; 95% confidence interval (CI) 0.764- 53.035; $P = 0.055$] (**Fig. 1**).

The percentage of grade (2-3) sensory and motor neuropathy was not significantly different

in patients with the G/G allele versus those with the G/A allele. Sensory neuropathy was 79.3% for the G/G allele versus 78.0% for the G/A-G/A allele; odds ratio (OR) = 0.928; 95% (CI) 0.290-2.970; $P= 0.899$. While motor neuropathy was 76.0% for G/G allele versus 73.0% for G/A allele; odds ratio (OR) = 0.853; 95% (CI) 0.265-2.747; $P= 0.789$.

Regarding the ABCG2 rs3114018 SNPs, there was no significant difference in the occurrence of OIPN in patients with the C/C allele (mutant genotype) versus those with A/C allele (Heterozygous) and the A/A allele (wild-type) ($P=0.309$) (**Fig. 2**).

The occurrence of OIPN in the A/A allele and the C/C allele was 80% versus 92.9%, odds ratio (OR) = 3.250; 95% (CI), 0.465- 22.711; $P = 0.235$.

The occurrence of OIPN in the A/A allele and A/C-A/C allele was 80 % versus 82.1%; odds

ratio (OR) = 1.150; 95% (CI), 0.185- 7.144; $P = 0.881$.

The percentage of grade (2-3) sensory and motor neuropathy was not significantly different in patients with A/A allele versus those with C/C allele or A/C-A/C allele.

Grade (2-3) sensory neuropathy was 62.5% in the A/A allele versus 82.1% in the C/C allele; (OR) = 0.365; 95% (CI) 0.07- 1.896; $P = 0.23$. While grade (2-3) sensory neuropathy was 62.5% in the A/A allele versus 78.3% in the A/C- A/C allele; (OR) = 0.463; 95% (CI), 0.081- 2.64; $P = 0.386$.

Grade (2-3) motor neuropathy was 57.1% in the A/A allele versus 84.8% in the C/C allele; (OR) = 0.238; 95% (CI) 0.04- 1.403; $P= 0.113$. While grade (2-3) sensory neuropathy was 57.1% in the A/A allele versus 63.6% in the A/C- A/C allele; (OR) = 0.762; 95% (CI), 0.135- 4.301; $P = 0.758$.

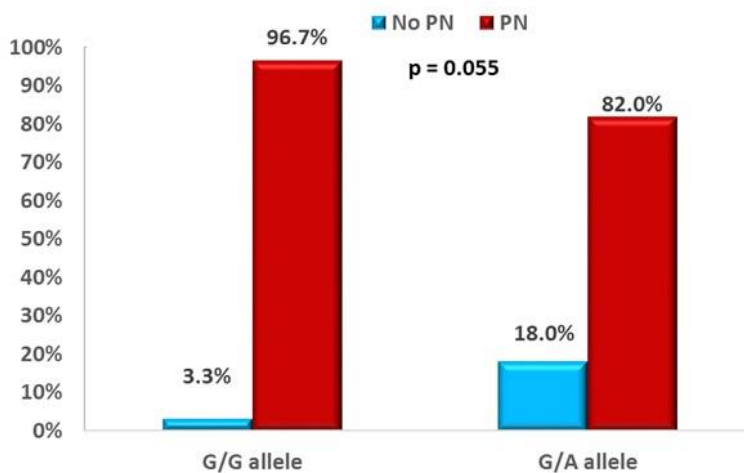


Fig. 1. The occurrence of peripheral neuropathy among patients carrying different alleles of ABCG2 at rs2231137

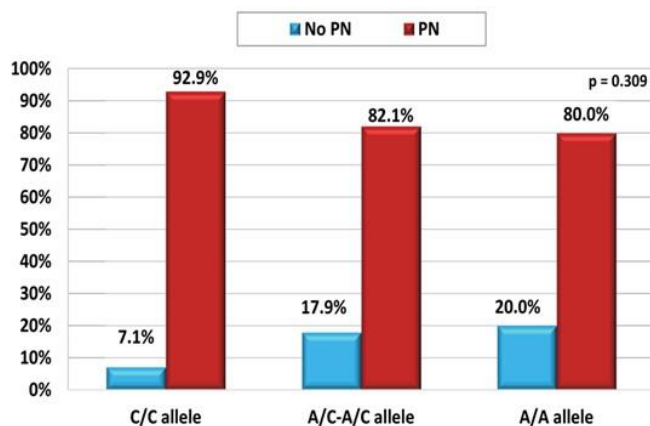


Fig. 2. The occurrence of peripheral neuropathy among patients carrying different alleles of ABCG2 at rs31140

Table 2. Demographic and clinical characteristics of the Patients

Parameter	Category	All patients	NO-PN	PN
		N= 80	N= 10	N= 70
Age	< 50 years	47	6(12.8%)	41(87.2%)
	≥ 50 years	33	4(12.1%)	29(87.9%)
mean ±SD		46.9±10.8	46.6±15.7	46.9±10.1
(range)		(25-73)	25-65	(25-73)
Gender	Male	33	4 (12.1%)	29 (87.9%)
	Female	47	6 (12.8%)	41(87.2%)
Weight	< 70 Kg	49	7(14.3%)	42(85.7%)
	≥ 70 Kg	31	3 (9.7%)	28 (90.3%)
mean ±SD		66.9±14.8	70.9±23.7	66.4±13.2
(range)		(38-114)	(42-114)	(38-96)
Tumor grade	Grade I	1(1.3%)	1(100%)	0(0.0%)
	Grade II	23(28.7%)	5(21.7%)	18(78.3%)
	Grade III	55(68.8%)	4(7.3%)	51(92.7%)
	Missing System	1(1.3%)	-	-
Oxaliplatin protocol	FOLFOX4	28(35%)	2(7.1%)	26(92.9%)
	FOLFOX6	52 (65%)	8(15.4%)	44(84.6%)
Dose (mg/m²) / Cycle	Mean± SD	142.9±21.1	142 ±23.9	143±20.8
	Range	90-230	100-170	90-230

N, Number; kg, kilogram; CRC, Colorectal cancer; HTN, Hypertension.

4. Discussion

The current study's findings have shown a correlation between OIPN and polymorphism in the ABCG2 rs2231137 in Egyptian patients with colorectal cancer. As far as we know, this work is the first study to date to examine the role of pharmacogenetics in ABCG2 rs2231137 SNPs in OIPN in CRC patients.

A blood sample was used to extract genomic DNA to evaluate the ABCG2 rs2231137 and rs3114018 SNPs for accessibility and probable application in clinical practice.

In the present study, a high rate of severe peripheral neuropathy (grade 2-3) developed during therapy; 78.5% of patients experienced sensory neuropathy, and 74.3% experienced severe motor neuropathy. Similarly, in a trial of 57 CRC patients treated with oxaliplatin-containing regimens, 77% of individuals receiving the oxaliplatin regimen (FOLFOX or XELOX regimens) had sensory neuropathy grade 2 or higher, according to Dault, R. et al. [15].

Contrary to the current findings, in the Mosaic trial, a grade 2 or higher estimated neuropathy was reported in 44% of patients undergoing adjuvant therapy for colon cancer with oxaliplatin, a FOLFOX-type regimen [16].

In addition, grade ≥ 2 sensory neuropathy was observed in 43.7% of CRC patients getting the modified FOLFOX 6 regimen and 48.9% of patients receiving the modified FOLFOX 6 plus bevacizumab, according to a study of 2710 patients receiving adjuvant treatment [17]. These studies all agreed that disturbance in activities of daily living, depression; dose reduction or treatment cessation, and increased mortality were common in OIPN patients [18-19].

The disparity between prior studies and the current study on the prevalence of grade ≥ 2 peripheral neuropathy might be attributed to a

variety of reasons, including a variation in sample size and the use of various neuropathy assessments with different versions of the CTCAE (Version 4 in the current study vs. version 3 or 1 in previous studies).

The ABCG2 protein is found in a variety of tissues, including the central nervous system and peripheral nerve pericytes. In general, the ABCG2 rs2231137 SNP is found in the exon 2 expressed portion of the gene, whereas the ABCG2 rs3114018 SNP is found in the intron non-expressed area.

This SNP regulates oxaliplatin transport on peripheral nerve cells by specific biochemical mechanisms that are unclear but the probabilities of exonic region polymorphisms producing biological effects on gene expression are greater. [20-21].

According to this trial's findings, patients with wild-type (G/G) SNPs of ABCG2 at rs2231137 had a greater incidence of OIPN than those with heterozygous (G/A) ABCG2 at the same location (96.7% G/G, 82.0% G/A, respectively). However, in the same SNPs of ABCG2 rs2231137, there was no significant difference in the occurrence of the various grades of sensory and motor peripheral neuropathy. Till today, no studies have addressed the association of ABCG2 rs2231137 SNPs with the occurrence of OIPN. While, in the case of the ABCG2 rs3114018 SNPs no statistically significant difference existed between patients carrying the A/A allele, A/C-A/C allele, and C/C allele, and the occurrence of OIPN (80.0% A/A, 82.1% A/C and 92.9% C/C respectively). Moreover, ABCG2 rs3114018 SNPs did not show significant differences in the incidence of various grades of peripheral neuropathy, either motor or sensory.

In contrast, the GEMCAD research looked at the ABCG2 SNPs at rs3114018 and the

prevalence of OIPN in 206 patients with stage II-III colorectal cancer. The study found that patients with the ABCG2 A/A genotype at rs3114018 had a greater frequency of grade 2-3 OIPN than those with ABCG2 with any C genotypes at the same location [22].

The difference between the current results and those in the GEMCAD group study could be attributed to several reasons that could have affected the outcome of their results, including regimens of oxaliplatin-based therapy, limited sample sizes Genetic variations related to ethnic background (Egyptian versus Caucasian patients).

Further, it should be highlighted that the majority of prior pharmacogenomics research, including our work, has concentrated on a single candidate gene, whose clinical importance is restricted and may not be sufficient to predict the frequency of severe oxaliplatin peripheral neuropathy and oxaliplatin resistance [22, 24, 25].

The current study has certain limitations because it was done at a single center and also addressed a small number of patients.

However, despite these limitations, our study comprised the ABCG2 rs2231137 that correlated to OIPN in colorectal cancer patients for the first time. The fact that all patients received the same oxaliplatin-based regimens according to the same guidelines is also an almost unique advantage. This presents a unique opportunity to determine the impact of specific polymorphisms on OIPN and avoids bias brought about by varying treatment regimens and the effects of previous therapies.

So, this study tries to know the reason for oxaliplatin induces different grades of peripheral neuropathy in patients treated for the same disease (colorectal cancer) with the same protocol (FOLFOX) and we found a genetic

correlation with G/G allele ABCG2(rs2231137) SNP.

Conclusion

In conclusion, the study found the patients carrying G/G alleles in ABCG2 (rs2231137) SNP were associated with severe OIPN in CRC patients. While the SNPs of ABCG2 at rs3114018 were similarly associated with OIPN in Egyptian colorectal cancer patients.

Declarations

Ethics approval and consent to participate

From the faculty of Pharmacy Ain Shams University serial number: master (no.38)

Consent to publish

All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article in the main manuscript.

Competing interests

The authors declare that no competing interests exist

Funding Statement

No funding source was received.

Authors' contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Inas Moawya Moukhtar Ahmed, Dr.Lamia Mohamed El Wakeel, Dr.Abdel Hady Aly Abdel Wahab, Dr.Amr Shafik Tawfik Saad, Dr.Raafat Ragaie Abdel-Malek and Dr.Ahmed Atef Emam. The first draft of the manuscript was written by Inas Moawya Moukhtar Ahmed and all authors

commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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