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Effects of Eugenol-Loaded Chitosan Biopolymer Nanoparticles on *CYP51*A and *CYP51*B Expression in *Aspergillus fumigatus*

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ABSTRACT

Backgrounds: *Aspergillus fumigatus* is a pathogen responsible for invasive aspergillosis and the main leading cause of death in immunosuppressed individuals. The present study aimed to evaluate the impact of eugenol-loaded chitosan nanoparticles on the expression of *CYP51a* and *CYP51b*, two well-known genes responsible for triazole drug resistance in *A. fumigatus*.

Materials & Methods: The minimum inhibitory concentration (MIC) of eugenol-loaded chitosan nanoparticles, chitosan, eugenol, and itraconazole was determined based on the Clinical and Laboratory Standards Institute M38-E3 method at concentrations of 4.6-2400, 11.7-12000, 2-2048, and 1-256 µg/mL, respectively. The expression of *CYP51A* and *CYP51B* was evaluated in *A. fumigatus* exposed to 0.5, 1, and 2× of MIC concentration of NPs and itraconazole using the real-time polymerase chain reaction.

Findings: The obtained results showed that eugenol-loaded chitosan nanoparticles sucessfully reduced *A. fumigatus* fungal growth at 300 µg/mL concentration. MIC of chitosan, eugenol, and itraconazole was measured to be 6000, 256, and 4 µg/mL, respectively. The results of real-time PCR also revealed that eugenol-loaded chitosan nanoparticles increased the expression of both *CYP51A* and *CYP51B* in a dose-dependent manner. The expression of fungal *CYP51A* and *CYP51B* at mRNA level was significantly increased 1.26, 1.93, and 3.1-fold as well as 1.2, 2.1, and 2.4-fold at concentrations of 150, 300, and 600 µg/mL, respectively (p<.05). However, it seems that the prepared nanoparticles had a lower impact on the expression of these genes compared to itraconazole.

Conclusion: Overall, these findings suggest that the treatment of *A. fumigatus* with eugenol-chitosan nanoparticles could increase the expression of the *CYP51* gene, suggesting the antifungal property of these nanoparticles.

Keywords: Eugenol-loaded chitosan nanoparticle, Aspergillus fumigatus, *CYP51A*, *CYP51B*, Gene expression.

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Introduction

With the advent of the first fungal infections and the identification of the harmful effects of these pathogens on human health, endless efforts have been made to control and manage fungal-associated diseases. It has been claimed that pathogenic fungi could infect more than one billion individuals each year and are the main leading cause of more than 1.5 million deaths around the world [1]Patients with suppressed immune system diseases, such as those with HIV/AIDs or those taking immunosuppressive drugs, are at higher risk of developing fungal infections and may lose their lives if not treated properly[2]. Among different pathogenic fungi, *Aspergillus fumigatus*, as the main pathogen responsible for invasive aspergillosis, seems to account for a considerable number of deaths in immunosuppressed patients[3]. Thus far, triazole drugs, as inhibitors of lanosterol 14-α-demethylase, have been widely used in the treatment *of A. fumigatus*-associated infections; however, in most cases, *A. fumigatus* has found a way to bypass the cytotoxicity of these drugs and develope a resistance phenotype. The alteration in the structure of the *CYP51* protein as a result of point mutations[4 ,5], gene amplification, or gene overexpression [6] is the most commonly reported mechanism in *A. fumigatus,* which could be related to triazole resistance. Through up-regulating the expression of these efflux pumps, *A. fumigatus* seems to prevent the accumulation of triazoles in the cells and thereby attenuate the toxicity of triazole drugs[7].

Given the failure of triazole drugs as a first-line treatment for invasive aspergillosis and the life-threatening impact of *A. fumigatus* on the quality of life of patients with immunosuppressed status, it is not surprising that many efforts are underway to propose the best therapeutic options for the treatment of this fungal infection with higher efficacy and fewer side effects. Thus far, much attention has been paid to the natural compounds for the treatment of invasive aspergillosis, as in many cases, these compounds have lower toxicity. Eugenol ($C_{10}H_{12}O_2$) is a major phenolic compound of clove oil, nominated as 4-allyl-2-methoxy phenol by IUPAC (Fig. 1), which has been shown to have potent antifungal, anti-inflammatory, and anti-parasitic functions [8-10].Moreover, eugenol has been shown to have synergistic effects with various anti-fungal agents such as methyleugenol and amphotericin B[11]. Interestingly, it has been suggested that eugenol could reduce the survival of drug-resistant strains of *Candida albicans* and *Streptococcus mutans* [12]. Although eugenol seems promising in the treatment of infections, its sensitivity to light and oxygen as well as its low solubility in water have muted the enthusiasm for the application of this agent in theraputic strategies[13]. However, the advent of new technologies has come into play and suggested that encapsulating eugenol with nanoparticles (NPs) could possibly be a way to not only increase the half-life of the compound but also more properly deliver the agent into the site of infection[13-15]. Chitosan as a natural cationic polysaccharide has been proposed to be the best biomaterial for fabricating NPs due to its biodegradability and non-toxicity (Fig. 2) [16]. Moreover, the potent antifungal activity of chitosan suggestes that copolymer of glucosamine and N-acetyl glucosamine units might be a good candidate to be used as a capsule for eugenol[17-19].

Objectives: Given the aforementioned reasons and based on the valuable anti-fungal efficacy of eugenol against drug-resistant fungal strains, the present study aimed to evaluate the impact of eugenol-loaded chitosan nanoparticles on the expression of

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CYP51a and *CYP51b* in *A. fumigatus*.

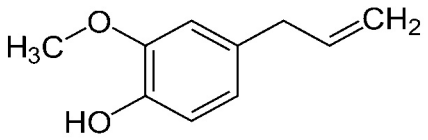

Figure 1) The molecular structure scheme of eugenol

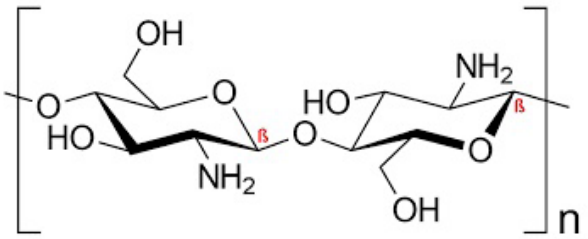

Figure 2) The molecular structure scheme of chitosan polymer

Materials and Methods

Synthesis and formulation of eugenol-loaded chitosan nanoparticles: The ionotropic gelation technique was used to manufacture eugenol-loaded chitosan NPs [20 ,17].In the first step, to prepare chitosan (CAS # 9012-76-4) solution, chitosan was agitated in the aqueous solution of acetic acid, the mixture was left overnight at room temperature and then centrifuged at 12000 rpm for 30 min at 4 °C by a laboratory centrifuge (TGL-20M, Lu Xiangyi, Shanghai, China). After filtering the supernatant using a Whatman quantitative No. 42 filter paper, Tween 80 (CAS # 9005-65-6) was added to the chitosan solution as a surfactant. To reach a homogenous solution, the resulting solution was stirred on an electric mixer (Froilabo: EC180 France) for 2 hrs. Then eugenol (99% CAS #97-53-0) was slowly dropped into the chitosan solution (20 mL) during homogenization. To synthesize the nanoparticles, TPP solution (20 mL) (CAS # 7758-29-4) was gradually dropped into the chiston-eugenol solution, and the mixture was sonicated for 4 min. The synthesized eugenol-loaded chitosan NPs had an average size of 300±100 and 330.9 ± 15.2 nm according to TEM images and Zeta sizer, respectively.

Fungal strain: A fluconazole-resistant clinical strain of *A. fumigatus* Af293 was obtained from the Department of Mycology, Pasteur Institute of Iran (http://fa1.pasteur.ac.ir/pages.aspx?id=1152) and cultured on Sabouraud dextrose agar (SDA, Merck, Germany) medium for 48 hrs at 37 °C.

Antifungal drug susceptibility and MIC determination: Antifungal activity of eugenol-loaded chitosan nanoparticles (NPs), chitosan, eugenol, and itraconazole was assayed according to the guidelines of the National Committee for Clinical Laboratory Standards CLSI M38-E3 method[21]. Briefly, *A. fumigatus* was adjusted to a final concentration of 0.5–2.5×10^5 CFU/mL in RPMI-1640 (Sigma-Aldrich, USA) plus MOPS (3-(N-morpholino) propanesulfonic acid) medium, and 100 µL of cell suspension was added to a 96-well plate. The stock solutions of the prepared NPs, chitosan, and eugenol were prepared by dissolving in RPMI-1640 (Sigma-Aldrich, USA), and two-fold serial dilutions were prepared in RPMI to obtain the final concentrations of 2400-4.6, 12000-11.7, and 2048-2 µg/mL, respectively. Serial two-fold concentrations of itraconazole (ITR) (256-1 µg/mL) were prepared from a stock solution of the drug (CAS # 84625-61-6, Sigma-Aldrich, USA) in DMSO (Merck, Germany). The plates were incubated at 35 °C for 72 hrs. All tests were conducted in triplicate in three independent experiments. The minimum inhibitory concentrations (MIC) of NPs, chitosan, eugenol, and ITR were determined based on the inhibition of fungal growth in 96-well microplates by visual assay. MIC was defined as the lowest concentration of the tested materials capable of interrupting any visible fungal growth [22]. MFC was the lowest concentration of the extract, which caused no fungal colony to appear on SDA plates.

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Table 1) The specific primer sequences and amplified product sizes

Primer	Sequence	Accession no.	Amplicon Size
CYP51A	5′- CGTGCAGAGAAAAGTATGGCG 5′- CTGAACGCCCAGGTAGACTG	XM_747044.1	77 bp
CYP51B	5′- CAACATGGGTGCTTGTTGGAA 5′- ATATCGCCATACTTTGCGCGG	XM_744041.1	202 bp
B-tubulin	5′- AATTGGTGCCGCTTTCTGG 5′- AGTTGTCGGGACGGAATAG	Xm-747363.1	281 bp

***CYP51A* and *CYP51B* genes expression analysis:** *A. fumigatus* spores suspension (0.5- 2.5 × 10^5 CFU/mL) was cultured in 100 mL of Czapek Dox broth (HiMedia, India) and treated with 0.5× of MIC concentrations of NPs and ITR. The flasks were kept in a shaking incubator at 32 °C for 72 hrs. Fungal cells were separated from the culture medium by centrifugation at 4000 ×g and frozen in liquid nitrogen until used for RNA isolation. Total RNA was purified from approximately 100 mg of crushed fungal cells in 1 mL of trizol (BioMerieux, France). Determination of total RNA quality was performed using a ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, US) and gel electrophoresis. The desired bands were observed on 1% agarose gel. The extracted RNA was stored in a freezer at -70 °C until used for cDNA synthesis[23], which was performed using cDNA synthesis kit made by Fermentas Company (Germany). The efficiency of specific primers was calculated by preparing 10^{-1} to 10^{-4} serial dilutions of each specific cDNA primer sample (Table 1). The beta-tubulin gene was used as a housekeeping gene. Quantitative real-time PCR was carried out using SYBR Green Master Mix (GENET BIO) in a final volume of 25 µL for each reaction using an ABI PRISM 7500 thermal cycler (Applied Biosystems). Two-step PCR conditions were as follows: after an initial incubation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s and 60 °C for 1 min were performed]23[. Expression of *CYP51A* and *CYP51B*genes at the mRNA level was investigated using the $2^{-\Delta\Delta ct}$ formula.

Statistical analysis: All statistical analyses were performed using SPSS software V. 28 and GraphPad Prism software V. 9.2.0. Data were analyzed using an unpaired student *t*-test. A *p* value of less than .05 was considered statistically significant.

Findings

Minimum inhibitory concentration (MIC) of the prepared nanoparticles against *A. fumigatus:* To evaluate whether eugenol-chitosan nanoparticles could exert antifungal activity, *A. fumigatus* (*Af293*) was treated with eugenol, chitosan, and eugenol-chitosan nanoparticles separately. As a positive control, the fungus was also treated with itraconazole. The inhibitory effect of these agents on *A. fumigatus* is shown in Table 2. As presented, the lowest concentration of itraconazole which could induce antifungal effects on *A. fumigatus* (Af293) was 4 µg/mL. The results also showed that eugenol-chitosan nanoparticles could reduce the survival of *A. fumigatus* at a lower concentration compared to chitosan or eugenol alone. While the lowest inhibitory

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Table 2. The minimum inhibitory and fungicidal concentrations of chitosan, eugenol, itraconazole, and eugenol-chitosan nanoparticles (µg/mL) on *A. fumigatus*.

Fungal strain	Chitosan		Eugenol		Nanoparticle		Itraconazole		Fluconazole	
	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
A. fumigatus (AF293)	6000	12000	256	1024	300	600	4	16	64<	256<

concentration of chitosan and eugenol each was 6000 and 256 µg/mL, respectively, the MIC of NPs was determined to be 300 µg/mL.

Effect of eugenol-chitosan nanoparticles on the expression level of *CYP51a* and *CYP51b* genes in *A. fumigatus:* The up-regulation of *CYP51A* in *A. fumigatus* is associated with the induction of drug resistance against itraconazole[24]. The results showed that when *A. fumigatus* was treated with itraconazole, the expression of *CYP51A* significantly increased, suggesting that *A. fumigatus* may exploit *CYP51A* to overcome itraconazole toxicity (Figure 3).

To evaluate whether the nanoparticles consisting of eugenol and chitosan could alter the expression of this gene in *A. fumigatus*, the fungus was treated with NPs at concentrations of 150, 300, and 600 µg/mL. The results showed that the nanoparticles could also increase the expression of *CYP51a* in a dose-dependent manner.

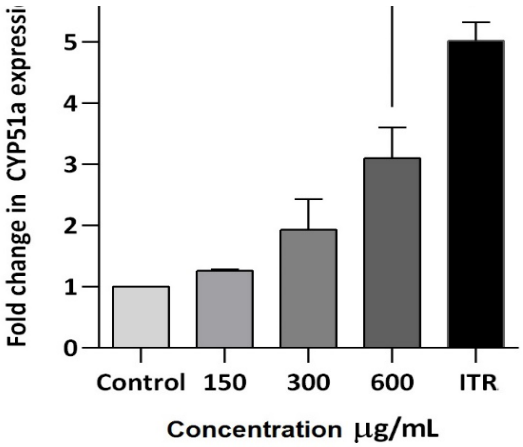

Figure 3) The effect of eugenol-chitosan nanoparticles on the expression of *CYP51A*. q-PCR analysis revealed that treatment of *A. fumigatus* with eugenol-chitosan nanoparticles and itraconazole increased the expression of *CYP51A*. The values are given as the mean ± standard deviation of three independent experiments. A *p*-value of less than .05 represents significant changes compared to untreated control.

As presented in Figure 4, the maximum effect of the nanoparticles on *CYP51A* expression was observed at the concentration of 600 µg/mL, which elevated this gene expression by a 3.1-fold change. As compared to itraconazole, which increased this gene expression by almost 5-fold, it seems that the highest dose of the nanoparticles had a lower impact on *CYP51A* expression. To strengthen this finding, the effect of itraconazole and the nanoparticles was evaluated on the expression of *CYP51B* as another gene involved in sterol 14-demethylase expression. In agreement with the results obtained from *CYP51A,* both itraconazole and eugenol-chitosan nanoparticles upregulated the expression of *CYP51B* (Figure 3 and 4). While itraconazole increased the expression of *CYP51B* by a 3.7-fold change, the nanoparticles at concentrations of 150, 300, and 600 µg/mL upregulated the expression of this gene by 1.2, 2.1, and 2.4-fold changes, respectively (Figure 4).

Discussion

The high incidence of invasive fungal infections, especially in patients with suppressed immune system conditions, has put the proper treatment of such infections

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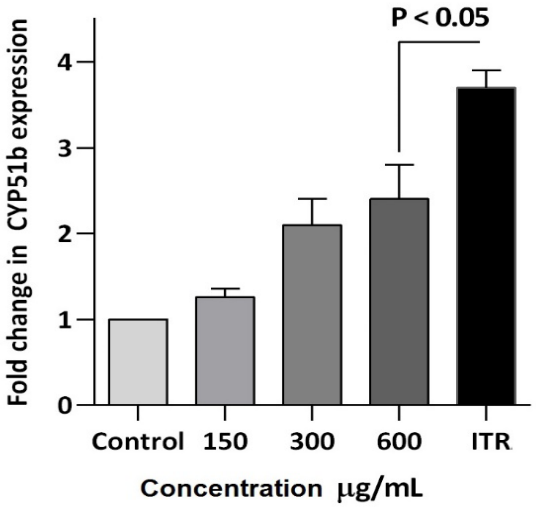

Figure 4) The effect of eugenol-chitosan nanoparticles on the expression of *CYP51B*. Both itraconazole and eugenol-chitosan nanoparticles elevated the mRNA expression of *CYP51B*. Values are given as the mean ±standard deviation of three independent experiments. A *p*-value of less than .05 represents significant changes compared to untreated control.

on a priority [25]. Among a wide range of pathogenic fungi, *A. fumigatus* as an airborne fungal pathogen has been shown to be responsible for the death of hospitalized patients, especially those with immune deficiency or those undergoing hematopoietic stem cell transplantation [26]. Even with the emergence of intensive treatments against this fungus such as triazole drugs like itraconazole and voriconazole, the mortality rate due to the induction of drug-resistance is still great. In the present study, itraconazole MIC (4 µg/mL) was found to be high against *A. fumigatus* Af293. In agreement with this finding, Verweij et al. (2007) also indicated that only high dosages of itraconazole, voriconazole, and ravuconazole could reduce the survival of different strains of *A. fumigatus* [27]. Despite their effectiveness, factors such as short half-life, insolubility, as well as difficulties in reaching the infection site have muted the enthusiasm for the application of such agents in the treatment of *A. fumigatus* [28]. Therefore, finding a drug with maximum antifungal effects against drug-resistant fungi has been turned into an active field, and recently, much attention has been paid to natural products with minimal side effects. The present study aimed to use eugenol in the form of nanoparticles to address its therapeutic value against *A. fumigatus*. Eugenol was loaded on chitosan, a natural cationic polysaccharide with anti-fungal activities [17-18].

The results showed that when *A. fumigatus* was treated with increasing concentrations of the prepared nanoparticles, the survival capacity of the fungus was reduced in a dose-dependent manner. More interestingly, the anti-fungal property of these nanoparticles was stronger compared to chitosan or eugenol alone. While the nanoparticles at a concentration of 300 µg/mL could significantly reduce the population of *A. fumigatus*, the lowest concentrations of chitosan and eugenol, which could significantly diminish the growth of this fungus were 6000 and 256 µg/mL, respectively. The survival rate of human cells treated with chitosan nanoparticles indicates the non-toxicity of these particles [29]. This finding clearly indicates that the application of nanoparticles made of organic and non-toxic materials could be a better option in the treatment of fungal infections, as they could exert stronger therapeutic impact. It should be noted that the differences in antifungal activity could be due to the fact that these particles are made in a one-to-one mode, and as a result the concentration of components is reduced by half. Thus far, many studies have emphasized the advantages of nanoparticles in the treatment of *A. fumigatus*, especially the green nanoparticles synthesized using natural polymers. It has been shown that amphotericin B–polymethacrylic acid nanoparticles have remarkable therapeutic effects and could prevent the destructive

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effects of aspergillosis on the lungs in murine models[30]. In a study, the effect of selenium nanoparticles was evaluated on *A. fumigatus,* and the results of MIC measurements revealed that at a concentration of 100 µg/mL, these NPs could reduce the population of the fungus[31]. These findings indicate that NPs could significantly be used as a therapeutic option in the treatment of fungal infection.

The induction of drug resistance is one of the main concerns in *A. fumigatus* treatment, and the results of previous studies suggest that this fungus recruits several mechanisms to attenuate the antifungal properties of different drugs[24]. Cytochrome P450s, also known as CYP51, is the target of azoles, which participates in the regulation of sterol synthesis. The importance of CYP51 in the survival capacity of *A. fumigatus* is to the degree that the simultaneous deletion/inactivation of *CYP51A* and *CYP51B*, as two isoforms of CYP51, is lethal for this fungus [32]. Although triazole drugs like itraconazole have been developed to inhibit the fungal CYP51, *A. fumigatus* has found a way to increase the expression of this gene and therefore attenuate the efficacy of these drugs. The up-regulation of *CYP51A* in *A. fumigatus* is associated with the induction of drug resistance against itraconazole[24]. The results showed that the expression of *CYP51A* (5-fold) was significantly increased in *A. fumigatus* treated with itraconazole, suggesting that *A. fumigatus* may exploit *CYP51A* to overcome itraconazole toxicity. Also, the expression of *CYP51A* gene (3.1-fold) in *A. fumigatus* treated with eugenol-chitosan encapsulated nanoparticles was significantly increased in a dose-dependent manner.

The expression of *CYP51B* was upregulated by a 3.7-fold change in *A. fumigatus* treated with itraconazole. The treatment of *A. fumigatus* with the nanoparticles at concentrations of 150, 300, and 600 µg/mL also caused a 1.2, 2.1, and 2.4-fold increase in *CYP51B* expression, respectively. A previosu study declared that the up-regulation of both *CYP51A* and *CYP51B* was associated with the induction of resistance to voriconazole in *A. fumigatus* species[33]. In agreement with this finding, the present study results also showed that the treatment of *A. fumigatus* with these nanoparticles was associated with a remarkable elevation in the expression levels of both *CYP51A* and *CYP51B,* which are involved in the induction of drug resistance mechanisms, as one of the main concerns, and in the regulation of sterol synthesis.

Mousavi et al. (2015) designed a siRNA against *CYP51A* mRNA and suggested that silencing *CYP51A* could reduce the MIC of itraconazole against *A. fumigatus* [34]. Based on the importance of CYP51 gene in the survival of *A. fumigatus*, we aimed to evaluate whether eugenol-chitosan nanoparticles could alter the expression of these genes. The results of qRT-PCR revealed that the expression of both genes increased in the presence of nanoparticles, suggesting the attempts of *A. fumigatus* to overcome the anti-fungal property of eugenol-chitosan nanoparticles. In contrast to this study findings, Bafghi et al. (2021) reported that selenium NPs were able to reduce the expression of *CYP51* in *A. fumigatus*[35]. This discrepancy in the results could be due to the nature of the nanoparticles, as in contrast to our nanoparticles which were composed of natural compounds, the selenium NPs used in Bafghi's study were synthetic.

To the best of our knowledge and as far as we know, this was the first time that the effect of eugenol-chitosan nanoparticles on *CYP51* expression was evaluated. Overall, this study results suggested that eugenol-chitosan nanoparticles had stronger anti-fungal activity against *A. fumigatus* compared to their components. Although these nanoparticles increased the expression of the *CYP51* gene, further studies are required to evaluate

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whether this up-regulation could attenuate the toxic activity of these NPs against the fungus. Moreover, it would be more beneficial to examine the synergistic effects of eugenol-chitosan nanoparticles together with other anti-fungal agents as well as *CYP51* inhibitors.

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Ethical permission: This work was approved by the Ethics committee of TMU.

Conflict of interest: The authors declare no conflicts of interest.

Authors' contribution: Conceptualization: MS-G, MR-A; data curation: MS-G; formal analysis: AN-J, MS-G, MR-A; Investigation: AN-J, MS-G, MR-A; methodology: AN-J; project administration: MS-G; resources: MSG; supervision: MS-G; writing of the original draft: AN-J; writing-review and editing: MSG, MR-A.

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Consent to participate: Written informed consents were obtained from all participants.

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