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GENERAL

RATIONALIZED AND COMPLEMENTARY FINDINGS OF SILYMARIN (MILK THISTLE) IN PAKISTANI HEALTHY VOLUNTEERS

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Abstract: The aim of the work was to examine the influence of gender on pharmacokinetics of silymarin; a basic constituent of medicinal herb "milk thistle" (*Silybum marianum*). The presented work is the extension of published work of Usman et al. (16). The comparative parallel design pharmacokinetic study was conducted in Pakistani healthy volunteers (male and female) receiving a single 200 mg oral dose of silymarin. Sixteen subjects (8 males and 8 females) were enrolled and completed the 12 h study. Blood screening was done on HPLC and the pharmacokinetic parameters were calculated by APO, 3.2 Ver. software using non-compartmental and two compartment model approaches. A significant difference ($p < 0.05$) was observed in almost all calculated pharmacokinetic parameters of silymarin in male and female. Clinically, the silymarin has been underestimated in the previous study. Gender based clinical investigations should be directed in the future on other flavonolignans of 'milk thistle' as well.

Keywords: gender-based pharmacokinetics, milk thistle, parallel design, silymarin, two compartment model

There is a general belief amongst the consumers all over the world that herbal drugs are always safe because they are natural or near to nature but evidence suggests otherwise. In Pakistan, traditional healers (Hakims/Tabibs) are registered by the government under an Act of the Parliament but there is no regulatory control on the manufacture, sale, distribution etc. of traditional medicines (1). Requirements and methods for research and evaluation of the safety and efficacy of herbal medicines are more complex than those for conventional pharmaceuticals. A single medicinal plant may contain hundreds of natural constituents, and a mixed herbal medicinal product may contain several times that number. If every active ingredient would be isolated from every herb, the time and resources required would be tremendous. Such an analysis may actually be impossible in practice, particularly in the case of mixed herbal medicines (2). Female and male have different body compositions. The body fate percentage is larger and the body water content is smaller in female. Furthermore, these differences are age dependent, with body fate increasing in both genders with age. Body fat composition may affect the volume of distribution of many drugs. For lipophilic drugs such as opioids and benzodiazepines, the volume of distribution per kg body weight generally will be higher in females than in males. Conversely, the volume of distribution for water soluble drugs such as muscle relaxant may be lower in females than in males. Thus the same dose per kg body weight will result in a lower

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initial plasma concentration of lipophilic drugs in females, whereas the initial concentration of water soluble drugs will be higher (3). Generally, males weigh more than females, yet dosing of most drugs is not corrected for body weight. For the drugs evaluated by the FDA in the bioequivalence studies, not adjusting for weight resulted in 20%–88% higher AUCs in females compared with males in the dataset where there was a significant sex difference. For drugs with narrow therapeutic ranges or steep dose-concentration curves or both, this may cause significantly increased adverse events in females compared with males. Even within a group of females, not taking into account body weight can affect efficacy. Holt et al. (4) found that if a woman weighed >70.5 kg, she had a 1.6 greater risk of oral contraceptive (OC) failure. With a low-dose OC and weight >70.5 kg, the relative risk (RR) increased to > 4-fold (5). Milk thistle (*Silybum marianum* (L.) Gaerth) is an annual wintering plant belonging to Asteraceae family that reaches a height of 200-250 cm. The capitula are 5-8 cm in diameter, and ovate. The flowers are purple in color. The sunny, stony slopes of the Mediterranean region are the growing locations of warmth loving milk thistle. It is common in countries of Mediterranean region. The ripe fruit of milk thistle contains flavonoids. Silymarin, a flavonolignan from 'milk thistle' (*Silybum marianum*) plant is used from ancient times as a hepatoprotective drug. Along with hepatoprotective action, other actions include antioxidant, anti-lipid peroxidative, antifibrotic, anti-inflammatory, immunomodulatory and liver regenerating. Silymarin has clinical applications in alcoholic liver diseases, liver cirrhosis, Amanita mushroom poisoning, viral hepatitis, toxic and drug induced liver diseases, psoriasis, and has neuroprotective and neurotropic activity. The seeds of milk thistle contain approximately 70-80% silymarin flavonolignans and approximately 20-30% of chemically undefined fraction, composed of mostly polymeric and oxidized polyphenolic compounds. Silymarin is a complex mixture of four flavonolignan isomers, namely silybin, isosilybin, silydianin and silychristin with an empirical formula $C_{25}H_{22}O_{10}$. Among the isomers silybin is the major and most active component and represents about 60-70%, followed by silychristin (20%), silydianin (10%), and isosilybin (5%). The seeds also contain betaine, trimethylglycine and essential fatty acids that may contribute to silymarin's hepatoprotective and anti-inflammatory activities (6, 7). Their mechanisms of action are still poorly understood. However, the data in the literature indicate that silymarin and silibinin act in four different ways: (i) as antioxidants, scavengers and regulators of the intracellular content of glutathione; (ii) as cell membrane stabilizers and permeability regulators that prevent hepatotoxic agents from entering hepatocytes; (iii) as promoters of ribosomal RNA synthesis, stimulating liver regeneration; and (iv) as inhibitors of the transformation of stellate hepatocytes into myofibroblasts, the process responsible for the deposition of collagen fibers leading to cirrhosis. The key mechanism that ensures hepatoprotection appears to be free radical scavenging. Anti-inflammatory and anticarcinogenic properties have also been documented (8). The chemoprotective action of silymarin opened the newer application of silymarin in the field of cancer therapy. The incidence of urinary bladder neoplasms and preneoplastic lesions induced by N-butyl-N-(4-hydroxybutyl)nitrosamine were significantly reduced. Silymarin also significantly inhibited azoxymethane induced colon carcinogenesis in rats. Skin carcinogenesis induced by benzoyl peroxide or 12-tetradecanoylphorbol-13-acetate was also inhibited by silymarin (9). Noteworthy, there is recent evidence of the inhibition of hepatitis C virus (HCV) RNA polymerase by silymarin and results from several small clinical trials suggest that silymarin could be used as an adjunctive therapy for HCV infection (10). It may also help prevent toxin entry into cells or possibly be involved with toxin exportation. Its purported mechanism of hepatoprotection may also include modulation of both phase I and phase II detoxification pathways in a dose dependent manner. In *in vivo* mice models, silymarin was shown to stimulate the phase II detoxification pathway, increasing levels of glutathione and glutathione S transferase, in a dose dependent manner in several tissues, including liver, lung, stomach, small bowel, and skin (11). The bioavailability of enterally administered silymarin is limited; the compound is poorly soluble in water, and only 20-50% is absorbed from the gastrointestinal tract after ingestion. Absorption is significantly enhanced if silybin is administered in a complex with phosphatidylcholine. There is rapid absorption after an oral dose with the peak plasma concentration reached after two to four hours and an elimination half-life of six hours it undergoes extensive enterohepatic circulation. Three to eight percent is excreted in the urine, and 80% is excreted in the bile as glucuronide and sulfate conjugates. Bioavailability can vary up to three-fold depending on the formulation; the brand used in most European studies, Legalon® contains approximately twice as much available silybin as other preparations (12). Low water solubility (0.04 mg/mL) of silymarin is report-

ed. Solubility of silymarin in various other solvents like transcutol, ethanol, polysorbate 20, and glycerylmonooleate is 350.1, 225.2, 131.3 and 33.2 mg/mL, respectively. Silymarin possesses no lipophilic properties, even though its water solubility is poor (13). Apart from the role the physicians have to play in safeguarding the public health, pharmacist's interventions in the appropriate use of herbal medicines are necessary to make the overall health delivery system safe and effective. Pharmacists should therefore be knowledgeable about the medicinal plants, herbal therapies and other herbal based dietary supplements in view of their increasing popularity and utilization so as to be able to provide objective information to the consumers (1, 14). Major activity of Food and Drug Administration (FDA) Office of Women's Health (OWH) is ensuring the development of consistent regulatory policies relating to the participation of women in clinical trials, and analysis of the data to detect gender differences. Whenever appropriate, OWH ensures that this information is incorporated into product labeling (15). The presented work is the extension of Usman et al. study (16). In the previous work, pharmacokinetic study was conducted only on male. In the present work, both the gender, male and female, were included in the study to encompass any difference in pharmacokinetics of silymarin due to gender variation.

MATERIALS AND METHODS

Materials

Silymarin standard (98%, HPLC) was provided by Abbot Pharmaceuticals Pvt. Ltd. Karachi, Pakistan. Methanol (96%, HPLC), potassium dihydrogen phosphate (98%, analytical) and phosphoric acid (85%, analytical) were obtained from Merck, Germany. Silymarin 200 mg tablets manufactured by Abbott Pharmaceuticals Pvt. Ltd. Karachi, Pakistan were purchased from the local market. All reagents and solutions throughout the research were prepared in fresh stock (not older than one week) of distilled water.

Instruments

Water was distilled by Water Distillation Apparatus (Köttermann). The pH of distilled water was confirmed *via* Cyber Scan pH meter before and after use. The analysis was carried out using HPLC (Shimadzu LC.9, CSW 32 Ver. 1.3 Software, Japan) equipped with a degasser, quaternary pump, manual sampler and a UV detector (Shimadzu SPD-6AV) connected to data collection system.

Chromatographic conditions

The analytical column was a Brownlee MPLC 5 µm (220 × 4.6 mm, pore size 80 Å) with packing C18 (RP18, ODS, Octadecyl) from Perkin Elmer, maintained at ambient room temperature. The extraction and emission wavelength were adjusted at 288 nm.

Preparation of mobile phase

The mobile phase was prepared by dissolving 2.72 g of potassium dihydrogen phosphate in methanol quantity sufficient to make final volume of 1 L with pH 2.8 adjusted with phosphoric acid and filtered through 0.45 µm membrane filter prior to use.

Preparation of standard and working solutions

Working solutions were prepared in mobile phase by 0.3, 0.6, 1.25, 2.5, 5 and 10 µg/mL dilutions. A stock solution of silymarin standard was prepared freshly by dissolving 50 mg drug in 50 mL of methanol to give a final concentration of 1 mg/mL.

Extraction method

The extraction procedure and other factors were kept constant to see the influence of gender only on pharmacokinetics of silymarin. A hundred milliliters of human blood sample was collected from blood bank of UVAS. The plasma was separated from blood by centrifugation (17). The extraction procedure was carried out as described by Usman et al. (16). Briefly, 100 µL of acetate buffer at pH 5.6 and glucuronidase type HP-2 (30 µL) (Helix pomatia, Merck, Germany, 127300 units/mL) were added to a 100 µL of plasma sample and this mixtures were incubated at 37^{0}C for 2 h with periodical shaking. Then, 200 µL of borate buffer (pH 8.5) and 2 mL of diethyl ether were added to the mixture. The mixture was vortexed for 1 min and centrifuged at 3000 rpm for 2 min. Then, organic phase was transferred into a sample test tube and evaporated under nitrogen steam. The residue was reconstituted in a 130 µL aliquot of the mobile phase, vortexed for 30 s and centrifuged for 1 min at 2500 rpm, and then 100 µL of the solution was injected directly into the chromatographic system. The same extraction method was implemented on blood samples of the test subjects.

Subjects and materials

Sixteen healthy Pakistani volunteers (8 male and 8 female) ranging from 18 to 45 years and from 50 to 80 kg in age and weight, respectively,

were selected. Alcoholic, drug abused, hepatitis B & C positive, pregnant and known history of hypersensitivity with the drug under study volunteers were rejected according to the inclusion and exclusion criteria of therapeutic ethical committee of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. Additionally, the volunteers who had bleed and/or taking any medicine within three months before initiating the study as well as who were incapable of understanding written consent were also abandoned from the study. Written informed consent was taken from each volunteer who received a dose of silymarin 200 mg orally. Each subject was fasted after midnight (00:00 a.m.) before the administration of the drug in next morning (9:00 a.m.). The subjects continued to fast for 3 h after administration. Volunteers were housed at blood collection center throughout the period of blood sampling. Eight samples of 5 mL blood each were collected over a period of 12 h from each subject at predefined time schedule of zero, 0.5, 1, 2, 3, 5, 8 and 12 h after silymarin administration and stored in heparinized glass tubes. The plasma was harvested from blood cells by centrifugation and stored at -40^{0}C until analysis.

Pharmacokinetics and statistics

The absorption and elimination kinetics profile of silymarin was determined by software APO PC-Computer Program, MWPHARM version 3.02, MEDIWARE, Holland. The program determines compartmental and non-compartmental analysis in calculation of the bioavailability and elimination kinetic parameters. GraphPad Prism 5 was used to apply unpaired *t* test for statistical analysis of the data.

Validation and optimization

Some parameters tested during the validation process were: system suitability, selectivity and linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision.

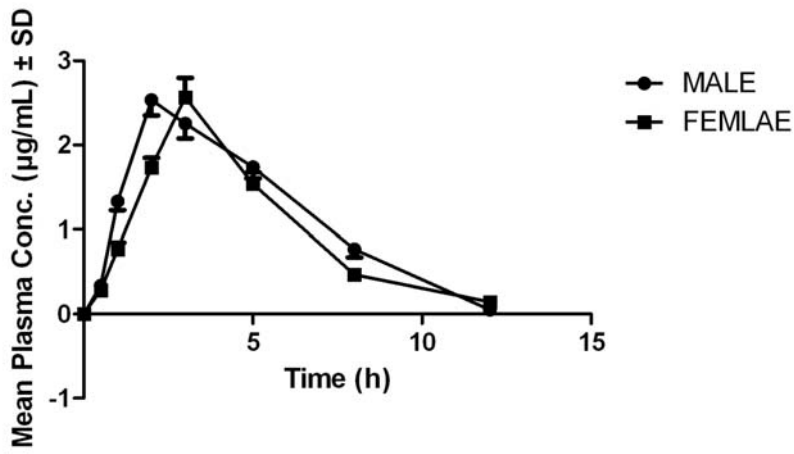

Figure 1. Mean plasma concentrations ± SD and time profile of healthy male and female volunteers

Table 1. Demographic data for volunteers (n = 8).

	Males				Females			
	Age (year)	Weight (kg)	Height (cm)	BMI	Age (year)	Weight (kg)	Height (cm)	BMI
Minimum	19	50	149	22	19	50	145	22
Maximum	29	68	169	24	27	62	162	25
Mean	23	58	158	23	23	56	154	24
SD*	3.6	6.3	7.8	0.81	2.4	4.6	6	1.1
CV**	15.50%	10.91%	4.93%	3.50%	10.46%	8.27%	3.87%	4.82%

*SD = standard deviation, **CV = coefficient of variance.

Table 2. Validated performance of treated samples (mean ± SD).

Parameters	Properties test	No. of treatment	Test conditions	Results	Acceptance criteria	Remarks
System suitability	Retention time (Tr) (min)	n > 3	Normal (predefined) and stress study (accelerated stability study)	[silychristin, silydianin, silybin A and silybin B] [4.62 ± 1.19 (%RSD 0.069), 12.09 ± 0.9 (%RSD 0.37), 12.32 ± 2.1 (%RSD 0.76), 14.4 ± 3.4 (%RSD 0.37, respectively)]	RSD = 2.0%	No interfering peaks
Selectivity and linearity	Goodness of fit test	n > 3	predefined	y = 13.87x + 5.7505, r^2 = 0.999	r^2 > 0.9	Results were reproducible within the stated range
LOD and LOQ (µg/mL)	Qualification and quantification	n > 5	predefined	0.036 ± 0.01, %RSD 7 0.06 ± 0.005, %RSD 0.7, respectively	%RSD ≤ 1.0%	-
Precision (% CV) and accuracy (%)	Intra-day and inter-day variations	n > 5	predefined	1.2-9.5%, ≥ 93%, respectively	% CV ≤ 10%, 85-105%	Higher % CV (≥ 10%) was observed at lower conc. (0.03 µg/mL)

Stability study

Chemical stability of silymarin in plasma was assessed in accordance with the Guidelines for industry: Bioanalytical method validation (18) (results were not shown).

RESULTS

The objective of proceeding extraction procedure of Usman et al. (16) was to encompass the influence of gender only. The demography of the volunteers has been shown in Table 1. The bioanalytical method was validated prior to quantify the drug in subject samples. Extraction efficiency (EE) of silymarin as silychristin, silydianin, silybin A and silybin B (chromatogram was not shown) was measured by expression: .

$$E.E = \frac{\Sigma(AUC)_{Extracted}}{\Sigma(AUC)_{Non\text{-}extracted}} \times 100$$

The % recovery (EE) was 89-103% with bias -13 to +17 at stated concentrations. Other parameters of validation process are given in Table 2. The results of the developed method were revalidated after one month and inter-day precision was defined. The mean plasma concentration ± SD *versus* time profiles of silymarin for the gender (male and female) is shown in Figure 1. The absolute percentage difference between the means of calculated pharmacokinetic parameters of male and female was 9.52-100. The mean values of time for maximum concentration (T_{max}), half lives (absorption, phase I and phase II), volume of distribution (V_d), volume of distribution in compartment 1 (V_{di}), volume of distribution steady state (V_{dss}), clearance (CL) and mean residence time (MRT) were higher in females while the values of peak plasma concentration (C_{max}), area under curve (AUC), AUC polyexponential, AUC trapezoidal, absorption rate constant (k_a), elimination rate constant from compartment 1 (k_{10}), transfer constants (k_{12} and k_{21}) and lag time were lower in females as compared to those in males (Table 3). There was significant difference ($p < 0.05$) in all calculated pharmacokinetic parameters between the male and female subjects except transfer constant k_{12} ($p > 0.05$). High significant difference ($p = 0.0001$) was observed in C_{max}, V_{di}, V_{dss} and AUC, especially if calculated by trapezoidal rule. If

we compare the result with those of Usman et al. (16), from minor to major variations were observed in the reported pharmacokinetics parameters (Table 4). In comparison with Silliver®, the results of T_{max} and AUC increased ~13% to ~19%, respectively, in male. A decline was observed in the values of C_{max}, CL, MRT and V_d and amounted: ~4, ~11, ~16 and ~62%, respectively. Comparing the results with Silimarin®, AUC, T_{max} and C_{max} were increased by ~15, ~19 and ~47%, respectively, while the results of CL, MRT and V_d were reduced by ~9, ~37 and ~69%, respectively, in male. On the other hand, in comparison of female with Silliver®, an increment of ~4 and ~37% was observed in AUC and T_{max}, respectively, in female. The results of MRT and CL were also increased (~5%) comparatively in female. A decline of ~36 and ~41% were observed in C_{max} and V_d, respectively, in female. Comparing the results with Silimarin®, CL and T_{max} were increased by~7 and ~44%, respectively, in female. The results of MRT and V_d were decreased ~23 and ~53%, respectively, in female. Zero to negligible change (<

Table 3. Pharmacokinetics of silymarin for male and female volunteers (mean ± SD).

Parameters	Males	Females	p value	Results
Peak concentration (C_{max}) [µg/mL]	2.79 ± 0.35	1.86 ± 0.12	< 0.0001	*sig
Time to peak (T_{max})	2.14 ± 0.26	2.6 ± 0.12	0.0004	*sig
Area under curve (AUC) [µg×h/mL]	12.82 ± 0.77	11.27 ± 1.01	0.004	*sig
AUC polyexponential (t = 12)	12.79 ± 0.75	11.21 ± 0.99	0.0029	*sig
AUC trapezoidal rule (t = 12)	14.23 ± 0.68	12.08 ± 0.94	0.0001	*sig
Half life phase I	1.19 ± 0.2	1.53 ± 0.09	0.0006	*sig
Half life phase II	1.19 ± 0.2	1.55 ± 0.1	0.0006	*sig
Absorption half life	1.19 ± 0.2	1.54 ± 0.1	0.0006	*sig
Absorption rate constant (k_a) [1/h]	0.6 ± 0.09	0.45 ± 0.03	0.0009	*sig
Rate constant (k_{10}) [1/h]	0.6 ± 0.09	0.45 ± 0.03	0.0009	*sig
Rate constant (k_{12}) [1/h]	0.75 ± 2.11	$2.53e^{-005}$ ± $7.10e^{-005}$	0.3343	**ns
Rate constant (k_{21}) [1/h]	0.6 ± 0.09	0.45 ± 0.02	0.001	*sig
Volume of distribution (V_d)	26.74 ± 3.6	39.74 ± 2.7	< 0.0001	*sig
Volume of distribution in compartment 1 (V_{di})	26.74 ± 3.6	39.68 ± 2.71	< 0.0001	*sig
Volume of distribution steady state (V_{dss})	26.74 ± 3.6	39.68 ± 2.71	< 0.0001	*sig
Clearance (CL) [l/h]	15.66 ± 0.89	17.87 ± 1.66	0.0051	*sig
Mean residence time (MRT)	3.85 ± 0.55	4.83 ± 0.27	0.0005	*sig
Lag time	0.42 ± 0.04	0.38 ± 0.02	0.024	*sig

*sig = significant, **ns = not significant.

Table 4. Comparison of pharmacokinetics of silymarin (200 mg orally administered in male and female) with published work of Usman et al. (16).

Present study			Usman et al. (16)	
Parameters	Silymarin 200 mg		Silliver®	Silimarin®
	Male	Female		
Peak concentration (C_{max}) [µg/mL]	2.79	1.86	2.9	1.9
Time to peak (T_{max}) [h]	2.14	2.6	1.9	1.8
Area under curve (AUC) [µg×h/mL]	12.82	11.27	10.8	11.2
Mean residence time (MRT) [h]	3.85	4.83	4.6	6.1
Volume of distribution (V_d) [L/kg]	0.46	0.71	1.2	1.5
Clearance (CL) [mL*h/kg]	270	319.11	303.5	297.4

1%) was observed in case of C_{max} and AUC, respectively, in female.

DISCUSSION

Numerous studies have shown a gender difference in the pharmacokinetics of many drugs. Pharmacokinetic differences arise because of differences in endogenous and exogenous hormones, differences in body size and fat compositions, and difference in liver metabolism. Approximately 50% of the drugs currently on the market are metabolized by cytochrome P-450 isozyme 3A4.Women appear to have higher levels of 3A4 than men (15, 19). The results of accuracy and precision showed that the extraction method was accurate and reproducible for plasma concentrations. It was inferred from the statistical analysis that pharmacokinetics of silymarin behaved differently in male and female that might be due to reduced liver blood flow and lower clearance of the drug in female volunteers as compared to male (3). As silymarin was the lipophilic extract of "milk thistle" that made it a potential candidate to reach in deep tissues of the body, especially in females that had high fat content as compared to male. This factor was led to increase of ~12, ~18, ~20, ~23 and ~33% in mean values of CL, T_{max}, MRT, half lives (phase I and phase II) and volumes of distribution (V_d, V_{di}, V_{dss}) in female (Table 2) (20, 21). Under very general assumptions, the area under the plasma or blood drug concentrations is a parameter that is closely dependent on the drug amount that enter into the systemic circulation and on the ability that the system has to eliminate the drug (clearance) (22). On the basis of higher mean values of AUC and C_{max} (12% and 33%, respectively) in male as compared to female, it is expected that much better bioavailability of silymarin will be achieved in male. As much of the drug was transferred from central compartment to deep tissue in female because of high fat contents that led to reduced amount to reach systemic circulation and resulted in low AUC and C_{max} in female as compared to male. The rate of absorption and distributions (k_a, k_{10}, k_{12} and k_{21}) was high in male as compared to female that might be due to difference in life style of Pakistani males and females. The ratio of fast food intake, use of tea, exercise etc., is high in Pakistani males as compared to females. All these factors directly influence the function of GIT. Two compartment model approach was used in this study to calculate pharmacokinetic parameters as compared to the previous study of silymarin by Usman et al. (16). The variation in pharmacokinetics of silymarin in comparison with the previous study might be due to demographic difference in gender.

CONCLUSIONS

On the bases of observed variations in pharmacokinetics of silymarin in male and female it will be right to say that gender based evidence should be provided if clinical study is reported especially on human being. It is evident that the dose of silymarin must be adjusted prior to administration according to demographic parameters of the patient. Furthermore, the presented work demands further research to conduct on larger population in the future to make a concrete decision about the dose adjustemnt of silymarin in male and female. It is also recommended by the authors that *In Vitro In Vivo* Correlation (IVIVC) shoud be developed for silymarin to predeict bioavalibity of the newly developed formulatons (23, 24).

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Conflict of interest

The authors declare that there is no financial support and conflict of interest related to the content of this manuscript.

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