Original Research

Fluorescence monitoring of a photosensitizer and prediction of the therapeutic effect of photodynamic therapy for port wine stains

Ying Wang[1], Ying Gu[1], Xiaohua Liao[2], Rong Chen[3] and Huiying Ding[1]

[1]Department of Laser Medicine, Chinese People's Liberation Army General Hospital, Beijing 100853; [2]Fujian Metrology Institute, Fuzhou 350003; [3]Institute of Laser, Fujian Normal University, Key Laboratory of Photonic Technology of Fujian Province, Fuzhou 350007, China
Corresponding author: Ying Gu, Department of Laser Medicine, Chinese People's Liberation Army General Hospital, No. 28 Fuxing Road, Haidian District, Beijing 100853, China. Emails: guyinglaser@sina.com, 25wy@sohu.com

Abstract

In this study, a fluorescence method was established to obtain the local concentration of a photosensitizer (PS) based on the realtime fluorescence measurement of skin with port wine stain (PWS) during photodynamic therapy (PDT). This algorithm corrected for the distortions of PS fluorescence spectra imposed by the absorption of melanin and hemoglobin in skin and other factors, which yields a semi-quantitative measurement of PS concentration. Based on this information, a therapeutic effect correlation index (TECI) was proposed as the area under the PS concentration–time curve during PDT. The correlation between TECI and PDT treatment outcome was analyzed from 31 PWS patients. The measured PS fluorescence spectra showed that under the same PS dose, there were clear variations in the concentrations of the PS during PDT. Statistical analysis showed that TECI has a positive correlation with PDT outcome. Patients with a higher TECI value had a better treatment outcome. These results suggest that fluorescence spectroscopy can be used *in situ* to monitor skin PS concentration during PDT and to provide a valuable diagnostic tool to predict PDT outcome.

Keywords: photodynamic therapy, port wine stain, photosensitizer, fluorescence spectroscopy

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Introduction

Photodynamic therapy (PDT) has been clinically used in the treatment of port wine stain (PWS) for 20 years.[1–3] This treatment method has an advantage over pulse laser treatment, which cannot clear the red color for most PWS patients and may induce thermal damage to the epidermis. It is therefore the preferred method for patients with PWS with yellow skin. However, the design of therapeutic regimens lacks objective outcome measurements and is mainly based on the experience of clinicians. PDT therapy requires the simultaneous presence of light, photosensitizer (PS) and oxygen. Therapeutic efficacy is dependent on light fluence rate, PS concentration and tissue oxygenation level. Optimal treatment outcome can be achieved only when these three parameters are closely matched. If local PS concentration can be monitored during PDT, and concomitantly the light dose can be adjusted, treatment efficiency can be greatly improved.

PWS is a congenital birthmark histologically characterized by ectatic vessels within the papillary and reticular dermis.[4] The goal of PDT for PWS is to clear the red color of PWS by selectively destroying the ectatic blood vessels without destroying the normal epidermis and dermis. In clinics, most PWS patients usually need at least three periods of treatment to destroy the ectatic vessels layer by layer. As the ectatic vessels are superficially located in the skin, the treatment laser (usually a yellow or green laser) can generally penetrate into the target vessels.[5] By adjusting laser wavelength or power density according to PWS depth, the deposition of laser energy in target tissues can be controlled.

To the best of our knowledge, there are no standard non-invasive methods of estimating the local concentration of PS *in vivo* during PDT for PWS. Previous attempts on monitoring PS concentration usually use fluorescence spectra from the tissue containing the PS. Unfortunately, accurate determination of PS concentration is very complicated, as the measured fluorescence emission intensity depends not only on the concentration of PS but also on the measurement geometry and the absorption and the scattering properties of tissue at the excitation and emission wavelengths. Hence, it is necessary to establish a reliable spectral

analysis method to eliminate the absorption of melanin and hemoglobin. Moreover, it is usually difficult to find out the relationship between PS fluorescence intensity and absolute PS concentration *in vivo*, and only the relative concentration of PS can be measured. The aim of this study is to establish a fluorescence method not only providing a semi-quantitative measurement of PS concentration but also predicting PDT efficacy.

In this study, the fluorescence spectra of PS during PDT were monitored, and an algorithm was established to correct the distortions imposed on the fluorescence spectra by the absorption of melanin and hemoglobin. Based on the relationship between measured fluorescence intensity of PS during PDT and treatment outcome, a therapeutic effect correlation index (TECI) was calculated. Data show that this index can help predict the therapeutic effect of PDT, which opens up many opportunities in adjusting the PS administration mode and dosage regimen design in the following treatment.

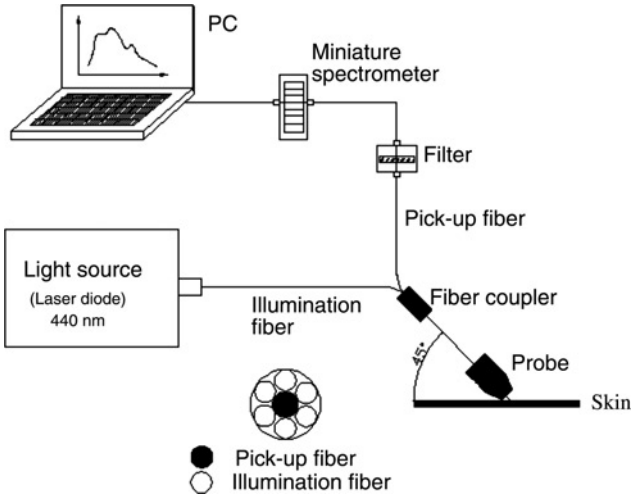

Figure 1 Fluorescence measurement system. A diode laser, Y-type fiber, filter and computer-controlled spectrometer form the fluorescence measurement system. The measurement arm consists of one pick-up fiber (in the center) and six illumination fibers (at the periphery). The tip of the fiber was held 45° to the surface of the skin during measurement

Materials and methods

Materials

PSD-007 (photocarcinorin) (10 mg/mL) was provided by the Institute of Pharmacochemistry of the Secondary Military Medical University and stored at -20°C.

Patients

Thirty-one patients (18 of them had one region treated in one PDT course and the other 13 had two regions treated in one PDT course, a total of 44 treatment regions) with congenital facial PWS were recruited for the study; all of them were undergoing PDT treatment at the Department of Laser Medicine of Chinese PLA General Hospital between October 2006 and May 2008. Informed consent was obtained from each patient.

Fluorescence spectrometer

This instrument (Figure 1) is composed of a 440 nm laser diode (B&W TEK Inc., Newark, DE, USA) with a stable output, an optical fiber and a USB2000+ miniature fiber optic spectrometer (Ocean Optics Inc., Dunedin, FL, USA). Fluorescence emission from the tissue was collected by a Y-type fiber, and after suitable filtering (437 ± 10 nm), the spectra from 400 to 800 nm were displayed (integration time 4 ms). Acquisition of the spectra was carried out with the software OOIBase32. During measurements the tip of the fiber was held 45° to the surface of the tissue to avoid the influence of mirror reflection. The acquisition of complete fluorescence emission spectra took less than 1 s, and any PDT effect generated by the excitation light was presumed to be minimal.

PDT treatment and fluorescence spectroscopy measurements

PSD-007 was administered by intravenous injection at a dose of 4–5 mg/kg body weight. Laser irradiation of 532 nm was performed immediately after PSD-007 injection. *In vivo* fluorescence spectra of PWS skin and normal skin were recorded (three different areas of the same plaque) before, immediately and 10, 20, 30 and 40 min after PSD-007 administration and irradiation.

Spectra analysis and calculation of TECI

According to the preliminary experiment, the observed fluorescence spectrum of PDT treated skin is the superposition of skin autofluorescence, fluorescence of PS and photoproducts.[6] It also depends on the absorption and scattering of excitation light by the tissue as well as the absorption of emitted fluorescence absorbed by the tissue. In skin, the major absorption components are hematoglobin and melanin, which can absorb part of the emitted fluorescence (400–800 nm) and part of the excitation laser (440 nm) (Figure 2). As different types of PWS usually have different

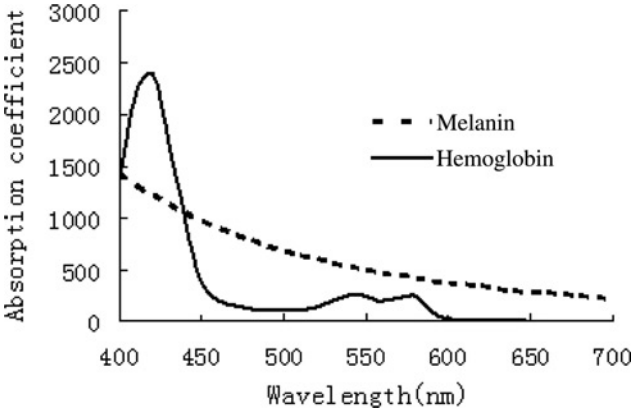

Figure 2 Absorption spectra of melanin and hemoglobin in water. Data were cited from reference[7,8]

hemoglobin content in skin, and the melanin content also differs, the measured primal PS fluorescence peak intensity does not present the real PS content, and cannot be compared between patients.

In the present algorithm, two processes were used to correct the distortions of absorbers on emission and excitation light, respectively.

First, the content or percentage of each component can be obtained by decomposing the fluorescence spectra into the contributions of individual components (fluorophores and absorbing chromophores). So each fluorescence spectrum was fitted to a linear combination of the three fluorescence components. Thus,

$$F_{Total}(\lambda) = c_S f_{Auto}(\lambda) + c_H f_{PS}(\lambda) + c_P f_{Pro}(\lambda) - aA_{Hb} - bA_{Mel} \quad (1)$$

where $f_{Auto}(\lambda)$, f_{PS} and f_{Pro} are the normalized fluorescence line shapes of normal skin, PSD-007 and photoproducts, respectively. A_{Hb} and A_{Mel} are the normalized absorbance spectra of hematoglobin and melanin. C_S, C_H, C_P, a and b present the fractional percentages of their respective fluorescence spectra. The autofluorescence spectrum of normal skin was obtained by the measurement of skin without PS, and the fluorescence spectrum of PSD-007 was red-shifted from that in solution by 5 nm. The line shape of photoproducts was obtained from the spectra of laser-irradiated PSD-007 solution. The absorbance spectra of hemoglobin and melanin were cited from reference.[7,8] The normalized fluorescence line shapes of each component were taken as the basic spectra of each component and were substituted into equation (1), and then the fractional percentages of each component were resolved by least-squares fitting.[9]

$$C = (\, c_S \quad c_H \quad c_P \quad a \quad b \,)' \quad (2)$$

Then, the absorption of emitted fluorescence (400–800 nm) by hemoglobin and melanin was removed.

Second, a ratio (FR) between c_H and c_S was calculated for each time point to remove the effect of hemoglobin, melanin and other factors on the excitation laser (440 nm). The use of this ratio corrected for the variation of incident light power arising from differences between absorbing chromophore content, and also corrected for the tiny variation of incident light power caused by the tiny change of the distance between fiber and skin surface during spectra measurement. This ratio can present a semi-quantitative PS concentration.

In this study, it was firstly proposed that during PDT, higher PS concentration will lead to a better treatment outcome. To describe the change of PS concentration during the whole treatment, the ratio (FR) was plotted as a function of time to yield a correlation curve for each patient. We calculated the area under the curve and denoted it as TECI. In the following experiments, we examined the correlation between TECI and PDT efficiency.

Clinical evaluation of PDT efficacy

The blanching of the red color of PWS was used as an indicator for PDT efficacy. Patients were recorded two or three months after PDT for PWS. Therapeutic effectiveness was evaluated according to the criteria[10] listed in Table 1.

Statistical analysis

Statistical analysis was performed using SPSS (Statistical Program for Social Sciences) software. The correlation between TECI and treatment outcome was analyzed using Spearman's rank correlation analysis. A difference was considered to be statistically significant with P values <0.05.

Results

PWS fluorescence spectra during PDT

The measured fluorescence spectrum of PDT treated PWS skin is the superposition of skin autofluorescence, fluorescence of PS and photoproducts. The typical fluorescence spectra of PWS skin before and during PS injection are shown in Figures 3a and b. Native skin autofluorescence peaks around 510 nm, and after PSD-007 injection, two peaks (around 625 and 685 nm) appeared. Fluorescence peaks of 625 and 685 nm were from the fluorescence peaks of PSD-007 in albumin solution, as indicated in Figure 3c. In Figure 3b, a small peak around 670 nm can be observed in PWS skin fluorescence spectra, which was the fluorescence signal peak of photoproducts generated after PSD-007 was irradiated, as shown in Figure 3d. The fluorescence of the photoproducts in skin was weaker than in solution.

The measured PWS skin fluorescence spectrum during PDT of one patient is shown in Figure 4. The PS fluorescence signal around 625 nm rose at first and then declined during PDT, while only a little fluctuation was observed in the autofluorescence signal of the skin.

Example of calculated TECI values from different PWS patients

The measured fluorescence spectrum showed that under identical conditions of PS dose and light dose, significant variability was found in the dynamic change of PS fluorescence signal. The curve of FR to treatment time and calculated TECI of four patients are listed in Figure 5; the absolute FR value at each time point varied, and the change trend (such as the time point that the FR value arrived at the tiptop) varied between patients. In these four patients, those with higher TECI had a better treatment

Table 1 Criteria of treatment outcome

Grade	Clinical description
I	Become normal skin
II	Slight residual color
III	Obvious lightening
IV	Minimal lightening
V	No change

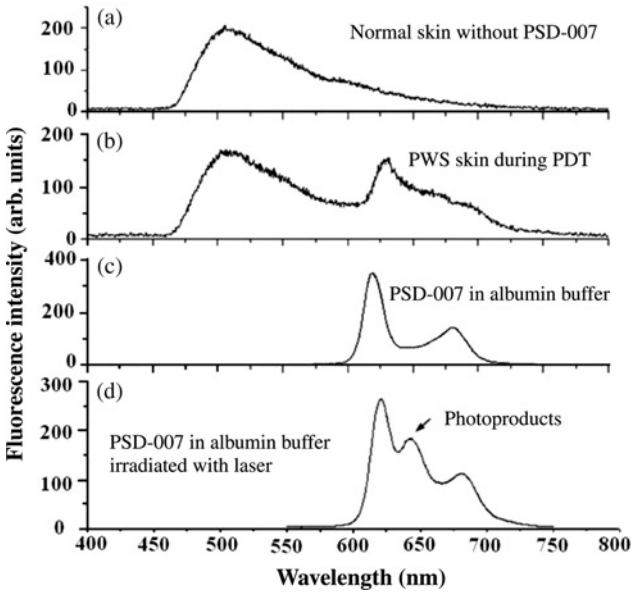

Figure 3 Fluorescence spectra of major fluorophores in skin. (a) Autofluorescence spectra of skin, with a peak around 510 nm. (b) Fluorescence spectra of skin during photodynamic therapy (PDT), with three peaks (at 510, 625 and 685 nm) and a tiny peak at around 670 nm. (c) Fluorescence spectra of photosensitizer (PSD-007) in albumin buffer (concentration of albumin is 50 μmol/L), with two peaks at 625 and 685 nm. (d) Fluorescence spectra of photosensitizer (PSD-007) irradiated with 532 nm laser. 670 nm is the peak of photoproducts generated by PSD-007

outcome, but those with lower TECI had a poorer treatment outcome.

Correlation between TECI and PDT treatment outcome

The calculated TECI values of 44 treatment regions from 31 patients are listed in Figure 6. They were sorted out according to the corresponding blanching grade. The following trend can be readily seen: in blanching grade II, patients had a higher TECI value. And with the TECI value increasing, the value of blanching grade decreased (i.e. treatment outcome became better). The higher TECI value indicated a more efficient treatment outcome.

TECI values were classified into four levels: 0–10, 11–20, 21–30 and 31–40. In each blanching grade, the number of treated regions was counted according to the four levels. TECI was positively correlated with the treatment outcome of PDT for PWS ($r = 0.7574$, $P < 0.05$).

Discussion

This study explored the utility of fluorescence spectroscopy for providing semi-quantitative measures of local skin PS concentration, and tried to correlate the measured fluorescence signals with the therapeutic effect of PDT. The results showed that TECI has a positive correlation with treatment outcome. Patients with TECI values between 11 and 20 usually get blanching grade between II and III. To get a better blanching grade, such as II, the TECI value is usually required to be above 20.

Previously, studies on the correlation between PS fluorescence information in tissue and PDT efficiency mainly focus on PDT for tumors.[11–13] Fluctuations in the degree of tumor destruction between patients were found to be related to mucosal drug uptake as measured by laser irradiated fluorescence.[12] As the measurement of absolute values of PS concentration by fluorescence detection *in vivo* is impracticable, the focus was then concentrated on the relative change of PS fluorescence in tissue. Scientists[14] found that the overall amount of PS photobleaching and the initial photobleaching rate correlate with the amount of PDT damage (i.e. yield of singlet oxygen). During PDT, reactive oxygen substances (ROS, singlet oxygen is the main reactive oxygen substance contributing to PDT damage of porphyrin-based PS) generated by the photochemical reactions between PS and laser may degrade PS itself. This degradation is often referred to as 'photobleaching', because the photoproducts will usually have less photosensitivity. Wilson *et al.*[15] proposed the concept of using PS photobleaching as an implicit dosimetric parameter for PDT, as they think the more singlet oxygen is generated, the more PS may be photobleached. However, Iinuma *et al.*[16] found that the photobleaching process of PS is complicated, with the possibility that there exist two different photobleaching processes for PS *in vivo*, one is oxygen-dependent (singlet oxygen is generated) and the other is oxygen-independent, and that the measured photobleaching amount is a composite of the two processes. Hence, the rate of PS photobleaching may not always be an appropriate monitor for singlet oxygen availability.

Moreover, in PDT for PWS, PS photobleaching is not a suitable indicator for PDT efficiency, even if the photobleaching process of PS is singlet oxygen-dependent. In PDT for tumors, laser irradiation starts at 24 or 48 h after PS injection, and the concentration of PS in the target

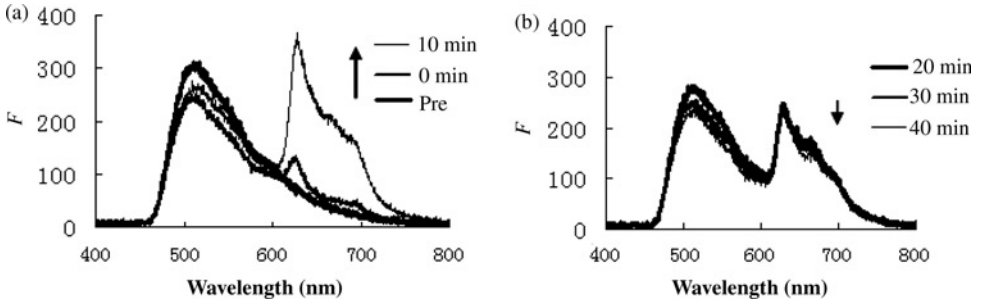

Figure 4 Change of port wine stain skin fluorescence spectra for one patient during photodynamic therapy. The photosensitizer fluorescence signal around 625 nm rose firstly and then declined during photodynamic therapy, while only a little fluctuation was observed in the autofluorescence signal of skin (510 nm)

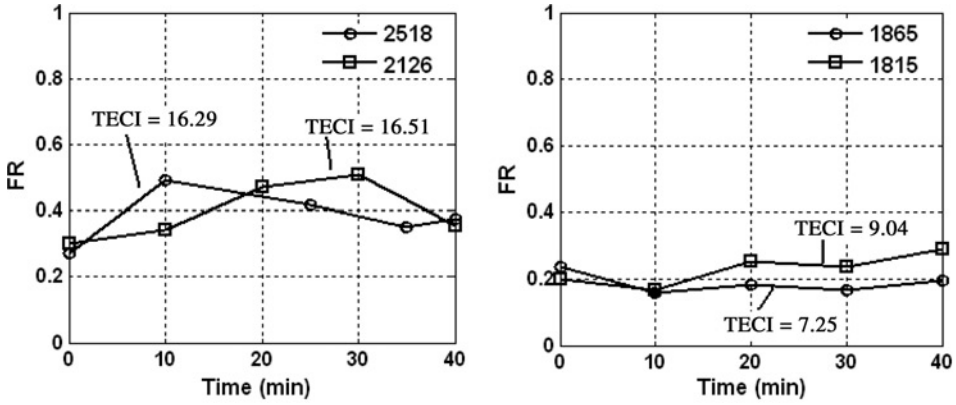

Figure 5 Change of photosensitizer fluorescence signal (FR) for four port wine stain patients during photodynamic therapy. The dynamic change of photosensitizer fluorescence signal was different for these four patients. There was also a significant variability in therapeutic effect correlated index (TECI) and treatment outcome. The TECI and treatment outcome of each patient (No. 2518, 1815, 2126 and 1865) were 16.29 (II), 9.04 (IV), 16.51 (III) and 7.25 (IV), respectively

tissue mainly depends on photobleaching. While in PDT for PWS, laser irradiation starts immediately after PS injection, the concentration of PS in target vessels depends not only on the photobleaching but also on the pharmacokinetics, PS accumulation and metabolism in target tissues. Photobleaching information is hard to extrapolate. Therefore, to implement realtime treatment supervision and feedback, other key parameters related to PWS treatment outcome should be defined.

In this study, a TECI was proposed and validated. Our results showed that TECI has a positive correlation with PDT outcome. Patients with a higher TECI value had a better treatment outcome. It is similar to the concept of AUC (area under the plasma concentration time curve, which is used to measure pharmacokinetic parameters of drugs). If the concentration of PS sustains at a higher level during the whole PDT process, more singlet oxygen can be generated to damage the target vessels.

As previously introduced, most PWS patients usually need at least three periods of treatment to destroy the ectatic vessels layer by layer to avoid destroying the normal epidermis and dermis, which may lead to scars. In clinics, for new PWS patients the treatment plan is based on prior experience of the physicians, according to the age, skin color and PWS type of the patient. For PWS patients treated before, adjustment of the treatment plan is made at each following treatment course, based on the response of treated skin, such as thickness of the scab, degree of pigmentation and degree of color blanching.[17]

Under the same light dosage and PS dosage, the results of PDT vary between patients. Good treatment outcome depends much on the experience of the doctor. Doctors with less experience usually make a treatment plan conservatively; this may increase the times of adjustment, extend the period of treatment and add more economical burden to patients. Hence, a realtime feedback system is ideally suited to achieve individualized optimization.

The fluorescence monitoring method of PS established in this study can provide semi-quantitative information of PS concentration, which can help doctors adjust PS administration mode and dosage regimen more objectively based on the PS concentration level during the whole treatment for every patient. To date, no other method is available to provide in-site treatment monitoring. There is still plenty of work needed to improve the current fluorescence monitoring method. The wavelength of the excitation laser (440 nm) may not be long enough to reach the deep ectatic vessels located in the dermis. A longer wavelength (such as 532 or 578 nm) can be used as the excitation laser in future studies. As they are the treatment wavelengths, measurement can be made using a treatment laser with stable output.

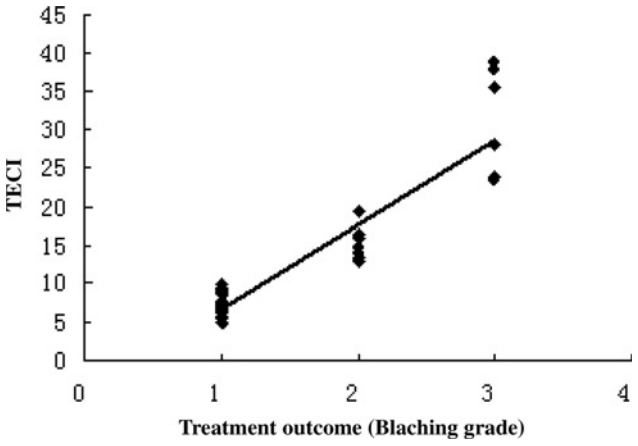

Figure 6 Calculated therapeutic effect correlated index (TECI) of 44 treatment regions and corresponding blanching grade (treatment outcome). The *X*-axis represents the four blanching grades. 1, 2, 3 and 4 represent VI, III, II and I, respectively

Conclusions

The calculated TECI values correlate positively with PDT outcome. These data suggest that fluorescence spectroscopy can not only be used to monitor skin PS concentration during PDT for PWS, but can also be used to predict treatment response. Realtime fluorescence measurements may improve the treatment for each patient by adjusting the individualized choice of PS dose and administration method in the following treatment.

Author contributions: YG, YW, RC and XL participated in the study design; YW and XL conducted the experiments, data collection and analysis of the data; RC supplied critical equipment (fluorescence spectrometer); YW wrote the manuscript; YG and HD contributed to manuscript preparation. All authors participated in interpretation of the studies and review of the manuscript.

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