The influence of PM$_{2.5}$ on lung injury and cytokines in mice

JIE YANG$^1$, YI CHEN$^2$, ZHI YU$^3$, HUI DING$^3$ and ZHONGFU MA$^2$

$^1$Department of Dermatology, Guangdong Academy of Medical Sciences (Guangdong General Hospital); $^2$Department of General Internal Medicine, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510080; $^3$Research Center of Intelligent Transportation System, School of Engineering, Sun Yat-sen University, Guangzhou, Guangdong 510275, P.R. China

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Abstract. Exposure to particulate matter ≤2.5 μm in diameter (PM$_{2.5}$) profoundly affects human health. However, the role of PM$_{2.5}$ on lung injury and cytokine levels in mice is currently unknown. The aim was to examine the effect of PM$_{2.5}$ pollution on lung injury in mice fed at an underground parking lot. A total of 20 female Kunming mice were randomly divided into control and polluted groups, with 10 rats in each group. The control group was kept in the laboratory, while the pollution group was fed in an underground parking lot. The concentrations of pollutants were measured using ambient air quality monitoring instruments. After 3 months of treatment, the lungs were collected and examined using electron microscopy, and the morphological structures were assessed using hematoxylin and eosin staining. The polarization of macrophages was evaluated by immunofluorescence. The concentration of interleukin (IL)-4, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β1 in peripheral sera were assessed by ELISA. The mRNA and protein levels of IL-4, TNF-α, and TGF-β1 in lung tissues were assessed by reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively. In the polluted group, the levels of CO, NO$_x$ and PM$_{2.5}$ were significantly higher compared with the control group. Compared with the controls, intracellular edema, an increased number of microvilli and lamellar bodies, smaller lamellar bodies in type II alveolar epithelial cells, and abundant particles induced by PM$_{2.5}$ in macrophages were observed in the polluted group. The lung ultrastructure changed in the polluted group, revealing exhaust-induced lung injury: The tissues were damaged, and the number of inflammatory cells, neutrophils, polylymphocytes and eosinophils increased in the polluted group compared with the control group. The authors also observed that the number of M1 and M2 macrophages markedly increased after the exhaust treatment. The levels of IL-4, TNF-α and TGF-β1 in the sera and tissues were significantly increased in the polluted group. PM$_{2.5}$ pollutants in underground garages can lead to lung injury and have a significant impact on the level of inflammatory cytokines in mice. Therefore, the authors suggest that PM$_{2.5}$ can activate the inflammatory reaction and induce immune dysfunction, leading to ultrastructural damage.

Introduction

With the rapid increase in the number of motor vehicles, the air quality in cities is increasingly deteriorating (1). Fine particles [particulate matter with an aerodynamic diameter smaller than 2.5 μm (PM$_{2.5}$)] cause ozone pollution, which leads to secondary pollution by oxidized pollutants under specific meteorological conditions, which in turn directly influence the health of most inhabitants in a polluted environment (1,2). Due to the characteristics of road traffic distribution, a considerable part of the population is under long-term exposure to serious PM$_{2.5}$ pollution in such a polluted environment (3,4). Thus, the health risks of PM$_{2.5}$ pollution cannot be ignored (1,2). Therefore, research on the effects of exhaust pollution on the human body, as well as the development of effective protection technologies, is necessary.

Diesel exhaust is not a single component, but a mixture of hundreds of gaseous and solid pollutants (5-7). Among them, the fine solid particles are mainly responsible for chronic lung injury. Pollutants in the range from PM$_{2.5}$ to PM$_{10}$ have been demonstrated to induce respiratory burst in RAW264.7 cells and could induce chronic lung injury (8-10). Previous studies showed that numerous acute toxicological experiments using single components of diesel exhaust confirmed that a variety of exhaust components can lead to an increased production of oxygen free radicals, thus directly damaging lung cells, and influencing digestive function, growth and development by negatively affecting Ca$^{2+}$ balance (11,12). In vivo studies have revealed that the expression of transforming growth factor (TGF)-β1 is positively correlated with the duration of exposure to and the amount of atmospheric particulate matter, such as PM$_{2.5}$, in the environment (13-17). It was also demonstrated that the serum levels of interleukin (IL)-4 and tumor necrosis factor (TNF)-α are positively correlated with the duration of

Correspondence to: Dr Zhongfu Ma, Department of General Internal Medicine, The First Affiliated Hospital of Sun Yat-sen University, 58 2nd Zhongshan Road, Guangzhou, Guangdong 510080, P.R. China

E-mail: 1362670488@qq.com; mzf7773@126.com

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exposure to air pollution, and that IL-4 levels were correlated with gaseous pollutants, such as NO₂, while TNF-α levels were correlated with particulate matter, such as PM₁₀ (18,19). In a study of the morphological changes in chronic lung injury of rats caused by gasoline exhaust pollution, inflammatory reactions were observed in the first 2 weeks, followed by lung fibroblast hyperplasia after 4 weeks (20). The hyperplasia of fibroblasts was increasingly evident with time and has been associated with chronic inflammatory reaction (21-23). However, the detailed changes in macrophage morphology and the macrophage M1-M2 balance remain unclear.

The air quality in the Central Laboratory of the First Affiliated Hospital of Sun Yat-sen University used by our group meets the requirements of GB3095-2012 standard class II of China (24), while the pollution levels exceed the national standard in underground parking lots. In the current study, the authors evaluated the levels of three representative indicators of pollution: CO, NOₓ, and PM₂.₅, in an underground parking lot and the laboratory. After living in the environment for 3 months, the ultrastructure in the lungs of mice was examined by electron microscopy, and morphological structures were detected by hematoxylin and eosin (H&E) staining. To study the changes in the macrophage M1-M2 balance, double fluorescence staining of adhesion G protein-coupled receptor E1 (F4/80) with human leukocyte antigen (HLA)-DR or cluster of differentiation (CD)68 was further performed in lung tissue sections.

Materials and methods

Animals. A total of 20 female Kunming mice, 8-10 weeks old, weighing 22-25 g, were provided and approved by the Experimental Animal Ethics Committee Center of The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China; animal certificate no. 4408500931). The mice were randomly divided into two groups: Control and polluted (10 mice per group).

Feeding location and exposure to pollution. The mice were housed as previously described (25). In brief, the mice were housed at the Central Laboratory of the First Affiliated Hospital of Sun Yat-sen University in a controlled environment (food and water available ad libitum, 21±1°C, 60% humidity and 12-h light/dark cycle). The mice in the polluted group were fed at the Second underground passage in a parking lot at the southeast corner of the First Affiliated Hospital of Sun Yat-sen University: At this location, there was a high flux of cars, and 12-h light/dark cycle. The mice in the polluted group were housed at the Central Laboratory of the First Affiliated Hospital of Sun Yat-sen University during the feeding period, all mice were free to eat and drink. The mice were treated and fed as described for 3 months. Live mice were injected with inhalational anesthetics, and then were sacrificed humanely using carbon dioxide asphyxiation. The mice were necropsied soon following the completion of the exposure protocol. During the study the body weight was assessed every 3 days.

Determination of the concentration of pollutants. Measurements of the concentration of pollutants in the cages in the underground garage and in the laboratory were performed using three ambient air quality monitoring instruments. The concentration of NOₓ was measured at the designated time-points using a 42C chemiluminescence NO-NOₓ analyzer (Thermo Environmental Instruments; Thermo Fisher Scientific, Inc.). For the determination of PM₂.₅ concentration (28), a β-ray particle monitoring instrument (model FH62C14; Thermo Fisher Scientific, Inc.) was used to measure the attenuation of β-rays through the particles suspended in the air. CO concentration was determined using a gas correlation infrared CO analyzer (model 48C; Thermo Environmental Instruments; Thermo Fisher Scientific, Inc.). The concentrations of pollutants at the two feeding locations were measured 12 times a day.

Electron microscopy examination. Lung tissues samples were examined using electron microscopy as previously described (29,30). In brief, the mice were sacrificed, and the lung tissues were removed. Lung tissue samples were fixed with 2.5% glutaraldehyde for 2 h at 4°C followed by 1% osmium tetroxide for 1 h at room temperature. The samples were dehydrated at room temperature using a series of acetone solutions of increasing concentration. Subsequently, they were counterstained in a saturated solution of uranyl acetate for 2 h at room temperature, followed by lead citrate treatment for 40 min at room temperature. Finally, sections (60 nm) were examined using a transmission electron microscope at a magnification of x50,000. In addition, the morphological changes in type II alveolar epithelial cells and macrophages from the lungs were also observed by electron microscopy. The morphological features of type II alveolar epithelial cells include large nuclei and multiple vacuoles, and mitochondria and rough endoplasmic reticulum in the cytoplasm (31). The surfaces of macrophages were covered with multiple irregular folds, microvilli and pseudopods; the cytoplasm contained a large number of lysosomes, phagosomes and vesicles; and there were many microfilaments and microtubules near the cell membrane (32).

Hematoxylin and eosin staining. All samples were treated with 4% paraformaldehyde (cat. no. #P6148, Sigma-Aldrich; Merk KGaA) for 24 h at 4°C. The samples were then treated with hematoxylin (cat. no. ZLI-9609; OriGene Technologies, Inc., Rockville, MD, USA) for 10 min, 70% ethanol and 1% hydrochloric acid at 25°C. The sections (4-µm-thick) were stained with 0.1% eosin (Surgipath™; Leica Microsystems, Ltd., Milton Keynes, UK) for 30 sec, and dehydrated in 80%, 90 and 100% ethanol at 25°C. Ethanol was used to dehydrate the sections at 25°C and the results were examined under an optical microscope (Eclipse E100; Nikon Corporation, Tokyo, Japan; magnification, x100).
ELISA. At the end of treatment in mice, blood was collected from mice and rapidly centrifuged at 5,000 x g for 10 min at 4°C. The supernatants were stored at -20°C until analysis. ELISA kits for mouse IL-4 (cat. no. EK0405; Boster Biological Technology, Pleasanton, CA, USA), TNF-α (CSB-E04741m) and TGF-β1 (CSB-E4726m; both Cusabio Technology LLC, Wuhan, China) were used according to the manufacturers’ protocol.

**Double immunofluorescence staining.** Lung tissues collected from mice were fixed, cut into sections and stained using the standard procedure (33). The slides were examined and photographed using fluorescence microscopy (ECLIPSE TI-SR; Nikon Corporation) using a magnification of x100. The slides were blocked with 5% bovine serum albumin (cat. no. A7638; Sigma Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at room temperature. The slides were incubated with primary antibodies at 4°C overnight. The following day, the slides were incubated with fluorescence-conjugated secondary antibodies (1:200; cat. no. ab9811; anti-TNF-α (cat. no. ab6671) and anti-TGF-β1 (cat. no. ab92486; all 1:1,000; all Abcam). The following primary antibodies were used: Anti-CD68 (1:25; cat. no. ab201844), anti-F4/80 (1:100; cat. no. ab100790), anti-CD163 (1:10; cat. no. ab17051) and anti-HLA-DR (1:500; cat. no. ab201844) according to the manufacturer’s protocol. The qPCR was performed using the TB Green Premix Ex TaqII kit (cat. no. RR820A; Takara Bio Inc.) according to the manufacturer’s protocol. The qPCR was performed using the TB Green Premix Ex TaqII kit (cat. no. RR820A; Takara Bio Inc.) according to the manufacturer’s protocol. The qPCR was performed using the TB Green Premix Ex TaqII kit (cat. no. RR820A; Takara Bio Inc.) according to the manufacturer’s protocol.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** The RNA was extracted from the lung tissues by using TRIzol reagent (cat. no. #9109; Takara Bio Inc., Otsu, Japan) and its concentration was measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA by using the Reverse Transcription kit (cat. no. RR037A; Takara Bio Inc.) according to the manufacturer’s protocol. The qPCR was performed using the TB Green Premix Ex TaqII kit (cat. no. RR820A; Takara Bio Inc.) according to the manufacturer’s protocol. The qPCR was performed using the TB Green Premix Ex TaqII kit (cat. no. RR820A; Takara Bio Inc.) according to the manufacturer’s protocol. The qPCR was performed using the TB Green Premix Ex TaqII kit (cat. no. RR820A; Takara Bio Inc.) according to the manufacturer’s protocol.

**Statistical analysis.** All data were analyzed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA) with Student’s t-test and reported as mean ± standard error of the mean (n=10). P<0.05 indicated that the difference between groups was statistically significant.

**Results**

**NO2 and PM2.5 concentrations are markedly high, and CO concentrations markedly fluctuate in the underground parking lot.** The atmospheric pollutants, CO, NO2 and PM2.5, were examined (Fig. 1). In the cage in the laboratory, the levels of CO, NO2 and PM2.5 were stable at ~2, 0.01, and 0.08 mg/m³, respectively. In the cage in the underground garage, NO2 and PM2.5 fluctuated considerably and were markedly higher compared with those levels in the laboratory at all time-points (7:00 am to 6:00 pm), whereas CO levels were increased in the underground garage compared with the laboratory at 12:00, 16:00 and 17:00 pm.

**Atmospheric pollutants induce lung damage.** The morphological changes in type II alveolar epithelial cells and macrophages from the lungs were examined by electron microscopy (Fig. 2A and B). In the control mice, microvilli and lamellar bodies were abundant in type II alveolar epithelial cells, which did not exhibit intracellular edema (Fig. 2A). In mice fed in the underground garage, the microvilli and the lamellar bodies were markedly reduced in type II alveolar epithelial cells. The size of lamellar bodies also decreased. In addition, intracellular edema was present in the type II alveolar epithelial cells. These findings indicated functional impairment of the cells.

In the control mice, macrophages contained a few low-density particles, but not the high-density particles of inorganic substances (Fig. 2B). The observed particles could have been bacteria or products of decomposition, such as remnants of cell necrosis. In the polluted group, there was a large number of high-density inorganic particles in the lung macrophages. The particles deposited in the lungs were PM2.5 from diesel exhaust pollution, which may have activated the macrophages.

Thus, the changes in the morphology of type II alveolar epithelial cells and macrophages suggested that diesel exhaust induced lung damage. During the study, no changes in the
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Furthermore, morphological structures of lung tissue were assessed by H&E staining in the control and polluted groups (Fig. 2C). The tissues were comparatively complete and the nuclei were clearly visible in the control group, while the tissues were damaged in the polluted group. The number of inflammatory cells, including neutrophils, polylumphocytes

body weight or clinical parameters of the mice were observed (data not shown).

Figure 1. NO\textsubscript{x} and PM\textsubscript{2.5} concentrations are markedly high, and CO concentrations markedly fluctuate in the underground parking lot. The concentration of (A) CO, (B) NO\textsubscript{x}, and (C) PM\textsubscript{2.5} were measured in an underground parking lot and a laboratory 12 times a day. PM\textsubscript{2.5}, particulate matter with an aerodynamic diameter smaller than 2.5 µm.

Figure 2. Atmospheric pollutants induce lung damage. (A) The ultrastructures of type II alveolar epithelial cells were observed by transmission electron microscope in the control and polluted group. Magnification, x5,800. The arrows indicate cell surface villi (1), lamellar body (2) and cytoplasm (3). (B) The ultrastructures of macrophages were observed by transmission electron microscope in the control and polluted group. Magnification, x9,700. Arrows indicate intracellular phagocytes. (C) The morphological structures were detected by haematoxylin and eosin staining in the control and polluted group. Magnification, x100.
and eosinophils, increased in the polluted group compared with the control group (data not shown).

**HLA-DR, CD163 and F4/80 protein expression increases in the lungs of the polluted group.** To further confirm the lung injury, double immunofluorescence staining for HLA-DR and F4/80, which are expressed in M1 polarized macrophages (36), and for CD163 and F4/80, which are expressed in M2 polarized macrophages, was performed (Fig. 3). In the polluted group, HLA-DR and F4/80 expression levels were markedly increased, while CD163 and F4/80 expression levels were also markedly increased (Fig. 3A and B). The protein expression levels of HLA-DR and F4/80 were significantly increased in the polluted group compared with the control group, suggesting an increased number of M1 polarized macrophages, which express HLA-DR (Fig. 3C). Similarly, CD163 and F4/80 protein expression levels were significantly increased in the polluted group, suggesting an increased number of M2 polarized macrophages, which express CD163 (Fig. 3D). Thus, the numbers of M1 and M2 macrophages were increased, and M1 and M2 macrophages infiltrated the tissue. CD163 and HLA-DR could thus be used for monitoring the extent of lung injury in a polluted environment.
IL-4, TNF-α and TGF-β1 serum levels increase in the polluted group. The serum levels of the cytokines, IL-4, TNF-α and TGF-β1, were examined (Fig. 4). In the mice fed in the polluted environment, the serum levels of IL-4 (Fig. 4A), TNF-α (Fig. 4B) and TGF-β1 (Fig. 4C) were significantly increased compared with those mice fed in the laboratory.

IL-4, TNF-α and TGF-β1 mRNA and protein levels increase in the lungs of the polluted group. The mRNA and protein levels of the cytokines, IL-4, TNF-α, and TGF-β1, in lung tissues were assessed by RT-qPCR (Fig. 5) and western blotting (Fig. 6). Atmospheric pollutants significantly upregulated the mRNA levels of IL-4 (Fig. 5A), TNF-α (Fig. 5B) and TGF-β1 (Fig. 5C), as well as markedly (Fig. 6A) and significantly (Fig. 6A) upregulating the protein levels of IL-4, TNF-α and TGF-β1. Thus, atmospheric pollutants increased the levels of these cytokines in lung tissues.

Discussion

The authors of the current study demonstrated that the levels of three representative indicators of pollution, CO, NOx and PM2.5, in an underground parking lot were significantly higher compared with those in the laboratory. The air quality in the laboratory met the requirements of GB3095-2012 standard class II of China, while, in the underground parking lot, the pollution levels exceeded the national standard by far (37).

Numerous acute toxicological experiments using single components of diesel exhaust confirmed that a variety of these components can lead to an increased production of oxygen free radicals, thus directly damaging lung cells and influencing digestive function, growth, and development by negatively affecting Ca²⁺ balance (11). After feeding in the polluted environment for 3 months, findings revealed that atmospheric pollutants could induce lung damage. In addition, it was determined that the number of inflammatory cells increased, including neutrophils, lymphocytes and eosinophils.
These results support the hypothesis that lung injury can be induced by oxygen free radicals. Previous research has shown that particular matter exposure could change macrophage morphology (8,38), which is consistent with the observation of macrophages in lung tissue using electron microscopy. The deposition of a large number of dust particles in macrophages has been demonstrated to induce macrophage apoptosis, which is also mediated by an increase in oxygen free radicals and injury of mitochondria in macrophages (39-41). If the deposition becomes more serious, macrophages are overburdened, eventually leading to decreased phagocytosis, thus influencing immune defense function (42). The change in oxygen free radicals was not evaluated in the current study.

Research has demonstrated that M1-polarized macrophages expressing HLA-DR have a pro-inflammatory effect, while M2-polarized macrophages expressing CD163 have the function of immunosuppressive tissue repair (43). The present results indicated that the number of M1 and M2 macrophages increased, and there was a large number of macrophage aggregation and infiltration in lung tissues of mice that were fed in the polluted environment. Therefore, the increase of M1 and M2-polarized macrophages may be an important cause of immune dysfunction caused by pollution. Particles/dust are solid or liquid particulate matter in the atmosphere (44). According to particle size, particles can be divided into total suspended particles and inhalable particles. Inhalable particles can be divided into coarse particles (PM10: aerodynamic diameter between 2.5-10 µm) and fine particles (PM2.5 ≤2.5 µm) (45). As an important air pollutant, particle size, shape and composition are associated with health (46). Previous research showed that inhalable particles are responsible for chronic lung injury (47). A large number of studies have confirmed that, due to their very small size, PM2.5 particles easily enter the lungs (48,49). With adsorption of heavy metals and other toxic substances, the surface area of those particles becomes relatively large (50,51). As a result, PM2.5 induces a potent inflammatory effect by increasing intracellular Ca2+, as well as the phenotypic transition of macrophages to M1 macrophages (52). The pollutants in the range from PM2.5 to PM10 have been demonstrated to induce respiratory burst in RAW264.7 cells, promoting the formation of a large number of oxygen free radicals, the imbalance of the oxidant and antioxidant system, and epithelial cell damage with increased epithelium permeability, all eventually resulting in chronic lung injury (8-10). The lung injury in turn promotes the phenotypic transition of macrophages to M2 macrophages (53). The excessive increase in M2 macrophage numbers has been demonstrated to have negative consequences, including pulmonary immune suppression, and an increased incidence of pulmonary fibrosis and lung cancer (54,55).

The experimental results by ELISA demonstrated that the serum levels of IL-4, TNF-α and TGF-β1 in the polluted group were significantly higher compared with the control group. Similar results were observed in the lung tissues. The mRNA and protein levels of IL-4, TNF-α and TGF-β1 in the polluted group were also significantly higher compared with the control group. The increase in the level of proteins was evidently less compared with that of mRNAs, which may be due to the regulation of the expression at the translation level.

In vivo studies have revealed that the expression of TGF-β1 is positively correlated with the duration of exposure to and the amount of atmospheric particulate matter, such as PM2.5, in the environment (13-17). It was also demonstrated that the serum levels of IL-4 and TNF-α were positively correlated with the duration of exposure to air pollution and that IL-4 levels were correlated with gaseous pollutants, such as NO, while TNF-α levels were correlated with particulate matter, such as PM2.5 (18,19). Those results are consistent with our findings. The mechanism of increased levels of inflammatory cytokines in mice may be associated with the oxidative damage of the respiratory mucosa by gaseous pollutants, such as NO, the stimulation of the lung epithelial cells by PM2.5 and transition metals present in PM2.5 (56).

IL-4 is an important cytokine in the regulation of T lymphocytes and the synthesis of immunoglobulin (Ig) E by B lymphocytes (57). An increase in IL-4 promotes the secretion of IgE, which is key for the onset of allergic diseases (58). This is also consistent with epidemiological surveys that revealed that air pollution results in an increase in IgE levels and a higher incidence of chronic pulmonary diseases, including asthma and chronic obstructive pulmonary disease (59,60). TNF-α is an important inflammatory factor and immunoregulatory factor; its overexpression has been determined to promote the aggregation of a variety of inflammatory cells, leading to abnormal inflammatory response, immune dysfunction or a variety of inflammatory diseases (61). Many studies have revealed that TGF-β1 is a potent chemokine for fibroblasts (62-64). In lung tissues, TGF-β1 upregulates the expression of collagen and fibronectin to promote an increase of the extracellular matrix; meanwhile, it downregulates the secretion of proteases, but increases the secretion of protease inhibitors to inhibit the degradation of the extracellular matrix, thus participating in lung fibrosis (65). In a study of the morphological changes in the chronic lung injury of rats caused by gasoline exhaust pollution, inflammatory reactions were observed in the first 2 weeks, followed by lung fibroblast hyperplasia after 4 weeks. The hyperplasia of fibroblasts was increasingly evident with time and has been associated with chronic inflammatory reaction (21,22). Recent research has indicated that TNF-α and TGF-β1 promotes the development of inflammation and pulmonary fibrosis (66,67).

In conclusion, the sub-chronic exposure to a highly polluted environment, such as that with high PM2.5 levels, can lead to lung injury in mice. Although ultrastructural damage, and local and systemic immune imbalance was observed, further studies should focus on the protection of lungs against injury caused by polluted environments.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.
Authors' contributions

JY, YC and ZM conceived and supervised the study; YC, ZY and HD designed and performed experiments; JY, YC and ZM analyzed data, wrote the manuscript and made manuscript revisions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Experimental Animal Ethics Committee Center of The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China; animal certificate no. 4408500931).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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