Sufentanil Postoperative Analgesia reduce the increase of T helper 17 (Th17) cells and FoxP 3 + regulatory T (Treg) cells in Rat Hepatocellular Carcinoma Surgical Model: A randomised animal study

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Abstract
Background: Several investigations have indicated that T helper cell 17 (Th 17) and Foxp3+ regulatory T cells (Treg) cells adopt a pro-tumorigenic role. Opioids and pain exacerbate immunosuppression in immunocompromised cancer patients. Methods: A rat hepatocellular carcinoma (HCC) models was established by N-nitrosodiethylamine (DENA). 48 of them were randomly divided into 3 groups (n=16): surgery without postoperative analgesia (Control); surgery with morphine postoperative analgesia (Morphine); surgery with sufentanil postoperative analgesia (Sufentanil). The level of cluster of CD4+, CD8+, Th1, Th2, Th17 and Treg cells in blood were also detected to assess immune function using flow cytometry on d0, d3 and d7. The rats’ survival situation of each group left after three days of surgery were observed. Results: The CD4+/CD8+ ratio and Th1 cells levels were significantly higher while Th2, Th17 and Treg cells levels were significantly lower in sufentanil and morphine postoperative analgesia rats compared with that without postoperative analgesia. Compared with morphine postoperative analgesia, sufentanil postoperative analgesia rats seem have higher CD4+/CD8+ ratio, Th1 cells, while lower Th2, Th17 and Treg cells level. Conclusions: Sufentanil and morphine postoperative analgesia show better immune function than that without postoperative analgesia by analyzing CD4+/CD8+ ratio, Th1, Th2, Th17 and Treg cells. Sufentanil postoperative analgesia demonstrated a favorable impact on immune function compared with morphine postoperative anesthesia in hepatocellular carcinoma rats undergoing left hepatectomy operation.

Background
Surgery-related pain and opioid analgesics are factors known to adversely affect the anti-tumor immune defenses which may promote tumor growth and metastasis\(^1\). In view of the growing interest in the immune system in control of neoplasia, further efforts toward the discovery of a good analgesia agent with a reduced impaction on immunity for postoperative pain treatment are urgently needed. The helper T cells were mainly divided into T helper 1(Th1), Th2, Th9, Th17, Th21, T follicular helper (Thf) and regulatory T (Treg) cells according to the function and phenotype\(^2, 3\). Among them, Th1, Th2, Th17 and Treg cells are more concerned in tumor immunity. Hepatocellular carcinoma (HCC) is...
the third cancer-related mortality worldwide\cite{4}. Th17 and Treg cells have been extensively studied during the past ten years in tumor immunity. Th17 cells play an important role in the inflammatory response, but in recent years, it is found that Th17 cells also could increase tumor progression by activating angiogenesis and immunosuppressive activities\cite{5,6}. Treg cells can reportedly inhibit the tumor-specific T cell-mediated immune response and have been observed increased quantity in tumor tissues or peripheral of patients or animal models with gastric cancer\cite{7}, ovarian cancer\cite{8}, breast cancer\cite{9} and hepatocellular carcinoma\cite{10}. Morphine and sufentanil are widely used in postoperative analgesia. Previous studies manifested that morphine can decreases the expressions of peripheral T lymphocytes (CD3\(^+\), CD4\(^+\), CD8\(^+\)) and natural killer cells (CD3\(^+\), CD56\(^+\)) in vivo\cite{11} and increases the ratio of CD4\(^+\)/CD8\(^+\) T cells and Treg populations in vitro\cite{12}. The Epidural postoperative analgesia with ropivacaine plus sufentanil significantly decreased B lymphocytes, T-helper cells and Natural killer cells compared with patient-controlled IV analgesia with morphine in patients after major spine surgery\cite{13}. However, little or nothing is known concerning the effect of sufentanil postoperative analgesia on Th17 and Treg cells.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, characteristic of relatively poor overall survival and increasing morbidity and mortality\cite{14}. DENA is one of the most widely hepatotoxic chemicals that are used to produce the HCC animal model so far. Its carcinogenesis process goes through liver injury, liver fibrosis, liver cirrhosis and ultimately develops to HCC, which is very similar to the developing process of human’s HCC\cite{15}.

This study was performed to compare the effects of sufentanil and morphine postoperative analgesia on the immune function by CD4\(^+\)/CD8\(^+\), Th1, Th2, Th17 and Treg cells quantification using flow cytometry in HCC rats undergoing left hepatectomy.

**Methods**

**Ethics**

All animal procedures were approved (Permit Number: 2015001) by the Institutional Animal Care and Use Committees of Hunan Cancer Hospital, Changsha, China (Chairman Committee: Jingshi Liu) on 27
March 2015, and were performed in strict accordance with recommendations of the Guide to the Care and Use of Laboratory Animals of the National Institutes of Health.

**Animals**

80 male Sprague-Dawley rats (100±20g; Center of Experimental Animals of Hunan Cancer Hospital, Hunan, China) were used in this experiment. Rats were housed under controlled conditions with a temperature of 25±2°C, relative humidity of 60±10%, room air changes of 12-18 times/h and a 12 h light/dark cycle and were acclimated for 7 days before experiments. They were allowed free access to food and water.

**Model Establishment and Experimental Protocol**

80 Sprague-Dawley rats were intraperitoneal administrated with 0.19% N-nitrosodiethylamineDENA(50 mg kg\(^{-1}\)) every 3 days for a total of 16 weeks to make HCC models\(^\left(15\right)\). After sixteen weeks, 58 of these rats were successfully modeled, 48 rats randomly selected from HCC rats by digital random method were stochastically assigned to 3 groups by digital random method (n=16): surgery without postoperative analgesia (Control); surgery with morphine postoperative analgesia (Morphine); surgery with sufentanil postoperative analgesia (Sufentanil). All animals undergoing surgery were given a standard left hepatolobectomy under isoflurane anesthesia (2-3%). Rats’ abdominal region was shaved and thoroughly cleaned with complex iodine. A 2 cm midline incision was made in the abdomen. After reaching the abdomen cavity, the left lateral leaf of the liver was exposed, and the left leaves were ligated from the root and excised. A implanted osmotic minipumps (volume 2 ml, pump speed 10 μl h\(^{-1}\) for 72h, Alzet, USA) was placed in the abdominal cavity for postoperative analgesia, Morphine Group per mouse was administered in a single dose of 0.25 mg Kg\(^{-1}\) h\(^{-1}\) for 72h (morphine dose was chosen under the document\(^\left(16\right)\) and associated with the average effective dose used clinically), Sufentanil Group per mouse in a dose of 0.25 ug Kg\(^{-1}\) h\(^{-1}\) per mouse was administered for 72h (the dose of sufentanil was calculated in accordance with its analgesic potency in comparison to morphine), Control Group per mouse 0.9% saline was administered 10 ul h\(^{-1}\) per mouse for 72h. Finally, the muscle and skin were closed with sterile
sutures. During surgery, the rats' temperatures were maintained using a thermal insulation blanket.

We measured the following parameters in each operated rat to comprehensive evaluate the postoperative analgesic effect on one day before surgery (d0), the first, second and third day after surgery (d1, d2, d3): Mechanical pain threshold assessed using standard von frey monofilaments; Locomotor activity tested using open field test; Body weight, daily food and water consumption. We randomly sacrificed four rats per group on one day before surgery (d0), six rats on the third day after surgery (d3), and all of the remaining rats on the seventh day after surgery (d7) to collect blood samples by cardiac puncturing method. All rats undergoing operation received general anesthesia of isoflurane (2-3%) and started surgery after no movement of clipping the tail, and all the rats were euthanized by the method of cervical vertebra decoupling under anesthesia after collecting blood samples. The rats’ survival situation of each group left after three days of surgery were observed. The serum alanine aminotransferase (ALT) and aspartate transaminase (AST) were measured to assess liver function, and the level of cluster of CD4+, CD8+, Th1, Th2, Th17 and Treg cells in blood were detected to assess immune function using flow cytometry on d0, d3 and d7.

**Mechanical pain threshold (MPT)**

The abdominal pain threshold was measured using Mechanical pain threshold (MPT) on d0, d1, d2, d3. Detection of MPT along the abdominal incision was assessed using standard von frey monofilaments. Rats were placed in test cages prior to the experiment and allowed to fully acclimate to the environment for 3 hours. A 0.1 to 12 g single fiber test needle was used to stimulate the position of the rat's abdominal incision about 0.5 cm perpendicular to the skin surface until the filament was slightly curved in an S shape for 5-6 seconds. The MPT for this region was measured using the Chaplan up-down method. If the rat appears to be licking or scratching the stimulated area during the stimulation time or removing the von Frey filament, or a sudden withdrawal or jump occurs, it is recorded as a positive behavioral response.

**Locomotor activity—Open field test**

The locomotor activity was surveyed using open field test on d0, d1, d2, d3. Rats were individually
exposed to the same open field (100 cm × 100 cm) for 5 min trials with an interval of 30 min between each trial. The open field behavior was videotaped using a camera that was placed above the arena. The videos were subsequently analyzed digitally using Noldus software (Noldus, The Netherlands). Parameters measured were the total distance traveled throughout the arena.

**Body weight, daily food and water consumption**

During the measured period, rats were housed in individual cages. Body weight, food and water consumption were assessed daily on d0, d1, d2, d3.

**Assessment of liver function**

Blood samples were collected and sera were obtained by centrifugation in low temperature on d0, d3, d7. Serum AST and ALT were measured using the modified Jaffe rate reaction in the clinical laboratory of The Hunan Cancer Hospital, Changsha, China.

**Flow cytometry studies**

Fresh heparinized blood samples of rats were collected on d0, d3, d7. Then Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood by standard density gradient separation using Ficoll density gradient. Each specimen is divided into five equal parts in testing CD4+ , CD8+ , Th1, Th2, Th17 and Treg cells. Isolated cells were washed three times with phosphate buffer saline and used for flow cytometry. A total of 1 × 10^5 PBMCs prepared for were acquired for each sample. Each sample was surface stained with CD3-PE, CD4-FITC plus PE-Cy7-labeled anti-rat CD8 to detect CD4+ T cells, CD8+ T cells at room temperature for 15 min (avoid light). The subsets detection needed analyze CD4 combined with specific cytokines such as CD4+ IFN-γ+ for Th1, CD4+ IL-4+ for Th2, and CD4+ IL-17+ for Th17. For the Th1, Th2, Th17, samples were surface stained with CD4-FITC at room temperature for 15 min (avoid light), and subsequently stimulated for the intracellular cytokines with PE-labeled anti-rat IFN-γ, PE-labeled anti-rat IL-4, PE-labeled anti-rat IL-17A respectively according to the manufacturer's instructions. The CD4+ Foxp3+ phenotype was recommended for identifying the Treg. Though, samples were surface stained with CD4-FITC at room temperature for 15 min (avoid light), and subsequently intracellularly stained with a PE anti-rat Foxp3 staining kit without stimulated
according to the manufacturer's instructions. Cells were detected by flow cytometry using a FACS Calibur, and data were analyzed by FlowJo.

**Statistical analysis**

Data are shown as mean± SD for normally distributed data. Probability values<0.05 were considered statistically significant. Then the data was transferred to the computer using SPSS 25 software, normally distributed data were analyzed by using a one-way ANOVA followed by a post hoc S-N-K test (Equal variances assumed) and Tamhane T2 test (Equal variances not assumed) to compare the three groups at each time point. When postoperative data and preoperative data in each group were compared, an independent samples-T test was employed. The descriptive findings were compared using Fisher’s exact test with P<0.0001.

**Results**

**There was no significant difference of analgesic effect between sufentanil and morphine**—There were no statistically significant differences in mechanical pain threshold, locomotor activity, body weight, food and water consumption among the three groups on d0 (p>0.05). No significant difference was found between Sufentanil and Morphine group in the five measures on d1, d2 and d3 (p>0.05) (Supplement.1). These results indicated that there was no significant difference of analgesic effect between sufentanil and morphine.

**Postoperative analgesia with sufentanil or morphine showed better liver function**—A significant increase in ALT and AST was observed in Control group in comparison to Sufentanil and Morphine group on d7 (p<0.05). No significant difference was found between the Sufentanil and Morphine group on d7 (p>0.05) (Supplement. 2).

**The survival situation**—Though we did not find statistically significant differences in mortality rate between postoperative analgesia rats and without analgesia rats (P=0.245, Fisher's Exact Test). We did observe that two rats of Control group died respectively on fourth and fifth day after surgery, one rat of Morphine group died on sixth day after surgery, and no rat died in Sufentanil group.

**Sufentanil postoperative analgesia rats have high CD4⁺/CD8⁺ level in blood**—Fig. 1A shows the flow cytometric analysis of CD4⁺ and CD8⁺ cells. Fig. 1B shows the statistical analysis of
CD4+/CD8+ ratio. There was no statistically significant difference in CD4+/CD8+ ratio among the three groups on d0 (P>0.05). A significant increase in the CD4+/CD8+ ratio was observed in Sufentanil group in comparison to Morphine group and also in Morphine group in comparison to Control group on d3 (P<0.05). A significant increase was observed in Sufentanil group and Morphine group in comparison to Control group on d7 (P<0.05). The CD4+/CD8+ ratio significantly increased on d3 and recovered on d7 within Sufentanil group (P=0.036, P=0.287 respectively) in comparison to d0. The CD4+/CD8+ ratio significantly increased on d3 and d7 within Control and Morphine group in comparison to d0 (Control group: P=0.000, P=0.000 respectively; Morphine group: P=0.002, P=0.003 respectively).

**Sufentanil postoperative analgesia rats have high Th1 cells in blood**—Fig. 2A shows the flow cytometric analysis of Th1 (CD4+IFN-γ+) cells. Fig. 2B shows the statistical analysis of Th1 cells. There were no statistically significant differences in the percentage of Th1 cells in blood between the three groups on d0 (p>0.05). Rats showed higher Th1 cells in Sufentanil group and Morphine group compared with Control group on d3 and d7 (p<0.05). There were no statistically significant differences in proportion of Th1 cells between Sufentanil group and Morphine group on d3 and d7 (p>0.05). A significant increase was observed in proportion of Th1 cells within the three groups on d3 and d7 in comparison to d0 (Sufentanil group: P=0.006; Morphine group: P=0.018; Control group: P=0.000).

**Sufentanil postoperative analgesia rats have low Th2 cells in blood**—Fig. 3A shows the flow cytometric analysis of Th2 (CD4+IL4+) cells. Fig. 3B shows the statistical analysis of Th2 cells. There were no statistically significant differences in the percentage of Th2 cells in blood between the three groups on d0 (p>0.05). Rats showed lower Th2 cells in Sufentanil group and Morphine group compared with Control group on d3 and d7 (p<0.05). There were no statistically significant differences in proportion of Th2 cells between Sufentanil group and Morphine group on d3 and d7 (p>0.05). The proportion of Th2 cells significantly increased on d3 and recovered on d7 within Sufentanil group (P=0.038, P=0.109 respectively) and Morphine group (P=0.036, P=0.061 respectively) in
comparison to d0. A significant increase in the proportion of Th2 cells was observed within Control group (P=0.002, P=0.036 respectively) on d3 and d7 in comparison to d0 (P<0.05).

**Sufentanil postoperative analgesia rats have low Th17 cells in blood**—Fig. 4A shows the flow cytometric analysis of Th17 (CD4^+IL17-A^+) cells. Fig. 4B shows the statistical analysis of Th17 (CD4^+IL17-A^+) cells. No significant difference was found between the 3 groups in proportion of Th17 cells on d0 (P>0.05). A significant decrease in proportion of Th17 cells was observed in Sufentanil group in comparison with Control group on d3 and d7 and also in Morphine group in comparison with Control group on d7 (P<0.05). No significant difference was found within Sufentanil group (P=0.891, P=0.726 respectively) in proportion of Th17 cells on d3 and d7 in comparison to d0. The proportion of Th17 cells significantly increased on d3 and recovered on d7 within Morphine group (P=0.015, P=0.066 respectively) in comparison to d0. A significant increase in the proportion of Th17 cells was observed within Control group (P=0.007, P=0.001 respectively) on d3 and d7 in comparison to d0 (P<0.05).

**Sufentanil postoperative analgesia rats have low Treg cells in blood**—Fig. 5A shows the flow cytometric analysis of Treg (CD4^+Foxp3^+) cells. Fig. 5B shows the statistical analysis of Treg (CD4^+Foxp3^+) cells. No significant difference was found between the 3 groups in proportion of Treg cells on d0 (P>0.05). A significant decrease in proportion of Treg cells was observed in Sufentanil group in comparison with Control group on d3 (P<0.05). A significant decrease in proportion of Treg cells was observed in Sufentanil group in comparison with Morphine group on d7 and also in Morphine group in comparison with Control group (P<0.05). No significant difference was found within Sufentanil group (P=0.655, P=0.957 respectively) and Morphine group (P=0.063, P=0.075 respectively) in proportion of Treg cells on d3 and d7 in comparison to d0. A significant increase was observed in Control group on d3 and d7 in comparison to d0 (P=0.000, P=0.000 respectively).

**Discussion**

This study is the first to directly compare sufentanil and morphine continuous infusion in postoperative pain on peripheral CD4^+/CD8^+, Th1, Th2, Th17 and Treg cells. The MPT, Locomotor
activity, daily food and water consumption results supported that sufentanil (0.25 ug Kg⁻¹ h⁻¹) and morphine (0.25 mg Kg⁻¹ h⁻¹) continuous administered by osmotic pumps into the abdominal cavity showed similar pain control efficacy in rats HCC surgical model. The possible impact of different pain intensity between Sufentanil group and Morphine group on postoperative outcome and immune variation can be ruled out.

There is such a view that the interaction between CD4⁺ and CD8⁺ T lymphocyte mediates the control of tumor growth(20). In a clinical study, the 5-year survival rate of cervical cancer patients with high CD4⁺/CD8⁺ ratio was higher than that of patients with low CD4⁺/CD8⁺ ratio, increasing the CD4⁺/CD8⁺ ratio can slow the progression of cervical cancer and improve its prognosis (21). It is generally believed that Th1 enhances tumor immune surveillance of tumor while Th2 associated with the tumor immune evasion can suppress the function of Th1 cells(22). Th17 can promote tumor angiogenesis in the promotion of tumor progression(6). Th17 cells in peripheral blood are positively correlated with the progression of liver cancer(23). Treg cells play a vital role in maintaining immunological homeostasis and exert major immunosuppressive activity(24). It was found that the 5-year survival rate of cervical cancer patients who had a high percentage of Treg was significantly lower as compared with those who had a lower percentage (21). A recent study has indicated that the percentages of CD4⁺CD25⁺FOXP3⁺Treg cells and CD4⁺IL-17⁺Th17 cells were significantly higher in HCC patients than in the healthy individuals; Moreover, the increased percentages of Treg and Th17 cells were closely related to the tumor stage and tumor size of HCC(10). Therefore, high CD4⁺/CD8⁺ ratio, high Th1, and low Th2, Th17, Treg should be an immune state index to inhibit tumor progression. In this study, we analyzed distribution of CD4⁺, CD8⁺, Th1, Th2, Th17 and Treg cells in blood by flow cytometry. This study found that rats showed decrease CD4⁺/CD8⁺ ratio and Th1 level while increase Th2, Th17 and Treg cells level after surgery. The CD4⁺/CD8⁺ ratio and Th1 cells levels were significantly higher while Th2, Th17 and Treg cells levels were significantly lower in sufentanil and
morphine postoperative analgesia rats compared with that without postoperative analgesia.

Compared with morphine postoperative analgesia, sufentanil postoperative analgesia rats seem have higher CD4+/CD8+ ratio, Th1 cells, while lower Th2, Th17 and Treg cells level. These results showed that surgery and postoperative pain suppress immune function while sufentanil and morphine postoperative analgesia alleviate this immunosuppression. The serum ALT and AST value in Sufentanil group were significantly lower in sufentanil postoperative analgesia rats compared with morphine postoperative analgesia rats and without postoperative analgesia. There were no statistically significant differences in mortality rate between postoperative analgesia rats and without analgesia rats, but we did observe the difference in the number of deaths between rats with analgesia and without analgesia. These results suggested that postoperative analgesic rats with sufentanil and morphine would be better than those without postoperative analgesia on liver function and survival situation.

Conclusions
The current results show that the postoperative analgesia with sufentanil or morphine can reduce immunosuppression thus may improve the behavioral consequences of surgery and survival rate.

Compared to morphine, postoperative analgesia with sufentanil may be much less immunosuppression. Recently, increasing studies have shown that there is a close positive correlation between recurrence and metastasis to the inhibition of immune system$^{(25, 26)}$. The findings of the presented study provide guidance for the clinical use of drugs in the field of postoperative analgesia.

Reasonable selection of analgesics may influence immune functions and postoperative outcomes.

Abbreviations

| Abbreviation | Description                      |
|--------------|----------------------------------|
| ALT          | represent serum alanine aminotransferase |
| AST          | represent aspartate transaminase   |
| DENA         | represent N-nitrosodiethylamine    |
| HCC          | represent hepatocellular carcinoma |
| Th           | represent T helper cell            |
| Thf          | represent T follicular helper      |
Treg represent regulatory T cells
MPT represent Mechanical pain threshold
PBMCs represent Peripheral Blood Mononuclear Cells

Declarations

Ethics approval and consent to participate All animal procedures were approved (Permit Number: 2015001) by the Institutional Animal Care and Use Committees of Hunan Cancer Hospital, Changsha, China (Chairman Committee: Jingshi Liu) on 27 March 2015, and were performed in strict accordance with recommendations of the Guide to the Care and Use of Laboratory Animals of the National Institutes of Health.

Consent for publication Not applicable

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests

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Authors’ contributions
YP helped design and conduct the study; sought ethical approval; design and perform the research; acquisition, interpret, and analyze the data; write the manuscript; and revise the manuscript. DG helped perform the research and acquisition of data. CZ helped acquisition, interpret, and analyze the data. HS helped design and conduct the study; sought ethical approval; acquisition, interpret, and analyze the data; SZ and QZ helped perform the research and acquisition of data. YZ, KL and KC helped write and revise the manuscript. JY helped propose the study concept, design and conduct the study; sought ethical approval; design the research; write the manuscript; read and approved the final manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

The effect of each treatment group on CD4+/CD8+ ratio. A. Detection of CD4+/CD8+ ratio in each group on one day before surgery, on the third and seventh day after surgery. B. Statistical analysis of the ratio of CD4+/CD8+. * Indicates p<0.05, ** Indicates p<0.01, *** Indicates p<0.001. ¥ Indicates a statistically significant difference vs the Control group; ¥ Indicates p<0.05.
The effect of each treatment group on proportion of Th1 cells. A. Detection of Th1 cells in each group on one day before surgery, on the third and seventh day after surgery. B. Statistical analysis of Th1 cell proportions. * Indicates p<0.05, ** Indicates p<0.01, *** Indicates p<0.001; # Indicates a statistically significant difference vs the Control group; # Indicates p<0.05, ## Indicates p<0.01, ### Indicates p<0.001.
The effect of each treatment group on proportion of Th2 cells. A. Detection of Th2 cells in each group on one day before surgery, on the third and seventh day after surgery. B. Statistical analysis of Th2 cell proportions. * Indicates p<0.05, ** Indicates p<0.01, *** Indicates p<0.001; # Indicates a statistically significant difference vs the Control group; # Indicates p<0.05, ## Indicates p<0.01, ### Indicates p<0.001.
The effect of each treatment group on proportion of Th17 cells. A. Detection of Th17 cells in each group on one day before surgery, on the third and seventh day after surgery. B. Statistical analysis of Th17 cell proportions. * Indicates p<0.05, ** Indicates p<0.01, *** Indicates p<0.001; # Indicates a statistically significant difference vs the Control group; ## Indicates p<0.05.
The effect of each treatment group on proportion of Treg cells. A. Detection of Treg cells in each group on one day before surgery, on the third and seventh day after surgery. B. Statistical analysis of Treg cell proportions. * Indicates p<0.05, ** Indicates p<0.01, *** Indicates p<0.001; # Indicates a statistically significant difference vs the Control group; # Indicates p<0.05.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

Supplement1.pdf
NC3Rs ARRIVE Guidelines Checklist (fillable).pdf
Supplement2.pdf