METHODS

Q-Plex human cytokine ELISA

The assay was conducted according to the manufacturer’s protocol with some modifications. An eight-concentration standard curve was prepared for each cytokine using 1:3 serial dilutions. The concentration ranges for each cytokine standard were 4.59-7,600 pg·mL\(^{-1}\) for interleukin (IL)-1\(\alpha\), 0.10-4,700 pg·mL\(^{-1}\) for IL-1\(\beta\), 1.35-2,700 pg·mL\(^{-1}\) for IL-2, 0.22-415 pg·mL\(^{-1}\) for IL-4, 1.89-3,700 pg·mL\(^{-1}\) for IL-6, 0.10-2,500 pg·mL\(^{-1}\) for IL-10, 7.08-7,100 pg·mL\(^{-1}\) for IL-12, 3.06-13,000 pg·mL\(^{-1}\) for interferon gamma (IFN\(\gamma\)), and 3.27-4,500 pg·mL\(^{-1}\) for tumour necrosis factor. The intra-assay and inter-assay coefficients of variations of the standards were both below 15%. To perform the Q-plex ELISA, 50 \(\mu\)L of samples (1:2 diluted) or standards were added to the ELISA plate in duplicate and shaken on an orbital shaker at 750 rpm at room temperature (RT) for two hours to allow for cytokine binding to the primary antibodies. The samples were then decanted from the plate. Wash buffer 300 \(\mu\)L was added to each well, decanted (x 3) and inverted, and blotted dry on paper towels. Detection mix 50 \(\mu\)L containing the biotinylated detection antibody was added to the plate and incubated for one hour at RT on the orbital shaker at 750 rpm. This was followed by three cycles of washing using the method described above. The light-sensitive DyLight® IRDye®—which attaches to biotinylated antibody and emits infrared radiation in the 800 nm range—was then added to each well. After incubation for 15 min and washing the wells six times, the plate was scanned in the LI-COR Odyssey® Infrared Imaging System at the 800 nm channel. The intensity, resolution, and focus offset were set at 7-10, 84 \(\mu\)m, and 3.9-4.0, respectively, and the lowest quality of scan was selected. The image was then imported to Q-View™ Software for data analysis. Standard curves of each cytokine were
plotted using the software’s built-in 5 parameter logistic (5 PL) regression program. Final cytokine concentrations in each sample were then calculated based on their pixel intensity and interpolation from the standard curves.

**Albumin assay**

Albumin concentrations (g·L⁻¹) in plasma and cerebrospinal fluid (CSF) samples were determined using a bromocresol green (BCG) based assay (QuantiChrom™, BioAssay Systems, Hayward, CA, USA). To perform the albumin assay, 5 µL of samples or standards (0, 0.5, 1.0, 1.5, 3.0, 3.0, 4.0, and 5.0 g·dL⁻¹ for plasma and 0, 0.05, 0.2, 0.5, 1.0, 1.5, and 2.0 g·dL⁻¹ for CSF) and undiluted CSF or one in two diluted plasma samples were added in duplicate to a 96-well clear bottom plate. Working reagent 200 µL containing BCG was then added to each well. After five-minute incubation at RT, the plate was read at 620 nm in a 96-well plate reader. Standard curves of the absorbance vs concentration values were generated by non-linear regression. The concentration of albumin in each sample was determined from the Michaelis-Menten equation \( Y = \frac{V_{\text{max}} \cdot X}{K_m + X} \), where \( Y \) is the sample absorbance, \( X \) is the albumin concentration, \( K_m \) is the Michaelis-Menten constant, and \( V_{\text{max}} \) is the maximum absorbance.

**Morphine extraction and quantification**

External standards were prepared by spiking pooled baseline plasma or CSF samples with morphine, morphine-3-glucuronide (M3G), and morphine-6-glucuronide (M6G) at a series of concentrations. For plasma, the final concentrations of external standards were 0, 1.0, 5.0, 10.0, 50.0, 100.0, 500.0, 1,000.0, and 2,000.0 ng·mL⁻¹. For CSF, the concentrations were 0, 0.5, 2.5, 5.0, 25.0, 50.0, 250.0, 500.0, and 1,000.0 ng·mL⁻¹. Deuterated morphine (M-D3) and M3G
(M3G-D3) were utilized as internal standards at a concentration of 1,000.0 ng·mL⁻¹ for plasma samples or 10 ng·mL⁻¹ for CSF samples.

To extract morphine and its metabolites, 96-well Oasis® MCX (30 µm) µElution solid phase extraction plates were used (Waters Corporation, Milford, MA, USA). The plates were conditioned with methanol 200 µL per well, followed by vacuum filtration. This conditioning step was then repeated with 200 µL of distilled-deionized water. To each well, 100 µL of sample or standard were added together with 100 µL of internal standards and 200 µL of 4% phosphoric acid. The solutions were vacuum filtered to allow morphine, M3G, and M6G to bind to the sorbent. To remove impurities, the plate was washed with 2% formic acid 200 µL per well, vacuum filtered, washed with methanol 200 µL, and then followed by vacuum filtration. Each well was eluted into a corresponding well of a 96-well collection plate in 2×50 µL of 5% ammonium hydroxide in 90% methanol. The 96-well plate containing the eluted samples was evaporated in the EZ-Bio personal evaporator using the program “BP < 75” and with the maximum temperature set at 40°C. The plate containing the dehydrated samples was sealed and stored at -70°C and then delivered on dry ice to our collaborator, Dr. Lekha Sleno in Montreal, for determining the concentrations of morphine and its metabolites by mass spectrometry.

The quantitation of morphine, M3G, and M6G in plasma and CSF samples was performed based on a previously described liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay developed and validated by our research group.(1) Briefly, the purified extracts were injected onto the LC-MS/MS system consisting of a Nexera ultra high performance liquid chromatograph (UHPLC) (Shimadzu, Japan) and a QTRAP® 5500 hybrid quadrupole-linear ion trap mass spectrometer (AB SCIEX, Concord, ON, Canada) in multiple reaction
monitoring (MRM) mode. The compounds were separated on a ZORBAX Eclipse® XDB-C18 (Agilent Technologies) 50×2.1 mm column packed with 1.8 µm particles, with a flow rate of 0.4 mL·min⁻¹. Gradient elution was achieved using water (mobile phase A) and methanol (mobile phase B), each containing ammonium acetate 10 mM with initial conditions at 3% B (held isocratic for 1.5 min) increased to 25% in 2.5 min, followed by a ramp to 85% within two minutes and held for two minutes (total gradient of eight minutes). Monitored MRM transitions were m/z 286-201 for morphine, m/z 465-286 for M3G and M6G, m/z 289-201 for M-D3, and m/z 468-289 for M3G-D3. The QTRAP mass spectrometer was operated in positive turbo-ion spray mode with source voltage at 5 kV, curtain gas at 35 psi, source gas 1 (GS1) and GS2 set at 50, declustering potential of 100 V, and a source temperature of 500°C. Collision energy was 35V for morphine and 40V for glucuronide metabolites, with a collisionally activated dissociation (CAD) gas setting of 6 (arbitrary units).

**Data analysis (area under the curve [AUC] calculations)**

The plasma and CSF AUCs between each two adjacent serial time points (interval AUCs) for each cytokine and morphine and M3G and M6G were calculated using the formula: $\frac{C_{avg\ interval}}{\Delta t}$, where $C_{avg\ interval}$ is the average concentration determined from the adjacent serial time points, and $\Delta t$ is the time elapsed between the adjacent serial time points. The interval AUCs were then summed to provide the cumulative AUC from 0 to each serial time point up to the last measured time point for each subject. For the cytokines, the cumulative AUC values for each serial time point were then divided by the corresponding time to provide an average hourly cytokine plasma or CSF exposure, which was used in subsequent correlation analysis.
RESULTS

Supplemental FIGURE The effect of open and endovascular aortic aneurysm surgery on plasma and cerebrospinal fluid (CSF) on cytokine concentrations. Plasma and CSF cytokine concentrations were measured in subjects undergoing open surgical procedures with or without cardiopulmonary bypass (CPB) or in subjects undergoing endovascular surgical procedures. Interleukin-1α (A, B), interleukin-1β (C, D), interleukin-2 (E, F), interleukin-4 (G, H), interleukin-12 (I, J), interferon γ (K, L), and tumour necrosis factor (M, N). The symbols and error bars depict the mean measured raw cytokine concentrations and standard deviations vs average sampling times.