SURGERY-INDUCED INFLAMMATION REDUCES MORPHINE DISTRIBUTION INTO CEREBROSPINAL FLUID

by

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ABSTRACT

Background: Inflammation-induced alterations in drug disposition during inflammatory conditions such as infection and surgery are common and may lead to altered drug responses and/or toxicities. Animal studies have shown that inflammation alters drug disposition into the brain, which has been attributed to regulatory effects of proinflammatory cytokines on blood-brain barrier drug efflux transporters, such as the ATP-binding cassette transporter P-glycoprotein. It is not known if such cytokine-blood-brain barrier drug transporter interactions occur and are important in humans.

Objective: To advance knowledge in this area by investigating the effects of inflammation on drug distribution across the blood-brain barrier in humans. Specifically, the goal was to determine the effects of surgery-induced inflammation on morphine distribution across the blood-brain barrier.

Methods: Patients undergoing elective aortic surgery that received morphine in their standard treatment were divided in two groups: cardiopulmonary bypass, CPB (n = 18) and non-CPB (n = 18) group, based on the surgery they received. Blood and cerebrospinal (CSF) samples were collected before, during and after the surgery. Plasma and CSF morphine, morphine-3 and morphine-6 glucuronide concentrations were determined by mass-spectrometry. Cytokines were quantified with Q-plex multiplex ELISA to characterize the surgical inflammatory response and albumin was measured in both plasma and CSF samples as a marker of passive blood-brain barrier permeability.

Results: The plasma C_{max} and area under the curve from 0 to 24 h ($AUC_{0\text{-}24h}$) of interleukin-6 (IL-6), a known regulator of blood-brain barrier drug transporters were higher ($P < 0.05$) in the CPB group (1045 ± 1421 pg/ml and 9299 ± 12940 pg/ml*h) than in the non-bypass group (162.0 ± 135.4 pg/ml and 2069 ± 2053 pg/ml*h) indicating that individuals receiving CPB had a more robust systemic inflammatory response. The CSF/plasma morphine $AUC_{0\text{-}24h}$ ratio was significantly lower in the CPB than in non-CPB groups (1.09 ± 0.52 and 2.65 ± 1.97, respectively). Albumin CSF/Plasma ratio was increased in the CPB group following surgery, indicating possible increase of blood-brain barrier permeability.

Conclusion: CSF morphine exposure is lower in individuals undergoing aortic aneurysm with CPB. This effect may be due to IL-6-mediated changes in blood-brain barrier drug uptake or efflux transporter function and may be one explanation for altered drug effect in the critically ill patient.

Implications: Altered drug disposition can cause adverse drug reactions (ADRs), which will lead to higher mortality, prolonged hospitalization and increased medical costs. Patients with cardiovascular diseases are particularly at high risk of ADRs and opioid analgesics are frequently associated with ADRs. By studying patients receiving cardiac surgeries and morphine, we have obtained a better understanding of morphine's distribution across blood-brain barrier under inflammatory conditions, which may help reduce ADRs in future clinical practice.

LIST OF ABBREVIATIONS USED

ABC	ATP-binding cassette
ADR	Adverse drug reactions
ADME	Absorption, distribution, metabolism and excretion
AED	Antiepileptic drugs
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
BCG	Bromocresol green
BCRP	Breast cancer resistance protein
CNS	Central nervous system
CPB	Cardiopulmonary bypass
CSF	Cerebrospinal fluid
DDI	Drug-drug interaction
ET	Endothelin
GABA	Gamma-aminobutyric acid
GAT	Gamma-aminobutyric transporter
GLUT	Glucose transporter
IFN	Interferon
IL	Interleukin
LAT	L-type amino acid transporter
LPS	Lipopolysaccharide
M3G	Morphine-3-glucuronide

M6G	Morphine-6-glucuronide
MCT	Monocarboxylate transporter
mRNA	Messenger RNA
MRP	Multidrug-resistance protein
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PAT	Proton-coupled amino acid transporter
PD	Pharmacodynamics
PGP	P-glycoprotein
PK	Pharmacokinetics
PKC	Protein kinase C
PXR	Pregnane X receptor
TNF	Tumor necrosis factor
SD	Standard deviation
SGLT	Sodium glucose co-transporter
SLC	Solute carrier family
TEER	Trans-endothelial electrical resistance
UGT	UDP-glucuronosyltransferase

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CHAPTER 1 INTRODUCTION

1.1 Drug Transporters

Drug transporters are functional proteins located in biological membranes. They are responsible for the cellular uptake or extrusion of drug molecules and endogenous biological molecules, including hormones, nucleotides inorganic ions, fats, carbohydrates and amino acids or peptides. Currently, more than 400 drug transporters have been identified in humans. Most of these belong to the ATP-binding cassette (ABC) superfamily (e.g. P-glycoprotein) or the solute-carrier (SLC) superfamily (e.g. organic cation transporters).[1]

Drug transporters can be referred to by their common names, which reflect their function or by standard nomenclature where drug transporters can be referred to as the gene coding them. For example: p-glycoprotein (PGP) is named as ABCB1 which stands for ABC superfamily, family B member 1; organic cation transporter 3 (OCT3) is referred to as SLC22A8, which means SLC superfamily, family 22 member 8. In addition, efflux transporters are also named after the role they play in drug resistance. For instance, PGP is also referred to as multidrug resistance 1 (MDR1), ABCG2 and ABCC1 are named as breast cancer resistance protein (BCRP) and multidrug resistance protein 1 (MRP1) respectively.

In this dissertation, I chose to use the common names that better indicate the function or the role of transporters, such as OCTs and BCRP. References to standard nomenclature,

such as SLC22A8 and ABCG2, are only used when discussing genes coding the specific transporters. For PGP/ABCB1/MDR1, I shall refer to it as PGP from here.

Membrane carrier proteins exist in various cells, tissues and organs. For example, OCTs are found in brain, liver, intestine and kidney[2]; the glucose transporter 4 (GLUT4) is expressed in muscle and adipose tissues[3]; the monocarboxylate transporters 1, 2 and 4 (MCT 1, 2 & 4) are located within brain astrocytes and neurons[4]. Drug transporters carry molecules through active (against a concentration gradient and requiring energy) or facilitated (not consuming energy) processes.

Depending on the direction of transport, drug transporters are grouped into two categories: the influx transporters, which move solutes from the extracellular space into the cells, and the efflux transporters, which pump solutes from the cytoplasm to the cell exterior. Most members of the ABC family are efflux transporters while members in the SLC family could be influx, efflux or even bi-directional transporters.[5]

Drug transporters in general play an important role in drug absorption, distribution, metabolism and elimination[6] (ADME). Drug uptake transporters located in the intestinal epithelium can affect how much of an oral drug dose is absorbed into the circulation by the patient. As an example, the transport of vigabatrin (an anti-epileptic drug) from the intestinal lumen into the portal blood by the highly capable proton-coupled amino-acid transporter 1 (PAT1) at the human intestinal epithelial brush-border membrane likely contributes to the rapid intestinal absorption and high oral bioavailability (80-90%) of this drug.[7]

At the blood-placenta barrier, membrane drug efflux transporters, such as PGP, BCRP and MRPs pump drug molecules from the fetal to maternal direction thereby preventing

drugs from distributing into the fetal circulation.[8] For example, in female mice and rats, BCRP has been shown to impede the entry of fatty acid amide hydrolase inhibitor III URB937 into fetoplacental unit.[9] At human placental brush border membrane, rosiglitazone and metformin are effluxed primarily by PGP.[10] With human placenta perfusion, researchers found that the inhibition of PGP resulted in increased transfer of saquinavir by 6 to 11 fold, suggesting the important role of PGP in protecting the fetus.[11]

In the liver, drugs need to be carried into the hepatocyte cytoplasm before the intracellular enzymes metabolize them. For example, the cationic drug morphine, is first taken up into the hepatocytes via organic cation transporters (OCTs), particularly OCT1.[2] Once in the hepatocytes, the metabolizing enzyme UDP-glucuronosyltransferase (UGT) turns morphine into morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G).[12] These glucuronosyl-conjugates and unmetabolized morphine are then secreted into the bile duct or transported back into circulation system by efflux transporters MRPs and PGP.[13]

In drug development, membrane transporters are key factors that must be considered and studied in both pre-clinical and clinical phases, because they are not only important in reaching the desired therapeutic effects of the drugs, but can also be involved in drug-drug interactions (DDIs) that can be the cause of ADRs and multi-drug resistance (MDR), which can lead to failure of drug therapy (e.g. chemotherapy). In addition, it is becoming increasingly appreciated that specifically targeting the function of membrane drug transporters is therapeutically important. For example, anti-epileptic drug Gabitril® (tiagabine) selectively blocks the g-aminobutyric acid (GABA) transporter 1 (GAT1)[14] and thus slows down the rate of GABA reuptake; the novel anti-diabetic agent

canagliflozin specifically inhibits sodium glucose co-transporter 2 (SGLT2) and thus increases glucose excretion by the kidney.[15]

The importance of drug transporters in pharmacology and therapeutics is receiving rising attention from not only the laboratories in universities and pharmaceutical companies but also regulatory agencies such as U.S. Food and Drug Administration (FDA). In 2006, the FDA guidance for industry about drug interaction studies focused merely on one transporter, PGP.[16] However, in February 2012, 6 more transporters (BCRP, OATP1B1, OATP1B3, OAT1, OAT3, OCT2) were added to the latest version of guidance for drug interaction studies.[17]

In summary, membrane transporters are important determinants of drug disposition in humans and can also serve as drug targets. The influence of drug transporters must be well studied and seriously considered in academic, industry and clinical settings.

1.2 The Blood-Brain Barrier

The blood-brain barrier (Figure 1) separates the circulating blood from the cerebral neurons and functions as the gatekeeper of the central nervous system (CNS). It is almost impermeable for all large molecules, such as proteins and polysaccharides, and strictly regulates the entry of small molecules, such as amino acids and drugs. The permeability of the blood-brain barrier is altered by various medical conditions and diseases including hypoxia–ischemia, HIV-induced dementia, multiple sclerosis, epilepsy and Alzheimer's disease.[18] Within the pharmaceutical industry, drug permeability at blood-brain barrier is also a very important factor that must be considered in drug development.[19, 20] On one

hand, drugs targeted for action in the CNS must penetrate the blood-brain barrier successfully to exert therapeutic effects in the desired location. On the other hand, it is advantageous for peripherally acting drugs to be blocked at the blood-brain barrier so that drug accumulation in the brain and adverse CNS drug effects are prevented.

The blood-brain barrier is comprised of brain capillary endothelial cells, astrocytes and pericytes.[21] As the primary component of the blood-brain barrier, the polarized brain capillary endothelial cells have special physical features that distinguish them from peripheral capillary endothelia in other organs such as the liver, kidney and muscle, which are relatively leaky. For instance, the brain capillary endothelial cells are closely linked with tight junctions that strictly limit passive diffusion of drugs between cells.[22] In contrast, fenestrae are present in peripheral capillary endothelia, which allow paracellular drug transport more freely. In addition, pinocytosis is almost absent at the brain capillary endothelium, which further restricts blood-born compounds from entering the brain.[23]

The blood-brain barrier is also a selective biological barrier comprised of various membrane transporters, including uptake transporters such as organic anion transporting polypeptides (OATPs) and L-type amino acid transporters (LATs) and efflux transporters of the ABC superfamily such as PGP, BCRP and MRPs.[24] Influx transporters bring drug molecules and vital nutrients into the brain. For example, L-DOPA (3, 4-dihydroxy-l-phenylalanine), a commonly used drug for treating Parkinson's disease, resembles the endogenous substance dopamine and is transported into the brain at the blood-brain barrier by LAT1.[25, 26, 27]

Conversely, efflux transporters pump drug molecules from the brain capillary endothelial cells into the capillary lumen; thus, preventing harmful substances from

accumulating in the brain. ABC superfamily transporters, especially PGP, play critical roles in eliminating drugs from the CNS.[28] This critical function has been best illustrated through drug pharmacokinetic studies in ABC transporter knockout mice. As an example, the maximum brain concentration of tacrolimus, a PGP substrate, was ten times higher in abcb1 knock-out mice compared to wild-type mice 1 hour after administration and the extremely high brain accumulation of tacrolimus was maintained for 24 hours.[29] Similar results were reported with other PGP substrates, such as vinblastine and quinidine.[30, 31]

Other important efflux transporters at the blood-brain barrier include BCRP and MRPs. For example, 2 hours after administration, the brain penetration of imatinib mesylate (Gleevec®) was 2.5-fold higher in abcg2 knock-out mice than in wild-type mice.[32] Similar to PGP, BCRP impedes a chemically diverse set of drugs including cimetidine, alfuzosin and dipyridamole from penetrating the human blood-brain barrier *in vitro*.[33] For MRPs, MRP1, 2, 3, 4, 5 & 6 are all expressed at the blood-brain barrier and affect disposition of various therapeutic agents.[28] For example, in abcc4 knock-out mice, the cerebrospinal fluid (CSF) concentration of anti-cancer drug topotecan was about 10-times higher than in wild-type mice 6 hours after administration.[34]

Overall, various membrane transporters, brain endothelium specific tight junctions and the absence of pinocytosis make the blood-brain barrier a highly selective interface between the peripheral compartment and the CNS. Thus, the blood-brain barrier effectively protects the brain from potentially harmful or toxic substances, while allowing access for important nutrients like glucose, amino acids and nucleic acids etc.

1.3 P-glycoprotein (PGP/MDR1/ABCB1)

In 1976, PGP was discovered as a modulator of drug permeability in Chinese hamster ovary cancer cells, where it was found to mediate cellular resistance to several cytotoxic agents.[35] This phenomenon is known as multidrug resistance (MDR) and is why PGP is also named as MDR1. Following the initial observations by Juliano and Ling, many other studies have established a clear role for PGP in mediating MDR in a variety of cancers.[36] Besides cancer cells, PGP is also expressed in various organs and cells, including brain endothelial cells, hepatocytes, intestinal enterocytes and kidney proximal tubules.[37]

The molecular weight of PGP is 170 KDa. Its structure consists of two transmembrane domains and two intracellular ATP-binding domains. A central pore is found at the cytoplasmic face of the membrane, which forms an aqueous chamber within the membrane and is believed to accommodate substrates of PGP.[38] PGP transports its substrates from the cytoplasm to the extracellular fluid by turning into an outward-facing configuration and releasing its substrate to the cell exterior when ATP binds to its intracellular domain. The protein reverts to the inward-facing structure to take another solute from the cytoplasm when the hydrolysis product (ADP and inorganic phosphate) is disassociated from the ATP binding site (Figure 2).

A broad spectrum of drugs (digoxin, loperamide, doxorubicin, vinblastine, paclitaxel) is pumped from the cytoplasm to extracellular fluid via PGP. For its critical role in drug absorption, disposition and clinical drug-drug interactions (Table 1), PGP is regarded as the most important efflux transporter discovered so far. Regulatory agencies list it as the top drug transporter that must be investigated in drug-drug interactions. In addition, PGP

is also studied as a potential therapeutic target for patients with multidrug resistance cancers, immunomodulation or epilepsy.[39, 40, 41, 42, 43]

In 1989, the gene coding PGP, ABCB1, was found in human brain endothelial cells for the first time.[44] At the blood-brain barrier, PGP is primarily expressed on the luminal membrane of brain capillary endothelial cells. Recent studies revealed that PGP is also expressed on astrocytes, pericytes and neurons.[45, 46] While the extrusion of PGP substrates from the brain is primarily performed by PGP localized at the brain capillary endothelium, PGP localized within adjacent astrocytes, pericytes and neurons is thought to protect these cells from taking up drug molecules.

The effect of PGP on drug distribution across blood-brain barrier was demonstrated by a number of studies. In 1994, abcb1 knock-out mice were reported to be 100 times and 3 times more sensitive than the wild type mice to the anthelmintic drug ivermectin and the anticancer drug vinblastine, respectively.[47] These seminal findings established the functional role of PGP at the blood-brain barrier. Several follow-up studies confirmed these findings in PGP-deficient animals with other PGP substrates, such as digoxin, dexamethasone and cyclosporine A.[48] For example, the brain concentration of (R)-and (S)-methadone was 15- and 23-fold higher in abcb1 knock-out mice than in wild-type controls.[49] The observations that PGP substrates were found to be at higher concentrations in the brain of PGP-deficient animals indicated its important role in eliminating drugs from the CNS. Similar effects were observed in higher mammalian species as well. After the administration of the opioid and PGP substrate loperamide, Collie dogs with a natural mutation in PGP that renders the transporter non-functional experienced opioid-like sedation while the wild type dogs stayed conscious.[50]

In humans, the role of PGP at the blood-brain barrier was demonstrated with PGP inhibitors *in vivo* and *in vitro* as well. Due to the lower expression of tight junctions in human brain capillary endothelial cells, animal kidney cells transfected with human ABC genes are usually used as a tool to study human blood-brain barrier transporters. On monolayers of porcine LLC-PK1 cells transfected with human ABCB1 gene, the basal-to-apical (B-to-A) transport of cortisol was enhanced compared with control cells and this B-to-A transport of cortisol was attenuated by PGP inhibitor LY335979, indicating that human PGP actively transports its substrate drug cortisol from basal membrane to apical (luminal) membrane in cell monolayer.[51] In some patients with refractory epilepsy, the brain expression of PGP was higher than in healthy subjects, suggesting that less antiepileptic drugs (AED) enter the brain of these patients.[52] A commonly used AED is levetiracetam which is also a substrate of PGP.[53] By co-administrating levetiracetam with the PGP inhibitor verapamil to patients with intractable epilepsy, the interval between two hospitalizations was prolonged and nighttime seizures were less frequent, suggesting that PGP limits the entrance of anti-epileptic drugs into the CNS at the absence of PGP inhibitor.[54, 55]

In addition, the function of PGP in the human blood-brain barrier was demonstrated with its gene polymorphisms. Two widely reported gene polymorphisms of PGP are 2677G>T (exon 21, Ala893Ser) and 3435C>T (exon 26, silent): the TT alleles at position 2677 and 3435 were both associated with decreased expression and activity of PGP. Although the mutation at 3435 is silent, it is in significant linkage disequilibrium with 2677 and thus may change PGP function via mutation at 2677.[56, 57] In epilepsy patients with the mutation CC at position 3435, the CSF concentration and the CSF/serum ratio of

phenobarbital was significantly lower than those of patients with TT or CT genotype, however, this effect was not seen in patients with mutations at 2677. This indicates that the CC genotype at position 3435 is associated with higher PGP activity and thus patients with CC genotype eliminate phenobarbital from the brain more efficiently. In 3435 TT homozygous patients receiving morphine therapy after caesarean section, the survival time of wound pain was longer than that of patients with CT or CC genotype, suggesting that TT genotype is associated with lower activity of PGP and thus patients with TT genotype have a higher brain accumulation of morphine.[58]

In summary, PGP is one of the most influential transporters of drug disposition and is a critical determinant of blood-brain barrier permeability for its substrates in rodents and higher mammalian species including humans.[59]

1.4 Regulation of Transporters by inflammation

Inflammation is a protective process produced as an important part of innate immunological response to harmful stimuli, such as infectious organisms, physical trauma and particular irritants. Inflammation involves various types of cells including monocytes, macrophages, T-cells, mast cells and nonhematopoietic cells, as well as a number of blood- and cell-derived mediators such as thrombin, histamine, interferon gamma (IFNγ) and tumor necrosis factors (TNF). These immune cells and mediators all have different functions. For example, histamine produced by mast cells causes arteriole dilation and increases vascular permeability; interleukin 8 (IL-8) produced by

macrophages activates and chemo-attracts neutrophils and other granulocytes to the wounded site and IFN produced by T-cells has potent anti-virus and anti-tumor activity.

The mechanism of inflammation is complex and could be multi-phasic. When the human body is exposed to stressors such as infections, trauma and surgery, immune sensors including macrophages and monocytes are recruited to recognize the pathogen. The recognition is accomplished by pattern recognition receptors (PRRs), which bind to conserved pathogen-associated molecular patterns on the surface of bacteria or virus. Pro-inflammatory cytokines, including interleukin-1 (IL-1), TNF and IFNγ that are expressed and released to the circulation, trigger a series of downstream cellular cascades, and exacerbate the severity of inflammation.[60] To counteract the effects of pro-inflammatory modulators, anti-inflammatory cytokines, such as IL-4 and IL-10 are also produced as an inflammatory response.[61] Depending on how the human body responds to these inflammatory mediators, patients could either recover from an acute inflammation, die or develop a chronic inflammation.

The association between inflammation and altered drug disposition into the brain was first recognized in the 1960s and predated the discovery of blood brain barrier drug efflux transporters. In one study, higher concentration of ethambutol in cerebrospinal fluid was reported in patients with tuberculous meningitis.[62] Similar results were shown in meningitis patients treated with rifampin. While no rifampin was detected in the healthy volunteers' cerebrospinal fluid, extremely high concentrations of rifampin were found in meningitis patients.[63]

Subsequent studies in animals proved that inflammation was associated with altered drug disposition at blood-brain barrier. In rats with localized CNS inflammation induced

by lipopolysaccharide (LPS), the brain level of digoxin was significantly higher than control rats at 6 and 24 hours after the administration, the brain/plasma distribution of digoxin was significantly higher at 48 hours after the administration and the mRNA level of PGP in the brain and liver was down-regulated 6 hours after the administration of LPS.[64] In rats with carrageenan-induced inflammatory pain (CIP), the analgesic effect of morphine was reduced while the expression of PGP was significantly increased 3 hours after CIP, suggesting that upregulated PGP activity contributed to the reduced pharmacological effects of morphine.[65] Other studies support that the observed changes in morphine pharmacodynamics could most likely be explained by cytokines' regulation of the blood-brain barrier efflux transporters under inflammatory conditions.[66]

In vitro studies with the human brain capillary endothelial cells and astrocytes revealed that drug transporters at blood-brain barrier are regulated by inflammatory cytokines. In the *in vitro* model of human blood-brain barrier, hCMEC/D3 cell line: IL-1β, IL-6 and TNF significantly reduced the transport activity and mRNA expression of BCRP. In comparison, the PGP mRNA level was elevated by IL-6 but decreased by TNF.[67] In primary cultures of rat astrocytes, the protein expression level of PGP was increased 1.6-fold by IL-1β, 2.9-fold by TNF and 8.9-fold by IL-6.[68] Interestingly, the regulation of membrane transporters by cytokines could be biphasic under chronic inflammation: in rat brain capillary endothelial cells, the activity of PGP was decreased rapidly (within 1 hour) in the presence of TNF or endothelin (ET-1), however, an increase in PGP activity was found after prolonged exposure (from 3 to 6 hours) to TNF and ET-1.[69]

Combined, the observations in humans, animals and brain cell lines, strongly indicate that drug disposition at blood-brain barrier is altered due to the regulation of transporters

by inflammatory cytokines. There are mainly two mechanisms that could explain the regulation of membrane transporters by inflammatory cytokines: signaling pathway through activation of transcriptional factors, such as nuclear factor-κB (NF-κB), or through the synthesis of nitric oxide (NO).[70] The first mechanism changes the activity of transporters by regulating their expression level, whereas the second mechanism alters the transport activity of the carrier protein directly (shown in Figure 3). In rat brain capillary cells, the decrease of PGP activity and expression caused by 1-hour exposure to TNF and ET-1 could be abolished by NOS inhibitor L-NMMA and PKC blocker BIM[71]; the increase of PGP activity and expression caused by 6-hour exposure to TNF and ET-1could be abolished by inhibiting nitric oxide synthase (NOS), protein kinase C (PKC) and NF-κB.[72] In a similar fashion, the down-regulatory effect of IL-6 on p-glycoprotein in human astrocytes was attenuated by an NF-κB inhibitor.[73]

In summary, inflammation can cause altered drug distribution across the blood-brain barrier due to the regulation of blood brain barrier drug transporters by cytokines. As this hypothesis has largely been proved in animals and *in vitro* studies, more clinical data are needed to confirm it occurs in humans.

1.5 Morphine Pharmacokinetics

Morphine can be administrated orally, intravenously, subcutaneously, intrathecally, epidurally and rectally. The most common route of administration is intravenously. After intravenous injection, assuming morphine is readily distributed into the systemic circulation, its apparent volume of distribution is 1-4.7 L/kg. The protein and muscle

binding rate of morphine is about 36% and 54%, respectively. The elimination half-life of morphine after intravenous administration is about 2.9 ± 0.5 hour.[74]

Morphine is primarily metabolized in the liver and to a much lower extent in other organs such as the lung[75], brain[76] or in fetus during pregnancy[77]. Conjugation with glucuronic acid catalyzed by UDP-glucuronosyltransferases (UGT) is the main route of morphine metabolism: the primary and second conjugation occurs at 3-hydroxyl group and 6-hydroxyl group, respectively. About 80-90% of administrated morphine is excreted in the form of M3G, about 10% of morphine is metabolized into M6G and 2-12% of morphine is eliminated in the unchanged form. The main metabolite morphine-3-glucuronide has no analgesic effects and may be neuroexcitatory[78] whereas morphine-6-glucuronide, as a minor metabolite of morphine, is an active opioid agonist and is much more potent than morphine.[79]

The excretion of morphine happens predominately in the kidney, where morphine and its glucuronide metabolites are expelled from the body in the urine. In patients with renal impairment, the elimination or plasma clearance of morphine was not significantly changed, however, the volume of distribution of morphine at steady state was significantly lower and the elimination half-life of M3G was significantly higher compared with control subjects.[80, 81, 82] Through the bile duct, about 7-10% morphine is excreted and eventually extruded from the body in feces. In addition, morphine is distributed and stored in the fat tissue and traces of morphine are also detected in breast milk and sweat.

As a commonly used anesthetic and analgesic agent, morphine targets primarily mu-opioid receptors in the brain. There are three mechanisms for morphine to penetrate the

blood-brain barrier: 1) limited passive diffusion; 2) unidentified, low-capacity active influx, and 3) primarily PGP-mediated active efflux.[83] The latter involvement of PGP is supported by studies in abcb1 knockout mice where the brain/plasma morphine concentration ratio was approximately 2-fold higher than the ratio in control mice.[84] In PGP inhibitor GF120918-treated rats, the brain/serum concentration of morphine is also significantly elevated.[85]

In comparison with morphine, M3G and M6G are much more hydrophilic and thus are restricted in their ability to cross the BBB by passive diffusion. This is supported by the longer time for these metabolites to accumulate in the CNS when compared to the more lipophilic morphine. [86, 87, 88, 89, 90] Similar to morphine, M3G and M6G are eliminated from CNS by efflux transporters: M3G and M6G are both substrates of MRP1 and 2, so in theory these transporters could contribute to efflux of the glucuronide metabolites from the brain to the circulation.[13,91] In addition, bidirectional transporter OATPs and the facilitated transporter GLUT1 might be involved in the transport of M3G and M6G across blood-brain barrier.[92, 93, 94]

1.6 The Effect of Inflammation on Morphine Distribution to the Brain

The brain exposure of morphine is altered when inflammation is present. In pigs with meningitis, the brain to blood area under the curve (AUC) ratio of morphine is significantly increased 3 hours after the induction of meningitis, implying the impaired function of the morphine efflux transporter PGP.[95] In rats, the CSF/plasma ratio of morphine decreased 4-hours following LPS-induced peritoneal inflammation.[96] It was

speculated that this involved enhanced drug efflux, but the specific transporter mechanisms were not determined. In patients with severe brain injury, the penetration of morphine and its metabolites into brain was altered compared with patients with normal brain, which was likely secondary to PGP downregulation or inhibition, however, the role of inflammation in the observed alteration in BBB function was not examined.[97] In our previous studies, patients with acute brain injury displayed increased level of M3G and M6G in the cerebrospinal fluid and the increase was proportional to the IL-6 concentration in the blood.[98] Based on these animal and clinical studies of morphine and its metabolites, it is possible that the brain distribution of morphine is altered in patients under systemic inflammatory conditions.

1.7 Experimental Design

In our study, we chose aortic aneurysm surgery as the clinical model of inflammation and morphine as the probe drug. The justification for this choice of experimental design is described in the details below.

1.7.1 Clinical Model of Inflammation – Aortic Aneurysm Surgery

We chose elective aortic surgery patients as our study population because 1) aortic surgical procedures produce a robust systemic inflammatory response with known onset time;[99, 100, 101, 102, 103, 104, 105, 106, 107] 2) cytokines such as IL-6 and TNF are produced during aortic surgeries[98] and studies have shown that these cytokines modulate drug transporters at blood brain barrier[71, 73]; 3) the chosen aortic surgical procedures require placement of

arterial and spinal catheters, which allows for plasma and cerebrospinal fluid collection, or in other words, drug sampling on both sides of the blood-brain barrier; 4) morphine is normally received for pain management in patients undergoing aortic aneurysm surgery; 5) aortic aneurysm surgery is a very common procedure, so the findings of our research have potential broad clinical implications.

1.7.2 Clinical Probe Drug – Morphine

We chose morphine as the probe drug in our clinical study because 1) morphine is transported by the most-well established and the most important drug transporter, PGP, at the blood-brain barrier and the pharmacokinetics of morphine have been well studied;[108, 109, 88, 89] 2) the methods to extract and quantify morphine and its metabolites from plasma and CSF samples are well established;[110] 3) the metabolites of morphine, M3G and M6G are transported by MRPs, which allows us to investigate other important drug transporters at the blood-brain barrier;[111, 93, 86, 112, 113, 114, 94] and 4) morphine is part of the routine treatment for patients undergoing aortic surgeries, so it will not alter the standard of care of the patients.

1.8 Objective

To determine the role of surgery-induced inflammation on the transport of morphine and its metabolites, M3G and M6G across the blood-brain barrier

1.9 Hypothesis

The distribution of morphine and its metabolites across the blood-brain barrier is altered during surgery-induced inflammation due to cytokine-mediated effects on blood-brain barrier drug efflux transporters.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

The QuantiChrom™ BCG albumin assay kits were purchased from BioAssay Systems (Hayward, CA). The Q-Plex™ human cytokine inflammation (9-plex) ELISA kits were products of Quansys Biosciences (Logan, UT). The Oasis 96-well MCX μelution plates were purchased from Waters Corporation (Milford, MA). Standard solutions of morphine (1mg/ml), M3G (1mg/ml) and M6G (0.1mg/ml), including D3-labelled morphine (1mg/ml) and M3G (0.1mg/ml), were purchased from Cambridge Isotope Laboratories (Tewksbury, MA).

2.2 General Description

2.2.1 Administrative requirements

The Research Ethics Board (REB), Capital District Health Authority, approved the study protocol (classified as minimum risk Category B) and the participant consent form. A Health Canada No Objection Letter was not required (waived). The names of subjects were coded and their personal information was kept confidential.

2.2.2 Study design

This was a sequential enrolment study design in which elective surgical patients presenting for repair of a descending thoracic aneurysm and fitted with a CSF drain as

part of their standard of care were approached for permission to draw blood samples at specified times during their hospital course. Concomitantly, samples of CSF were collected from the CSF drainage system which normally flows to waste. Morphine was used as the primary analgesic agent (this is within the standard of care). For each subject, 7 to 27 samples were collected at specified time intervals for 5-days or whenever the CSF drain was removed (whichever came first).

2.2.3 Patient population and requirements

A total number of 36 patients were recruited from three groups of patients who received a lumbar CSF drain as part of their surgical procedure:

Group 1: Patients undergoing percutaneous insertion of an aortic stent;

Group 2: Patients undergoing open thoracotomy and graft insertion;

Group 3: Patients undergoing open thoracotomy and graft insertion during cardiopulmonary bypass (CPB).

Since the invasiveness of the surgeries increases from group 1 to 3, we expected to see ascending levels of inflammatory response indicated by rising concentrations of inflammatory mediators, such as IL-6, IL-10, TNF etc. Due to the lack of a control (non-surgery) group, group 1 and 2 together (non-bypass group) therefore served as low-inflammation control for group 3 (bypass group), in which a higher degree of inflammation was expected.

2.2.4 Subject Selection

Inclusion criteria: 1) Subjects who underwent elective surgery for thoracic aortic vascular disease requiring insertion of a lumbar CSF drain and 2) who provided written informed consent.

Exclusion criteria: 1) Unwilling or unable to provide informed consent; 2) sensitivity or documented allergy to morphine; and 3) inability to place lumbar CSF drain.

2.2.5 Clinical Study Procedures

Perioperative management: Subjects were fitted with a lumbar CSF drain (inserted into the subarachnoid space) and a peripheral arterial catheter. Prior to administration of morphine, the research coordinator obtained 5–15 mL of CSF (collected into a dedicated reservoir) and 5 mL of blood (collected into a sodium citrate containing glass tube) for baseline analysis of inflammatory mediators (control). Subjects were then administered up to 10 mg of morphine followed by initiation of a morphine infusion (2 mg hr^{-1}) that was maintained for the duration of the surgery. Group 1 and 2 subjects had CSF (5-15 mL) and blood samples (5 mL) obtained at pre-incision, at wound closure, and then every 6 h for 5 days or until the lumbar CSF drain was removed (whichever came first). Group 3 subjects had CSF (5-15 mL) and blood samples (5 mL) obtained at pre-incision, prior to initiation of CPB, following return of native circulation post-CPB, at skin closure, and then every 4 hours for 5 days or until the lumbar CSF drain was removed (whichever came first). No attempt was made to alter the course of patient care other than to require the use of morphine as the primary narcotic analgesic agent while in the ICU. This does not represent a deviation from the standard of care. A data set of the underlying medical

conditions, demographics (including hospital course), and drugs used during the study period was compiled from the hospital medical records. Confounders such as degree of renal dysfunction[115] and concurrent medications that are known to interact with PGP and MRP1or 2[116, 117, 118, 119] were recorded. In the pilot study the presence of drugs that are major inhibitors/enhancers of transporter function had not been a major concern.

2.2.6 Sample analysis

Upon collection, CSF and plasma samples were divided into working aliquots and stored at – 70 °C prior to analysis. CSF and plasma samples were assayed for morphine, M3G, M6G, pro-inflammatory and anti-inflammatory mediators and albumin which is a marker of passive blood-brain barrier permeability.

2.3 Cytokine ELISA

To determine the concentration of inflammatory mediators at various time points after the surgery, multi-plex ELISA was employed to quantify 9 inflammation-related cytokines in CSF and plasma samples. The cytokines analyzed were IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IFN and TNF. We conducted this assay according to the manufacturer's protocol with some minor changes. An 8-concentration standard curve was prepared for each cytokine using 1:3 serial dilutions. The range of standards and limits of quantifications are shown in Table 2. The intra-assay and inter-assay coefficients of variations of the standards were both below 15%. Samples were diluted 1 in 2. To perform the Q-plex ELISA, 50 ul of diluted samples or standards were added to

the ELISA plate in duplicate and shaken on an orbital shaker at 750 rpm to allow for cytokine binding to the primary antibodies. After incubation for 2 hours, samples were decanted from the plate and 300 µl of wash buffer was added to each well. To wash the plate thoroughly, the wash buffer was aggressively flicked out into a waste container. After repeating the washing step 3 times, the plate was inverted and tapped forcefully against a paper towel to dry. Then 50 µl of detection mix containing the biotinylated detection antibody was added to the plate. After incubation for 1 hour on the orbital shaker, the plate was washed three times using the washing method described above. Then, the light-sensitive DyLight® IR dye was added to each well. This fluorescent molecule attaches to biotinylated antibody and produces detectable signals at 800nm. After incubation for 15 minutes and washing the wells 6 times, the plate was scanned in the LI-COR Odyssey imaging system at the 800nm channel. The intensity, resolution, focus-offset and quality were set at 7-10, 84 µm and 3.9-4.0, respectively; the lowest quality of scan was selected. The image was then imported to the Q-view software for data analysis. Standard curves of each cytokine were plotted using the software built-in 5PL regression program. Final cytokine concentrations in each sample were then calculated based on their pixel intensity and standard curves.

2.4 Morphine Extraction & Quantification

External standards were prepared by spiking pooled baseline plasma or CSF samples with morphine, M3G and M6G at a series of concentrations. For plasma, the final concentrations of external standards were 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 2

μg/ml; for CSF, the concentrations were 0, 0.0005, 0.0025, 0.005, 0.025, 0.05, 0.25, 0.5, 1 μg/ml. Deuterated morphine (M-D3) and M3G (M3G-D3) were utilized as internal standards at a concentration of 1 μg/ml for plasma samples or 0.01 μg/ml for CSF samples.

To extract morphine and its metabolites, 96-well Oasis MCX (30μm) μelution solid phase extraction plates were used. The plates were first conditioned with 200 μl of methanol per well, followed by vacuum filtration. This conditioning step was then repeated and with the same volume of double-distilled water. To each well, 100 μl of sample or standard was added together with 100 μl of internal standards and 200 μl of 4 % phosphoric acid. The solutions were then vacuum filtered, which allows binding of morphine and metabolites to the sorbent. The plate was washed with 200 μl of 2% formic acid per well and then the same volume of methanol, followed by vacuum filtration to remove impurities. Each well was eluted into a corresponding well of a 96-well collection plate twice with 50 μl of 5% ammonium hydroxide and 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 was sealed and stored at -70°C and then delivered on dry-ice to our collaborator, Dr. Lekha Sleno in Montreal, for determination of concentrations of morphine and its metabolites using mass spectrometry.

The quantification 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[98]. Briefly, the purified extracts were injected onto the LC-MS/MS system consisting of a

Shimadzu Nexera UHPLC and an AB Sciex (Concord, ON, Canada) QTRAP 5500 hybrid quadrupole-linear ion trap mass spectrometer in multiple reaction monitoring (MRM) mode. The compounds were separated on a Zorbax C18-XDB (Agilent Technologies) 50 x 2.1 mm column packed with 1.8 µm particles, with a flow rate of 0.4 mL/min. Gradient elution was achieved using water (mobile phase A) and methanol (mobile phase B), each containing 10 mM ammonium acetate 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 2 min and held for 2 min (total gradient of 8 min). Monitored MRM transitions were m/z 286-201 for morphine, m/z 465-286 for M3G and M6G, m/z 289-201 for d3-morphine and m/z 468-289 for d3-M3G. The QTRAP mass spectrometer was operated in positive turbo-ion spray mode with source voltage at 5 kV; curtain gas at 35 psi, GS1 and GS2 set at 50, declustering potential of 100 V, and a source temperature of 500°C. Collision energy was 35 V for morphine and 40V for glucuronide metabolites, with a CAD gas setting of 6 (arbitrary units).

2.5 Albumin Assay

Albumin is a marker for passive permeability across blood-brain barrier. Under normal conditions, albumin does not penetrate BBB. To confirm the integrity of blood-brain barrier, albumin concentrations in both plasma and CSF samples were measured as instructed by the manufacturer's protocol. This assay uses a compound called bromocresol green (BCG), which forms a colored complex specifically with albumin. The standard curve in this assay included 8 points: for plasma samples, the standard

concentrations were 0, 0.5, 1.0, 1.5, 3.0, 3.0, 4.0 and 5.0 g/dL; for CSF samples, the standard concentrations were 0, 0.05, 0.2, 0.5, 1.0, 1.5 and 2.0 g/dL. Since the plasma albumin concentration is much higher than CSF albumin concentration the plasma samples were diluted 1 in 2 with distilled water. To perform the albumin assay, 5 μl of samples or standards were added in duplicate to a 96-well clear bottom plate. Then, 200 μl of working reagent that contained BCG was added to each well. After 5-minute incubation at room temperature, the plate was read at 620 nm in a 96-well plate reader. Standard curves were generated by non-linear regression of the absorbance versus concentration values. The concentration of albumin in each sample was determined from the Michaelis-Menten equation $Y = Vmax*X/(Km + X)$, where Y is the sample absorbance, X is the albumin concentration, Km is the Michaelis-Menten constant and Vmax is the maximum absorbance.

2.6 Pharmacokinetic Analysis

Our primary metric of analysis was through calculations and comparisons of area under the curve (AUC) values for cytokines, morphine and M3G and M6G. Over 4 time intervals, 0-6, 0-12, 0-18 and 0-24 hours, plasma and CSF AUCs were determined for each individual cytokine, albumin, morphine, M3G and M6G using the trapezoidal rule in GraphPad Prism 5. (Plasma AUC of morphine, M3G and M6G from $0\text{-}t_n$ are shown in Figure 4 Panel B).

In addition, the maximum concentration (C_{max}) of cytokines was calculated to indicate the degree of individual inflammatory response. The CSF/plasma AUC ratio of morphine

and its metabolites was taken as an indicator of their penetration degree across blood-brain barrier. To examine correlations between systemic inflammation and drug accumulation in the brain, a linear regression analysis of the plasma AUCs of individual cytokines versus the CSF/plasma AUC ratio of morphine, M3G or M6G was performed.

To generate a more complete picture regarding the CSF uptake and elimination of morphine and M3G and M6G, a series of additional pharmacokinetic parameters were calculated.

The CSF uptake kinetics of morphine and M3G and M6G was determined by calculating the CSF uptake constant (k_{in}) and half-life ($t_{1/2\ in}$), which represents the initial rate of drug uptake into the CSF. To calculate k_{in}, the plasma AUC of morphine from baseline to the first time point when morphine is detectable in CSF (T_1 CSF) was calculated in GraphPad Prism 5 (Figure 4 Panel B). By dividing the plasma $AUC_{0\text{-}T1\ CSF}$ with the first detectable concentration of morphine in CSF (C_1 CSF, shown on Figure 4 Panel A), k_{in} was obtained.[120] The value of $t_{1/2\ in}$ then can be calculated from k_{in} using the equation $t_{1/2} = 0.693/k_{in}$.

The CSF elimination rate constant (k_{el}) was defined as the fraction of drug in the CSF that is eliminated per unit of time. To calculate k_{el} of morphine, the morphine elimination phase (from the peak to the end, shown on Figure 4 Panel C) on the time-concentration curve was first transformed to a logarithm scale and linear regression was used to calculate the slope which equals - $k_{el}/2.303$. The CSF elimination half-life ($t_{1/2}$), the time required for the amount or the concentration of drug in the body to decrease by h was calculated by using the equation $t_{1/2} = 0.693/k_{in}$ (shown in Figure 4 Panel D).

Clearance of a drug is defined as the volume of the body fluid from which the drug is apparently and completely removed by metabolism and/or excretion, per unit time. Plasma clearance of morphine and its metabolites was obtained by dividing $AUC_{0\text{-}24h}$ with total dose administered within 24 hours. CSF clearance was not calculated as the total morphine that entered the CNS was unknown.

2.7 Statistical Analysis

A nonparametric t-test, unpaired t-test and two-way analysis of variance (ANOVA) were used in the data analysis. When $P < 0.05$, the difference was considered statistically significant.

CHAPTER 3 RESULTS

3.1 Patients

Overall, we recruited 36 adult patients: 18 in the non-CPB group and 18 in the CPB group. The individual patient characteristics are shown in Table 3 and a summary of these parameters by groups is shown in Table 4. Patients were all Caucasians. Because aortic surgeries are commonly performed in the elderly, more than half of the patients were older than 70. Subjects in the CPB group were on average 12 years younger than those in the non-CPB group ($P = 0.02$ in unpaired t-test). Both groups were equally distributed with respect to male ($n = 13$) and female ($n = 5$) patients. According to the analysis from the unpaired t-test, there were no significant differences in body weight and height between the two groups. In the morphine analysis, 2 patients were excluded for administration of codeine (patient 1) and morphine (patient 6) before the cardiac surgery.

In the CPB group, the duration of morphine administration and ICU stay were generally longer, possibly due to more intensive patient care after more complex and invasive surgeries. Correspondingly, the cumulative dose of morphine was also higher in CPB patients. However, when it came to the average dosing rate (mg/h) of morphine, there was no significant difference between CPB and non-CPB groups. Among the 36 patients, 28 of them displayed normal recoveries following surgery and returned home. Eight subjects experienced adverse outcomes as a result of the surgical intervention. These included 2 deaths, 1 paralysis and 2 hospitalizations in the CPB group and 3 hospitalizations in the non-CPB group.

In addition, we screened concomitant medications for PGP inhibitors. In total, there were three potential PGP blockers. Patients taking these three inhibitors are shown in Table 5. Because patient 1 was excluded in morphine analysis, there were 4 patients in non-CPB group and 3 patients in CPB group that received PGP inhibitory drugs. These patients were not excluded from the morphine analysis.

3.2 Surgical-Mediated Inflammatory Response

3.2.1 General Description of the Cytokine Concentration-Time Profiles

In the non-CPB group, we analyzed a minimum of 8 time points: baseline, pre-incision, skin closure, arrival at ICU and post-surgery 6, 12, 18 and 24 hours; in the CPB group, a minimum of 10 time points were analyzed: baseline, pre-incision, pre-CPB, post-CPB, skin closure, arrival at the ICU and post-surgery 6, 12, 18 and 24 hours. The time points for sample collection are illustrated using plasma IL-6 in the non-CPB and CPB groups as an example (Figure 5). The profiles for mean cytokine concentration versus average time of sample collection are shown in Figure 6 (plasma) and 7 (CSF). Every patient had one or more low but detectable cytokines in their baseline samples. However, there were no statistical differences in the plasma or CSF baseline cytokine values between the CPB and non-CPB groups shown by two-way ANOVA with post-hoc multiple comparison tests (Figures 8 and 9). Therefore, any increase of cytokine concentrations during the clinical study or any differences of cytokine levels between groups could be attributed to the surgical procedure and not a pre-existing inflammatory condition.

Subsequent to baseline, during and after the surgical procedures the following cytokine profiles were observed. Comparison of plasma cytokine levels between groups showed (Figure 6): 1) concentrations of IL-1α, IL-1β and IL-4 were at similar levels over time in two groups, though IL-4 showed higher levels in CPB group at approximately 10 h (ICU arrival); 2) concentrations of IL-6, IL-10, IL-12 and TNF appeared to be higher in CPB groups and 3) concentrations of IL-2 and IFN-γ appeared to be higher in non-CPB groups. In the non-CPB group, plasma TNF and IL-10 showed clearly identified peaks at skin closure and ICU arrival, respectively. In the CPB group, plasma IL-6, TNF and IL-10 both reached peak levels at post-CPB time point. In addition, plasma IL-12 showed two peaks in CPB patients: first one at skin closure and second one at 18 hours after ICU arrival.

Comparison of CSF cytokine levels between groups showed (Figure 7): the concentrations of TNF appeared to be higher in CPB patients than in non-CPB patients at all time points; the concentrations of IFN-γ was lower in CPB patients than in non-CPB patients at most time points with the only exception at pre-incision. In CSF samples from the CPB group: IL-10 reached peak level at the beginning of the surgery (pre-incision); IL-6 and IL-12 both showed easily identified peaks at post-surgery 6 hours and IL-4 reached peak concentration 6 hours later, at post-surgery 12 hours.

In summary, an inflammatory response occurred in the circulation and in the CNS, as characterized by the elevated concentration of inflammatory mediators in both plasma and CSF following the selected surgeries. However, some of the cytokine profiles appeared different between CPB and non-CPB groups, suggesting that inflammatory responses might be impacted by the type of surgical procedure.

3.2.2 Baseline (C_0) Versus Peak Cytokine Concentrations (C_{max})

In the clinical study design, we predicted that patients undergoing the CPB procedure would experience a more robust inflammatory response. To characterize the degree of inflammatory response, we compared the maximum concentration (C_{max}) of every cytokine in the plasma and CSF to the baseline value (C_0) from each patient. For the initial analysis, a two-way ANOVA with factors surgical procedure (CPB versus non-CPB) and repeated measures for time (C_0 versus C_{max}) was used. Testing for the main effect of time irrespective of surgical procedure, it was found that IL-1α, IL-1β, IL-2, IL-6, IL-10 and TNF had significantly higher C_{max} values than their corresponding C_0 in plasma (Figure 8) and in CSF (Figure 9). The observation of significantly elevated cytokine levels in plasma at C_{max} following aortic aneurysm surgeries confirmed a systemic inflammatory response. When testing for the main effect of surgical procedure irrespective of time, IL-6 and IL-10 concentrations showed significantly higher plasma C_{max} (Figure 8) but not CSF C_{max} (Figure 9) in subjects following CPB procedure. Furthermore, there was a significant interaction between the surgical procedure and time for IL-6 and IL-10 in plasma, indicating that the C_{max} obtained for these cytokines was dependent on the surgical procedure (Figure 8). There was no significant interaction between surgical procedure and sampling time for any of the 9 cytokines measured in CSF (Figure 9).

For the cytokines that demonstrated a significantly higher C_{max} we completed a post hoc multiple comparison of C_0 to C_{max} by group for plasma (Figure 8) and CSF (Figure 9). In the non-CPB group, the plasma C_{max} of IL-1α, IL-2 and IL-10, was significantly higher than their corresponding baseline level. In the CPB group, plasma C_{max} of 3 cytokines, IL-6, IL-10 and TNF, was significantly higher than baseline. In the CNS, the CSF peak concentrations of IL-1α, IL-2, IL-6 and IL-10 were significantly higher compared with their baseline concentration in patients undergoing CPB procedures. However, in non-CPB group, no cytokines showed significantly higher CSF peak concentrations compared with their corresponding baselines.

As the ANOVA revealed that the change in IL-6 and IL-10 was dependent on the surgical procedure, the post-hoc analysis for these cytokines also included a comparison of the Cmax values between the CPB and non-CPB groups in plasma. This analysis confirmed that the mean plasma C_{max} values for IL-6 and IL-10 were significantly higher following the CPB procedure versus the non-CPB procedure. These results also indicate as we predicted that the systemic inflammatory response is more robust following the more invasive CPB procedure.

Overall, the data analysis of peak concentrations validated our clinical study design and demonstrated that 1) inflammatory mediators were released into the general circulation and CSF; 2) an inflammatory response occurred in both CPB and non-CPB patients and 3) systemic inflammation is more robust in CPB group.

3.2.3 Area under the Curve (AUC)

In addition to peak concentration, we compared the plasma and CSF AUC value of each cytokine between the CPB and non-CPB groups at 6 ($AUC_{0\text{-}6h}$), 12 ($AUC_{0\text{-}12h}$), 18 ($AUC_{0\text{-}18h}$) and 24 ($AUC_{0\text{-}24h}$) hours to provide an index of the overall cytokine exposure over time. The AUC data that were significantly different between groups are shown in Figure 10.

In plasma, the $AUC_{0\text{-}24h}$ values for IL-6, IL-10 and TNF were significantly higher in the CPB patients compared with the non-CPB subjects, indicating that the subjects undergoing CPB had significantly larger systemic exposure to IL-6, IL-10 and TNF. In addition, the systemic exposure to TNF as measured by AUC was significantly larger in CPB patients after 18 hours. In CSF, TNF was significantly affected by surgery type irrespective of time point. However, CSF AUC of other cytokines did not show any statistically meaningful difference between groups.

To summarize, in our data analysis of cytokines, we found that 1) the baseline cytokine levels were not significantly different between CPB and non-CPB patients; 2) peak plasma but not CSF concentrations of IL-6 and IL-10 were significantly higher in patients following the CPB procedure, indicating a more robust systemic inflammatory response was induced by CPB; 3) the total systemic exposure to IL-6, IL-10 and TNF as measured by plasma AUC values was significantly higher in the CPB group, indicating more robust inflammation in patients undergoing CPB.

3.3 Quantification of morphine and morphine glucuronides in the circulation and CSF

3.3.1 Concentration-Time Curves of Morphine and its Metabolites

The average concentration-time curves of morphine and its metabolites (M3G and M6G) are presented in Figure 11. In general, plasma concentrations of M3G and M6G were higher than morphine whereas the CSF concentration of morphine was higher or similar in comparison with M3G and M6G. In both groups, plasma M3G was at higher levels than M6G and morphine. In the CSF, morphine accumulated more quickly and was at higher levels than its two metabolites. In comparison between non-CPB and CPB group patients, the concentrations of morphine, M3G and M6G were higher in CPB patients at later point (around 15 hour) after the surgery because CPB patients generally received higher dose of morphine and for a longer period of time.

3.3.2 Metabolite to Parent Compound Ratio

Drug metabolism can be altered under inflammatory conditions. To examine the metabolism of morphine in patients following aortic aneurysm surgery, we calculated the metabolites to parent compound concentration ratio (M3G/morphine, M6G/morphine) in both plasma and CSF. The average ratios of M3G and M6G to morphine were calculated at 6, 12, 18, 24 hour. No significant difference was shown at any of the 4 time points. The average M3G and M6G to morphine ratios for the CPB and non-CPB groups are shown in Table 6. In both CPB and non-CPB patients, the plasma concentrations of M3G and

M6G were higher than morphine. However, there was no significant difference between the CPB and non-CPB groups.

3.3.3 AUC CSF to Plasma Ratio

To eliminate individual differences in morphine dosing, we used the AUC CSF/plasma ratio to describe the distribution of morphine across the blood-brain barrier. Similar to the cytokine AUC analysis, four time points were selected, including 6, 12, 18 and 24 hours (Figure 12). Using a two-way ANOVA, the morphine AUC ratio was affected significantly by the main effects of time (P=0.0038) and surgery (P=0.0003). There was also a significant interaction between the two factors (P=0.0329), indicating that the change in morphine CSF/plasma AUC ratio with time depends on the type of surgery performed. For the post-hoc multiple-comparison analysis, the morphine CSF/plasma AUC ratios between the CPB and non-CPB groups at the 6, 12, 18 and 24 hour points were compared. After 6 hours, the average CSF/plasma AUC ratios for morphine were similar in CPB compared with the non-CPB group, both below 1. From 12-24 hours, the CSF/plasma morphine AUC ratio in non-CPB group increased gradually whereas the CSF/plasma morphine AUC ratio in CPB patients did not change much. This resulted in the difference between CPB and non-CPB group reaching significance after 18 and 24 hours. Overall, these results indicated that less morphine distributed into the CSF relative to plasma of subjects undergoing the CPB procedure and that the difference was most pronounced at 24 hours after the first sample collection time.

Similar to the morphine data analysis, we compared the CSF to plasma AUC ratio of M3G and M6G between the CPB and non-CPB group (Figure 12). In two-way ANOVA: M3G was significantly influenced by time ($P<0.0001$) rather than surgery type ($P=0.4$); M6G was not dependent neither on time ($P=0.1$) nor on surgery type ($P=0.2$).

In summary, CPB affected the CSF distribution of morphine but not M3G and M6G as measured by the CSF/plasma morphine AUC ratio. Furthermore, CPB did not alter the metabolism of morphine as determined by the CSF and plasma M3G/morphine and M6G/morphine ratios.

3.3.4 Other Pharmacokinetic Parameters

A summary of all calculated PK parameters other than AUC ratio is presented in Table 7. These parameters included systemic clearance, CNS uptake and elimination half-life of morphine and its metabolites. The plasma clearance of morphine was not significantly different between groups, which further confirmed our conclusion that metabolism and renal elimination of morphine was not affected by CPB. The half-life for morphine uptake into the CSF was much more rapid than for M3G and M6G. There were no differences in the CSF uptake half-life of morphine M3G or M6G or CSF elimination half-life of morphine between groups. Because M3G and M6G uptake into the CSF was very slow we were not able to see a clear elimination phase within our sampling time frame. Therefore, the CSF elimination half-life of morphine metabolites was not available in our clinical study.

3.4 Correlations between Morphine and Cytokines

To better understand the relationship between morphine distribution across blood-brain barrier and the elevated cytokine concentrations in the blood, we performed a linear regression of morphine $AUC_{0\text{-}24h}$ CSF/Plasma ratio against the peak concentrations and $AUC_{0\text{-}24h}$ of IL-6 (Figure 13). The two figures were similar in data point distribution and we did not see a significant linear correlation in either case (r^2=0.06). Similarly a significant linear correlation was not observed if the surgical groups were analyzed separately (data not shown). In addition to plasma, CSF IL-6 was not correlated with morphine ratio either. Similar analysis was performed with IL-10 and TNF: again, no significant correlations were observed. Overall, morphine $AUC_{0\text{-}24h}$ ratio was not well correlated with AUC and C_{max} of IL-6, IL-10 or TNF.

3.5 Albumin

Albumin levels were determined in plasma and CSF at each time point. The average concentration-time curves of plasma and CSF albumin are shown in Figure 14. In both groups, the baseline plasma and CSF albumin concentrations were similar. The plasma and CSF concentration of albumin then tended to decline during the surgical procedure and stayed at comparable levels thereafter in both non-CPB and CPB groups. However, the minimum concentration was reached at different time points in two groups. In the non-CPB group, the plasma concentration of albumin was significantly lower (P = 0.0391) at the end of surgery (26.0 ± 10.35, n = 17) compared with plasma concentration prior to surgery (33.4 ± 10.1, n = 18). In the CPB group, the plasma concentration was

significantly lower ($P = 0.002$) at the end of CPB (20.1 ± 9.68, $n = 18$) compared to baseline (35.6 ± 11.5 g/L, $n = 16$). In CSF, the albumin level did not change substantially following surgeries in both groups. In non-CPB group, the CSF/plasma ratio of albumin did not differ significantly after the surgery although the plasma albumin level decreased significantly at the end of surgery. In the CPB group, the significant decrease of plasma resulted in significantly higher ($P<0.05$) albumin CSF/plasma ratio at the end of surgery (0.057 ± 0.041) than at baseline (0.027 ± 0.028), suggesting a possible increase in blood-brain barrier permeability (Figure 15).

CHAPTER 4 DISCUSSION

4.1 Overview

In critically ill patients, altered pharmacokinetic (PK) and pharmacodynamics (PD) are often seen for various therapeutic agents, especially analgesics and sedatives.[121] For CNS-active drugs such as analgesics and sedatives, the blood-brain barrier is an important factor in determining their PK/PD in the brain. As an important protective barrier of the CNS, the blood-brain barrier also limits the brain accumulation of many systemically acting drugs. A number of animal and cell-based studies have demonstrated that PGP function or expression is altered under inflammatory conditions, leading to an altered PGP substrate distribution across blood-brain barrier (multiple studies reviewed in reference[122]). So an interesting question concerning critically ill patients is to determine if inflammation–mediated changes in blood-brain barrier, especially changes of PGP function, is involved in the altered PK/PD of therapeutic agents. If blood-brain barrier and PGP are involved, there could be broad clinical implications for both CNS-active molecules and systemically-acting medications.

In our clinical study, we examined the distribution of morphine across the blood-brain barrier in patients following aortic aneurysm surgeries with or without CPB. We hypothesized that the aortic aneurysm surgeries that involved the CPB procedure would produce a more robust inflammatory response compared to the surgeries that did not require CPB. In turn, we hypothesized, based on results of animal studies, that the higher

degree of inflammation following CPB would alter morphine distribution into the brain due to a change in the function of the blood-brain barrier efflux transporter PGP.

In our results, both the CPB and non-CPB groups showed elevated inflammatory cytokine levels after surgery in both CSF and plasma. In support of our hypothesis, the CPB group had significantly higher IL-6 and IL-10 C_{max}, and higher IL-6, IL-10 and TNF AUCs in the plasma indicating they had larger overall systemic exposure to those cytokines throughout their stay in the intensive care unit (ICU). In CSF, no cytokine was significantly higher in the CPB group. Therefore, we believe that the primary pharmacokinetic observation of reduced disposition of morphine into the CSF relative to the plasma compartment in patients undergoing CPB procedure is primarily linked to the differential systemic inflammatory response rather that a CNS inflammatory response. The overall results indicated that the CPB procedure altered the blood-brain barrier handling of morphine, but the direction of the effect was opposite compared to some previous animal studies. Species difference in drug transporters; the type of inflammation and time course of study could be reasons why our human data is different from some of the previous animal data.

4.2 Importance of the Clinical Study on the Human Blood-Brain Barrier

In studies investigating the blood-brain barrier under inflammatory conditions, animal models and *in vitro* cell culture have been widely used. However, there are two major concerns when studying blood-brain barrier in these models: first is species difference in

animal *in vivo* models and animal cell lines; second is the low expression of tight junctions and drug transporters in human *in vitro* cell models.

A great number of studies have discussed the difference of PGP across species. First of all, the species difference could be shown as different brain concentration or brain-to-plasma concentration ratios of the same PGP drug substrate. By using positron emission tomography, the brain-to-plasma concentration of PGP substrate drug [11C] GR205171 was found to be 9-times higher in humans than in rats and the difference remained after treatment with the PGP inhibitor cyclosporin A, suggesting less functionality of human PGP in blood-brain barrier compared to rat.[123] Second, species differences could be seen in kinetic parameters. In seven cell lines transfected with the PGP gene from different species, the apparent Km values for diltiazem were about 16.5-fold different among species and the corrected Vmax/Km of diltiazem was over 5-time higher in humans versus canines.[124] Even in genetically similar species, a difference in PGP function is experimentally detectable. For example, the corrected Vmax/Km of cyclosporin A was 3.8-fold higher in cells transfected with human PGP than in cells transfected with monkey PGP[124] and the EC_{90} of PGP inhibitor, GF120918 was about 10-fold higher in guinea pigs than in rats or mice[125].

Given so many differences of PGP across species, human *in vitro* models seem to be a better choice. However, the properties of currently available human brain capillary cells are not perfect for studying drug permeability across blood-brain barrier. The first limitation of human blood-brain *in vitro* models is the significantly lower expression of tight junctions. The number of tight junctions determines the "tightness" of the blood-brain barrier and the "tightness" of endothelial cells could be described as trans-

endothelial electrical resistance (TEER). *In vivo*, the TEER of human blood-brain barrier was proposed to be between 1500 and 2000 $\Omega*cm^2$; *in vitro*, the TEER of human brain capillary endothelial cells co-cultivated with astrocytes is at most 70 $\Omega*cm^2$.[126] The over 20-fold difference in TEER makes it difficult to translate data obtained from human brain capillary to humans *in vivo*.

Besides the "leakier" property of human *in vitro* blood-brain barrier models, the lower expression of drug transporters *in vitro* in comparison with *in vivo* expression is another limitation when extrapolating *in vitro* data to *in vivo* data. Instead of human brain capillary endothelial cells, many researchers chose non-cerebral cell lines transfected human ABCB1 gene, such as human intestinal Caco-2 and dog kidney MDCK cells that are highly expressing human ABCB1 or other human drug transporters.[127] Considering these limiting factors, blood-barrier studies conducted in animal models and various *in vitro* models may shed new light on molecular pathways but are difficult to be translated to clinically relevant results. Therefore, studies such as ours that are conducted in humans are extremely important. In comparison to animal and *in vitro* studies, our study recruited patients that received common cardiac surgeries and were administrated with a widely used analgesic drug, morphine, thus, provided clinically relevant results.

4.3 Inflammatory Cytokines following CPB

Compared to surgeries without CPB, surgeries using the CPB procedure are more dangerous: they are associated with higher morbidity/mortality, multiple organ failures and various neurological complications.[128] The complex and unpredictable perioperative

syndrome caused by CPB is partially due to severe systematic inflammation after the surgery.[129] Previous studies have focused on correlating the levels of cytokines in the blood with the outcomes after surgeries with CPB. Some commonly-studied cytokines include pro-inflammatory cytokines IL-1β, IL-8, TNF as well as anti-inflammatory cytokine IL-10.[130, 131, 132] Among them, the plasma or serum levels of IL-6, IL-8 and IL-10 were found to be significantly elevated following the CPB procedure. It is believed that IL-6 and IL-8 may exacerbate the systemic inflammatory response syndrome (SIRS, serious systemic inflammation which is often accompanied by organ dysfunction and failure) after CPB. Additionally, TNF contributes to the initiation and maintenance of inflammatory response (reviewed in reference[133]) and leads to renal dysfunction and myocardial apoptosis after CPB (multiple studies reviewed in reference[134]). On the contrary, as an anti-inflammatory cytokine, IL-10 tends to attenuate the systemic inflammatory response syndrome. For example, patients with severe brain injury showed an increase of IL-10 in CSF that was correlated with the reduction of TNF in CNS, suggesting that the function of IL-10 opposed the effects of TNF.[135]

Consistent with these previous studies we found that the main differentiating factors in the inflammatory response between the CPB and non-CPB procedures were higher peak plasma concentrations and/or AUC of IL-6, IL-10, and TNF. Thus, it is possible that the elevated plasma concentrations of these cytokines could explain more robust and more complicated perioperative inflammatory syndrome in CPB patients. For instance in patient 29 who died from renal and hepatic failure, the plasma C_{max} of IL-6 (4779 pg/ml) was the highest in all patients (Mean plasma C_{max}=162 and 1045 pg/ml in non-CPB and CPB groups, respectively).

Our findings pertaining to IL-6 and IL-10 are also in agreement with previous studies investigating cytokine gene polymorphisms under inflammatory conditions. For example, the gene polymorphism IL6-572GG was shown to be associated with lower IL-6 peak concentrations after coronary artery bypass surgery[136] and IL10-592CC was reported to be related with higher production of IL-10 under pathological conditions[137, 138]. Following CPB, researchers found that IL6-572GG was associated with less adverse events whereas IL10-592CC was linked with higher frequency of adverse outcome.[139] These studies further confirmed our observation that IL-6 and IL-10 are two primary inflammatory mediators in CPB patients.

4.4 Effects of Cytokines on Morphine Distribution across Blood-Brain Barrier

In animal studies, up-regulated expression of PGP and reduced pharmacological effects of morphine has been reported under inflammatory conditions.[65] In our cytokine analysis, plasma IL-6, IL-10 and TNF were the cytokines that were significantly different between the CPB and non-CPB groups, indicating they could be involved in the altered CSF/plasma distribution of morphine in CPB group. While we cannot prove this directly in our study, this idea is supported by a number of other studies. For example, in rat brain capillary endothelial cells, TNF regulated the expression of PGP in a biphasic manner[69]. In astrocytes infected with human immunodeficiency virus (HIV), neutralization of IL-6 inhibited the down-regulation of PGP expression, suggesting that IL-6 is a key modulator of PGP[73]. Similarly, in human brain capillary endothelial cells hCMEC/D3, IL-6 reduced the expression and activity of BCRP while TNF down-regulated both BCRP and PGP.[67]

Plasma but not CSF IL-10 was rapidly elevated and significantly higher in the CPB group. However, a search of the literature revealed no previous studies investigating the effects of IL-10 on blood-brain barrier transporters. One study found that IL-10 up-regulated the expression of an ABC transporter, cholesterol efflux regulatory protein (CERP or ABCA1), in THP-1 macrophage-derived foam cells.[140] A more recent study also reported that IL-10 mediated the overexpression of MRPs in antimony-resistant *Leishmania donovani* infected macrophages.[141] Investigation of the possibility that IL-10 exerts similar effects on PGP or other transporters at the blood-brain barrier would represent a novel area for future research.

Some cell-based studies showed that pro-inflammatory cytokines such as IFN and TNF altered (primarily increased) the barrier permeability of various endothelial and epithelial cell lines by interfering with the structure of tight junctions (for a complete review, see reference[142]). In our study, we considered this possibility and introduced the measurement of albumin in our sample analysis. We expected to see a slight increase of the blood-brain barrier permeability. In our results, we observed a significant elevated CSF/plasma ratio of albumin in CPB group due to significantly lower plasma albumin in CPB patients. Our albumin data suggested that the passive permeability of blood-brain barrier was increased temporarily following CPB procedure. However, if the blood-brain barrier permeability increased, we should have observed increased CSF/plasma ratios of morphine. Instead the morphine CSF/plasma ratio decreased indicating that even if there was increased passive permeability of the blood-brain barrier it was not the rate-limiting factor in the CSF/plasma distribution of morphine.

4.5 Polarized Effects of Cytokines on Blood-Brain Barrier Drug Transporters

Previous published human studies that examined the inflammatory response produced by CPB surgery only took samples from the arterial catheter but not the CSF drain. To our knowledge, there is no previous study where cytokines levels were measured in the CSF samples from CPB patients. The inclusion of inflammatory cytokines measurement in the CSF differentiates our work and is a strong point of our study because it provides a more comprehensive understanding of cytokine response to CPB procedure.

From the CSF cytokine data, we found that different cytokines were elevated in CSF than in plasma, even in the same group: in the non-CPB group, IL-1α, IL-2 and IL-10 are elevated in plasma while no cytokines were increased in CSF; in the CPB group, IL-6 and IL-10 were elevated in both CSF and plasma, TNF was only increased in plasma, IL-1α and IL-2 were merely increased in CSF (shown in Figure 8 and 9). Considering that brain capillary endothelial cells are polarized, exposure to different cytokines at each side may lead to highly complicated change in term of properties and permeability of the blood-brain barrier.

In brain endothelial cells, researchers have found that the response to lipopolysaccharide (LPS) was in a polarized manner: when LPS was presented at the abluminal (brain) side, the secretion of IL-6 was more robust.[143] In rat brain capillary cells, the response to TNF was proposed to be generated from the abluminal side.[71] Thus, while the inflammatory response was more robust in general circulation in our study, cytokines released into the CSF could potentially regulate blood-brain barrier drug transporters via signaling at the abluminal membrane of brain capillary cells and altering

morphine distribution into the brain, together with cytokines released in the blood. Since a significant difference in IL-6 and IL-10 between the CPB and non-CPB groups was only observed in plasma, we believe that the observed change in morphine kinetics in the CPB group most likely involves inflammatory cytokine signaling on the luminal side of the endothelium. Regardless, more studies are certainly needed to further investigate the concept of polarized effects of cytokines on blood-brain barrier.

4.6 Time-dependent Effects of Cytokines on Blood-Brain Barrier Drug Transporters

It has been reported that the regulation of drug transporters by cytokines could be biphasic. In rat brain capillary cells, the activity of PGP was first decreased by 50 % one hour after exposure to TNF and endothelin-1 (ET-1). This was followed by a 2-3 hour stable period and then a rebound increase in the activity and expression level of PGP. After 6 h of cytokine treatment, the activity and protein expression of PGP were almost 2-times as high as control cells.[69] Such biphasic regulation of PGP was reported in animal models as well: in rats with seizures, the gene expression of PGP and MRPs at various sites of the brain decreased during the 1st day after the onset of seizure, then the expression level went back to a normal state and kept rising till reached its peak level approximately two days after the onset of seizure. At the same time, inflammatory cytokines including as IL-1β, IL-6 and TNF were significantly increased in the epileptic rats.[144]

In comparison with these two studies, our data is similar to the study conducted in rat cells. It is possible that at 6 hours post-surgery, the PGP function in both groups was

already up-regulated by inflammatory cytokines, resulting in a non-significantly different morphine CSF/plasma $AUC_{0\text{-}6h}$ ratio between groups. As time goes by, the inflammatory cytokines decreased in non-CPB patients more quickly as demonstrated by the significantly lower plasma AUC values of IL-6, IL-10 and TNF at 24 hours compared to the CPB group (Figure 10). We hypothesize that this results in a return to normal function of PGP in the non-CPB group. This idea is supported by the increase of morphine CSF/plasma AUC ratio from 12 to 24 hours in the non-CPB group. However, in the CPB group, patients were continuously exposed to larger amount of inflammatory cytokines, shown as significantly higher AUCs of IL-6, IL-10 and TNF. As a result, we predict the activity of PGP to be up-regulated for a longer duration in patients following CPB. This idea is supported by the significantly lower morphine CSF/plasma AUC ratio in CPB patients than non-CPB patients.

Considering the time-dependent regulation of PGP at the blood-brain barrier, we carried out our analysis at multiple time points. In our study, data were analyzed at four time points throughout the perioperative period of all patients. Accompanied by multiple time point analysis, our data are able to reveal the change of monitored inflammatory and pharmacokinetic parameters over time and to spot the time points when the alteration took place. For example, we pointed out that the morphine distribution into CSF of CPB patients remained at a low level during the first 24 hours after surgeries, whereas in the non-CPB group the distribution increased over time. The difference of morphine AUC ratio was significant at 18 and 24 hours. Similarly, we demonstrated that the exposure to IL-6, IL-10 were higher at 24 hours and the exposure to TNF was higher at 18 and 24 hours in CPB patients. Therefore, of all inflammatory cytokines measured in our study,

we predict that IL-6, IL-10 and TNF are more likely to cause the altered morphine distribution into CSF.

In the correlation analysis, we did not see a linear regression between morphine AUC ratio and C_{max} or AUC of IL-6, IL-10 or TNF. A possible reason is that the more than one cytokines are required to regulate the function or expression of PGP. Therefore, plotting morphine AUC ratio against single cytokine failed to reveal the correlation between cytokine concentration/exposure and morphine distribution across blood-brain barrier. Another explanation is that the regulation of PGP is not determined by cytokine's AUC or C_{max} alone, but affected by the two factors combined. In addition, genetic polymorphisms of PGP, UGT and cytokines could also contribute to the lack of linear correlation between morphine and cytokines. To better understand the regulation of PGP by cytokines, a correlation analysis that is more complex than linear regression and that takes more factors into account is needed.

4.7 Study Limitations

The first limitation was the inability to include healthy volunteers in our clinical studies for ethical reasons and the risks associated with insertion of spinal catheters into healthy patients. Therefore, we cannot determine the full magnitude in the change in morphine distribution that occurs following CPB. Given that the non-CPB patients also experienced an inflammatory response, there could also be difference in morphine distribution into the brain of these individuals compared to healthy individuals without inflammation. In our clinical study design, we used non-CPB patients as controls. But the problem is that non-

CPB patients are not inflammation free, most of them still produce moderate degree of inflammatory response. As our cytokine data showed, a number of inflammatory mediators were released in non-CPB patients' plasma and CSF samples. If, as we hypothesized, IL-6 affects the function of PGP in CPB group, then IL-6 may affect the function of PGP in non-CPB group as well. Similar argument could be made for IL-10 and TNF, which were also elevated in both CPB and non-CPB group. Therefore, a remaining question for us to answer is: is the morphine distribution across blood-brain barrier without interference of cytokines different from morphine distribution under inflammatory conditions.

The second limitation of our study is that we did not have a direct measurement of PGP. Although we used morphine as a probe drug of PGP, morphine is not exclusively transported by PGP alone. Other transporters such as BCRP share a number of substrates with PGP.[145,146] In human brain capillary endothelial cells hCMEC/D3: the mRNA expression and activity of BCRP was regulated by IL-1β, IL-6 and TNF; in contrast, the mRNA expression of PGP was mainly regulated by TNF but not IL-1β or IL-6 and the regulation by TNF showed no effect on the function of PGP.[67] As that study pointed to the regulation of BCRP but not PGP by inflammatory mediators, it is possible that BCRP rather than PGP was more important for the altered distribution of morphine in CPB patients.

As shown by our data (Figure 12), the variability of morphine AUC CSF/plasma ratio is substantial even within the same group: the SD (error bar) of each set of data is larger than half of the corresponding mean value. An important factor that is likely to contribute to this inter-individual variance in morphine distribution is genetic polymorphisms of

drug transporters and metabolizing enzymes. Currently, almost 30 single nucleotide polymorphisms (SNP) of PGP have been identified (multiple studies reviewed in reference[147]). The two most common polymorphisms, exon 26 (C3435T) and exon 26 (G2677T) have been associated with altered expression and function of PGP in a number of studies[148]. With respect to morphine, the mutation at exon 26 (C3435T) was found to be significantly correlated with the pain relief variability of morphine.[149] In addition, the mutation at exon 26 (G2677T) was reported to be significantly correlated with the side effects (drowsiness and confusion or hallucinations) variability in cancer patients receiving morphine.[150]

As a follow-up study, we would like to test the gene polymorphisms of PGP in patients. By genotyping patients for PGP polymorphisms, we would be able to distinguish individuals highly expressing/functioning PGP from individuals with normally expressing/functioning PGP. If as we proposed, altered morphine disposition at blood-brain barrier is due to PGP, patients with PGP mutations that result in higher expression/functionality may exhibit alteration of morphine disposition to a higher degree whereas patients normally expressing PGP will show the alteration at a moderate degree. Another possibility is that, if the PGP function is upregulated, those with higher expressing PGP may already be at maximum functionality, which leads to no further effects in these patients; at the meantime, patients with normal expressing PGP may show a more substantial induction of PGP. Either way, including PGP genetic polymorphisms into the analysis could provide us with a more thorough understanding of PGP regulation by inflammatory cytokines.

In addition to PGP, we could also test for genetic polymorphisms of the morphine-metabolizing enzyme UGT2B7. Currently, more than 10 single nucleotide polymorphisms (SNP) of UGT2B7 have been reported and some of them are associated with altered glucuronidation of morphine or other drugs.[151, 152, 153, 154] In our results, we did not see significant difference in morphine metabolism between the non-CPB and CPB groups, but it is possible that individuals carrying the more active UGT2B7 were included in both groups. If we could identify individuals with these UGT2B7 mutations, we would be able to group and compare our data according to the detected UGT2B7 genotypes to see if there is any impact of the degree of change in morphine distribution to the CSF. Similar to PGP and UGT, transporters that are responsible for the efflux of morphine metabolites such as MRP1 and MRP2 could be genotyped too. Overall, with the genetic make-up elucidated in patients recruited in the clinical study, a more solid conclusion regarding the effects of inflammation of specific drug transporters could be reached.

4.8 Future Directions

4.8.1 *In Vitro* Models

Despite the disadvantages of the human blood-brain barrier *in vitro* models discussed previously, they are still a convenient tool to study the mechanisms and pathways of how cytokines regulates drug transporters. The data we obtained clinically will serve as guidance for us to conduct future *in vitro* studies. By treating human brain endothelial cells with cytokines at concentrations we observed in this clinical study, we will mimic

the clinical conditions of patients as well as we can. For cytokine treatments, we will start with individual treatments of IL-6, IL-10 and TNF, as they were significantly higher in CPB patients. By performing real-time PCR, western blots and drug transporter efflux assays, we will determine the impact of these cytokine treatments on the mRNA and protein expression and function of PGP or other transporters. We will then be able to determine if these changes impact the cellular accumulation and/or efflux of radiolabeled morphine as a measure of PGP transporter function. After determining the effects induced by single cytokines, we will then proceed with multiple cytokine treatments to better model the complete inflammatory response that occurred *in vivo*. Overall, in the cell-based assays, we would like to focus specifically on interaction between cytokines and drug transporters to reaffirm our predictions generated from the clinical study.

4.8.2 S100B Assay

S100B is a calcium-binding protein that is mainly produced by astrocytes. It has been proposed as a biomarker of brain injury.[155] Damage of astrocytes and glial cells will result in paracrine and autocrine release of S100B.[156] In humans and animals, altered brain and serum concentration of S100B has been associated with abnormal behaviors and cognitive deficiencies. In patients with various brain injuries and brain diseases, S100B levels were elevated compared with healthy controls. For example, in patients with schizophrenia, blood concentration of S100B was significantly higher than in healthy volunteers.[157] In addition, the S100B level was believed to be a predictor of the postoperative outcome of patients with stoke after cardiac surgeries. It was reported that blood concentration of S100B after cardiac surgeries was correlated with the size of

infarcted brain tissue and higher S100B level was associated with a higher mortality rate following cardiac surgeries.[158]

At the current stage, we have measured the CSF S100B level in 11 patients. Among these 11 patients, patient 35 (CPB group) exhibited extremely high levels of S100B (C_{max} = 4581 pg/ml) after the surgery (T_{max}= 69.75 h) whereas other 10 patients had their Cmax from 246 to 1241 pg/ml. Given the adverse outcome of this patient (death), we think that CSF concentration of S100B may also be correlated with clinical endpoint and can be used as a predictor of patients' recovery after aortic surgeries using CPB. Our plan is to finish the quantification of CSF S100B in all patients. By plotting the concentration of S100B against time, we would like to get better understanding of the change of S100B over time. Since S100B is a biomarker of brain injury, we would like to relate our cytokine data and morphine data with the degree of brain injury. In the literature, most studies focused on S100B concentration in the blood. In contrast, we will add some novelty by assessing S100B levels in the CNS.

4.8.3 Animal Studies

In the clinical study, we were not able to compare the CPB patients with healthy volunteers. With proper animal models, we can potentially solve this problem. As CPB is a unique surgical procedure that produces a robust inflammatory response and causes more serious neurological injuries than other types of surgeries, it cannot be mimicked easily in animal models with any type of surgeries. However, there have been some reports of the establishment of animal models for CPB in rats [159, 160] and rabbits[161]. For example, CPB procedure under normal temperature was performed in rats via roller

pump and membrane oxygenator and assessment of their neurological outcome validated the effectiveness of this model because rats undergoing CPB showed clearly deteriorating neurocognitive ability compared with rats receiving other surgical procedure.[162] By collaborating with researchers who have experience with the CPB animal models, we are confident in the possibilities to further investigate the altered drug effects in patients undergoing this highly complex and invasive surgical procedure.

4.9 Conclusion

In our study, we found that the CPB procedure induced a more robust systemic inflammatory response than non-CPB surgery and the larger inflammation following CPB was associated with reduced morphine distribution across the blood-brain barrier as inferred from the reduction in the CSF/plasma morphine AUC ratio. The effect of CPB-induced inflammation on blood-brain barrier drug distribution is possibly due to the regulation of drug efflux transporters by inflammatory cytokines rather than regulation of drug metabolism. Our study agreed with some previous animal and *in vitro* studies, which predicted that PGP substrate drug disposition at the human blood-brain barrier would be decreased under inflammatory conditions due to up-regulation of PGP. Additionally, our data advanced the knowledge of inflammation that occurs systemically and within CNS following aortic aneurysm surgeries that include or do not include CPB. This information may ultimately help to reduce adverse outcomes and/or inflammation related ADRs in patients following cardiac surgery, especially surgeries using CPB procedure.

APPENDIX I. FIGURES

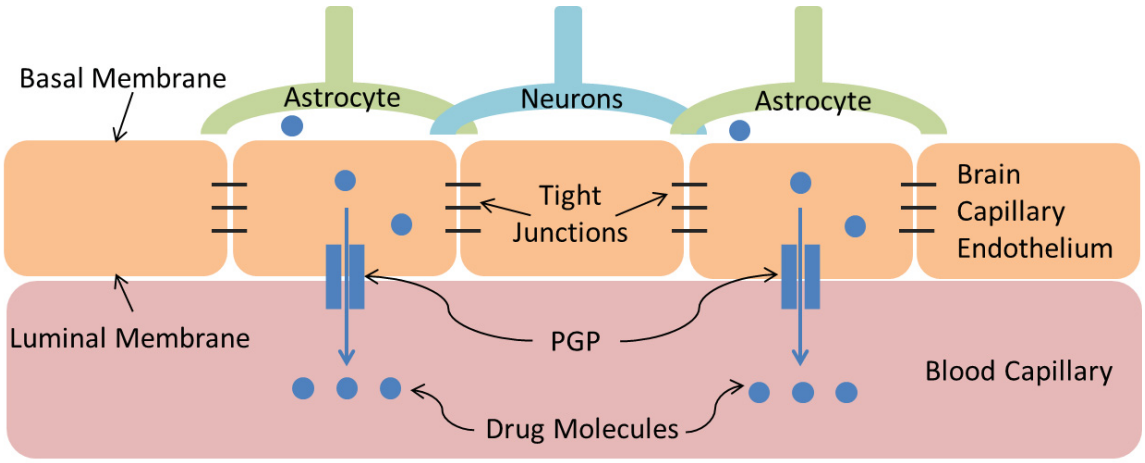


Figure 1. P-glycoprotein (PGP) function and location in the blood-brain barrier

This figure was based on figure 1B in reference[122].

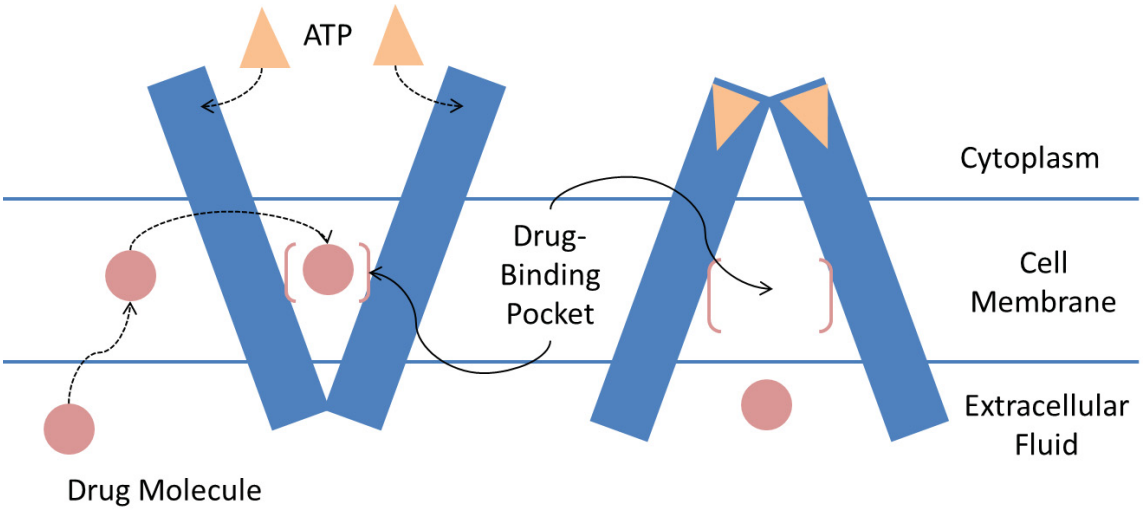


Figure 2. Mechanisms of p-glycoprotein (PGP) transporter function

This figure is based on reference[163]. First of all, the substrate enters the biological membrane and moves into the PGP drug-binding pocket. Then, ATP binds and is metabolized to ADP and inorganic phosphate, which release energy to pump out substrates to the extracellular space.

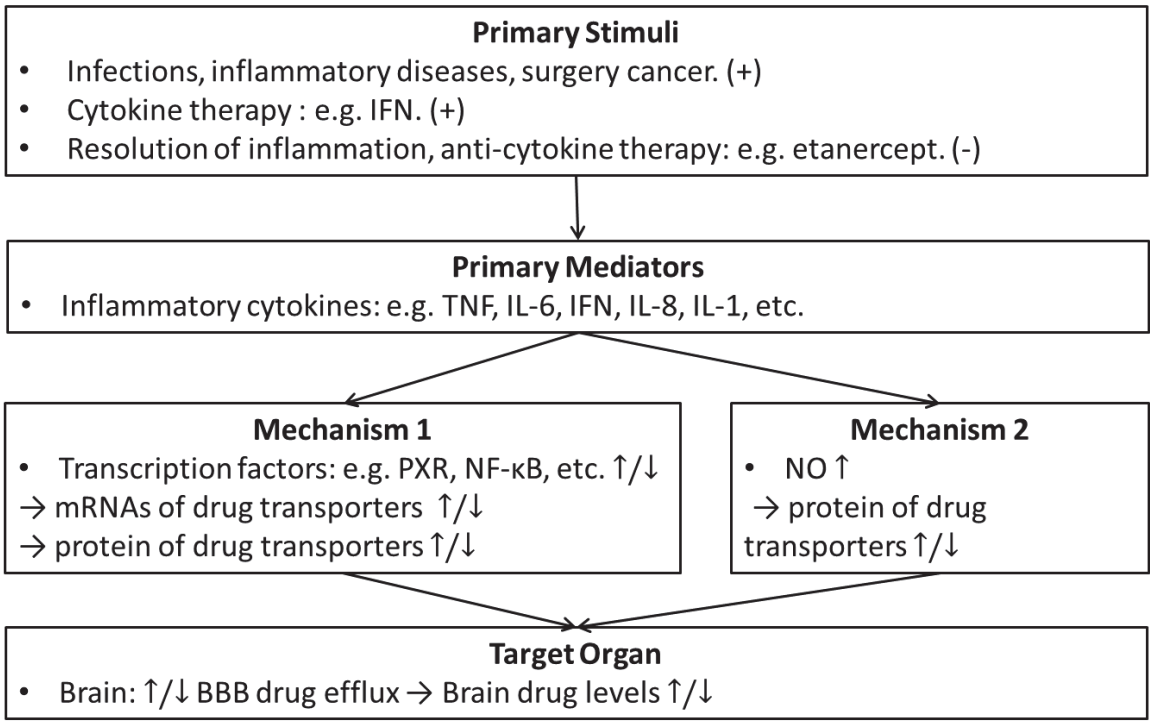


Figure 3. Summary of cytokine regulation of blood-brain barrier drug transporters

This figure was based on reference[164]. Up/down-regulatory effects were denoted with +/-. Increased or decreased level of the functional protein was denoted with ↑/↓. The sequence of pathway was shown with →. IFN, TNF, IL, PXR, NF-κB and NO are abbreviations of interferon, tumor necrosis factors, interleukin, pregnane X receptor, nuclear factor kappa-light-chain-enhancer of activated B cells and nitric oxide.

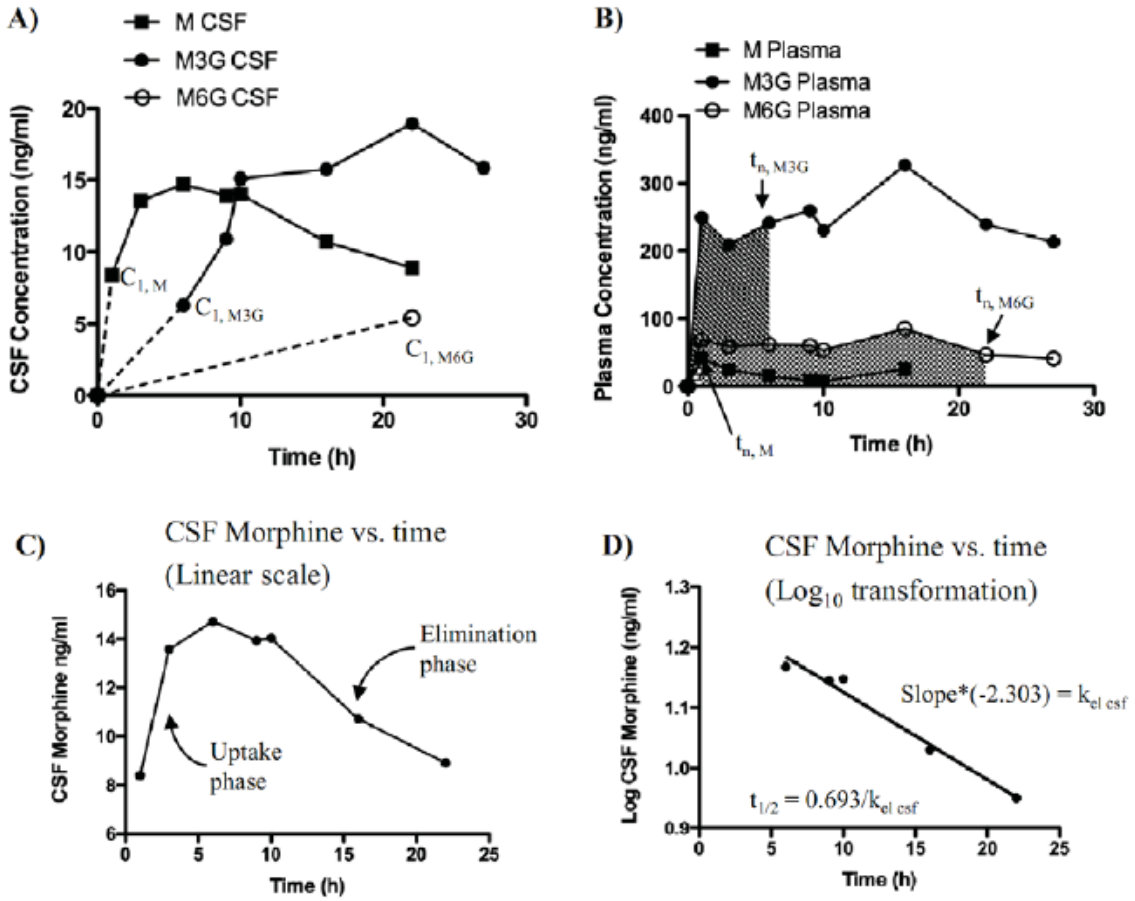


Figure 4. Graphical demonstration of initial rate constant for CSF uptake (k_{in}), the CSF elimination rate constant ($k_{el\ csf}$) and half-life of elimination from CSF ($t_{1/2\ csf}$)

A) The morphine (M), and morphine-3 and 6-glucuronide (M3G & M6G) CSF concentration-time curve are shown for patient 4. The first detected concentration of M, M3G and M6G in CSF were denoted as "$C_{1, M}$", "$C_{1, M3G}$" and "$C_{1, M6G}$". **B)** The M, M3G and M6G plasma concentration-time curves are shown for patient 4. The time point when morphine is started was denoted as t_0 and the time point where CSF M, M3G and M6G were first detected were denoted as "$t_{n, M}$", "$t_{n, M3G}$" and "$t_{n, M6G}$". The shaded area shows the plasma $AUC_{t0\text{-}tn}$ for M, M3G and M6G. As $k_{in}=C_{1\ csf}/AUC_{t0\text{-}tn\ plasma}$, k_{in} was calculated

based on curves shown in panel A and B. **C)** The M CSF concentration-time curve was shown for patient 4. **D)** The M concentrations corresponding to the elimination phase plotted on a semi-log_{10} scale demonstrated that elimination approximates a 1st order process (r^2=0.977). $k_{el\ csf}$ was calculated from the slope of the elimination phase multiplied by -2.303 (the conversion for Log_{10} to In). The elimination half-time was calculated following the formula $t_{1/2}=0.693/k_{el\ csf}$.

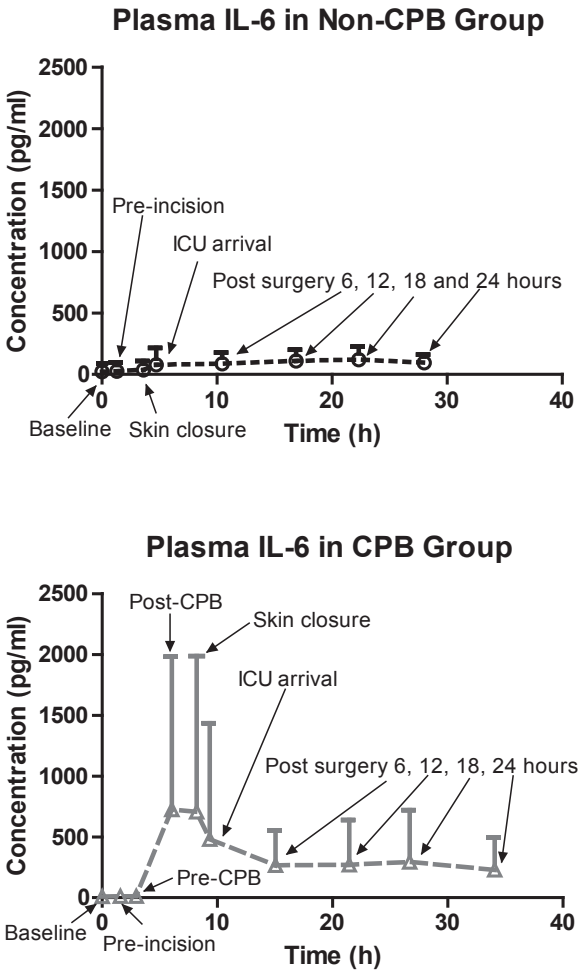


Figure 5. IL-6 mean plasma concentration versus time curves

This figure shows the 8 and 10 sampling time points in the non-CPB and CPB groups respectively, using plasma IL-6 as an example. CSF samples were collected at the same time with plasma samples. The concentration-time curves of other cytokines, morphine, M3G and M6G in plasma and CSF were all analyzed at time points shown on this figure.

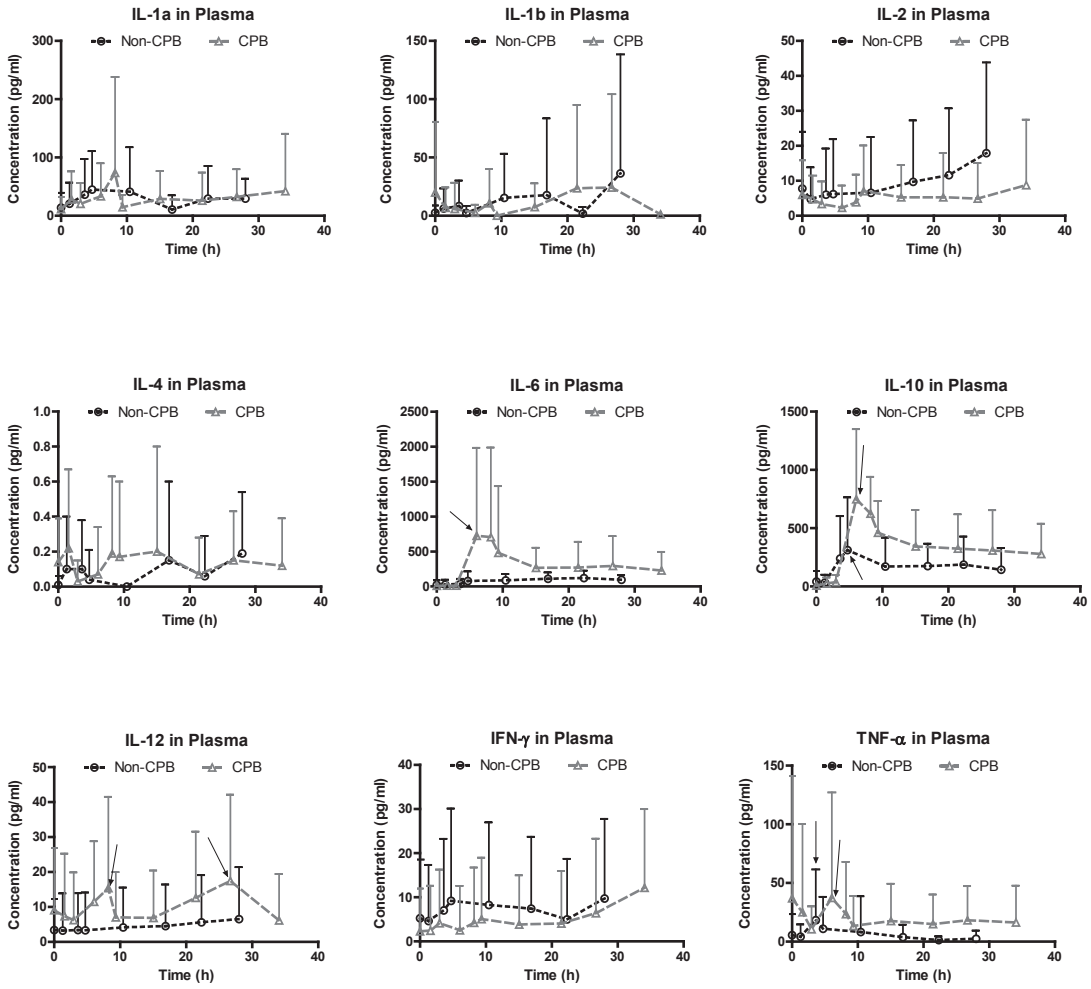


Figure 6. Mean plasma cytokine concentration versus average time curves

Each point represents the mean cytokine concentration. The error bars represents the SD. Arrows indicate peak concentrations. In both the non-CPB and CPB groups n=18. Time points shown for the non-CPB group are 1) baseline, 2) pre-incision, 3) skin closure, 4) ICU arrival, 5) post-surgery 6h, 6) post-surgery 12h, 7) post-surgery 18h and 8) post-surgery 24h; for the CPB group are 1) baseline, 2) pre-incision, 3) pre-CPB, 4) post-CPB, 5) skin closure, 6) ICU arrival, 7) post-surgery 6h, 8) post-surgery 12h, 9) post-surgery 18h and 10) post-surgery 24h.

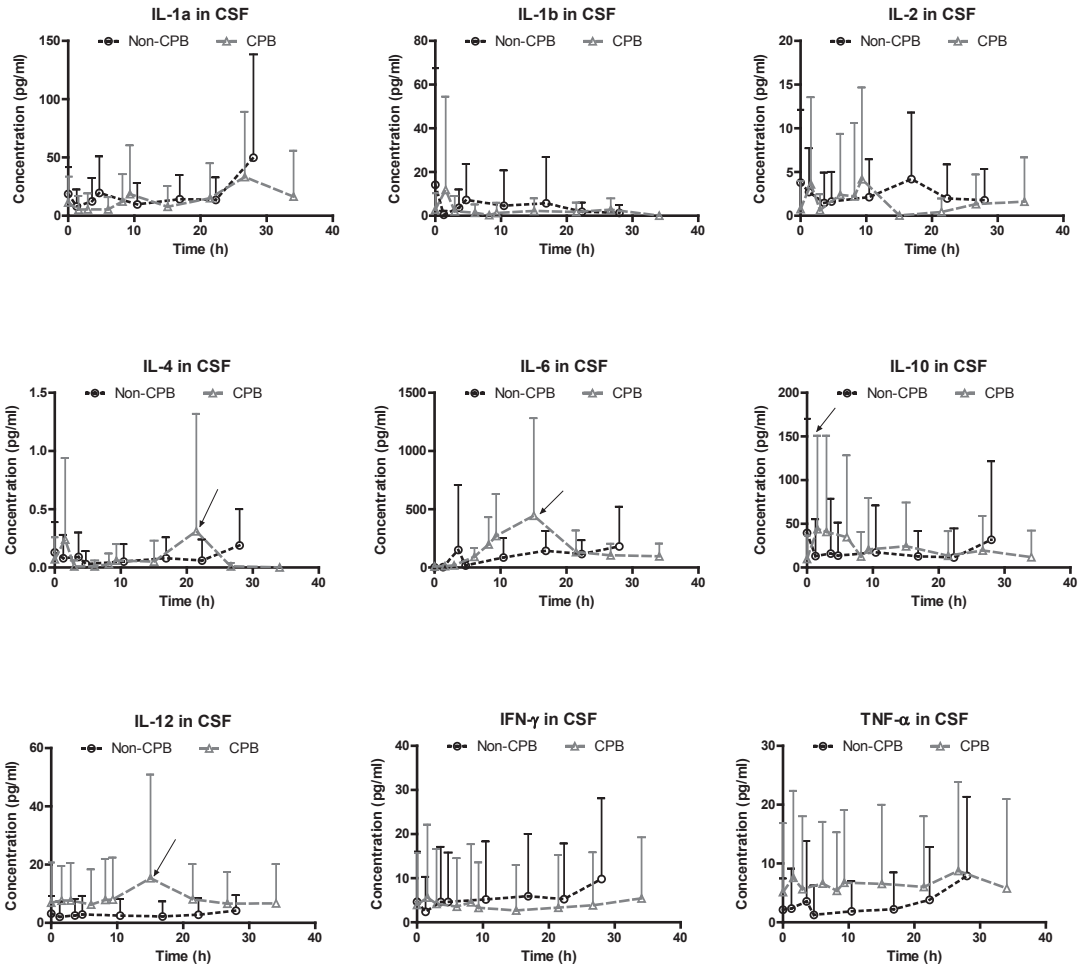


Figure 7. Mean CSF cytokine concentration versus average time curves

Each point represents mean cytokine concentrations. The error bars represents the SD. Arrows indicate peak concentrations. In the non-CPB group, n=18; in the CPB group, n=17. Time points shown for the non-CPB group are 1) baseline, 2) pre-incision, 3) skin closure, 4) ICU arrival, 5) post-surgery 6h, 6) post-surgery 12h, 7) post-surgery 18h and 8) post-surgery 24h; for the CPB group are 1) baseline, 2) pre-incision, 3) pre-CPB, 4) post-CPB, 5) skin closure, 6) ICU arrival, 7) post-surgery 6h, 8) post-surgery 12h, 9) post-surgery 18h and 10) post-surgery 24h.

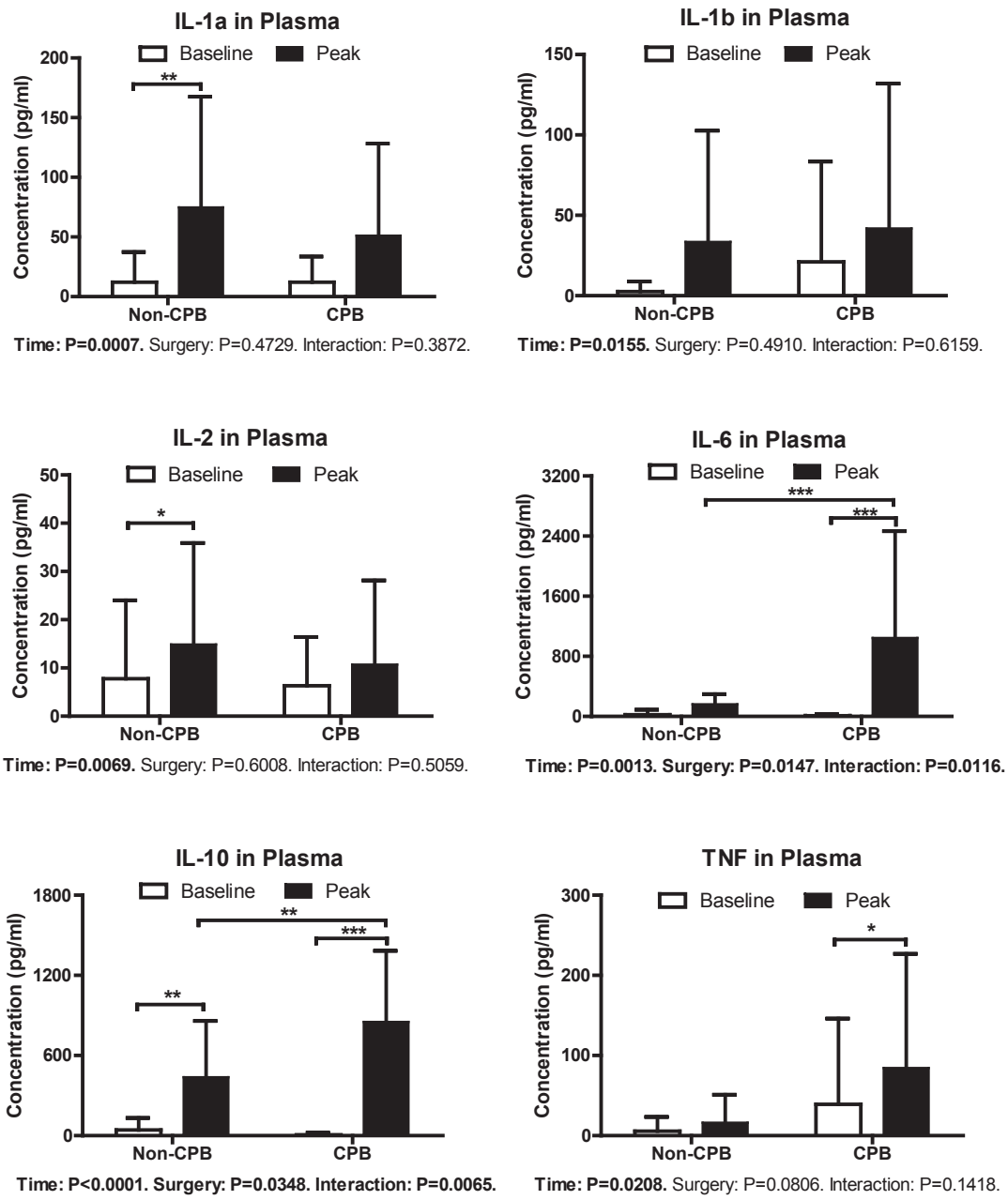


Figure 8. Plasma baseline and peak concentrations of each cytokine

Data are shown as Mean ± SD. In the non-CPB group, n=18; in the CPB group, n=17. P values below figures are calculated using a two-way repeated ANOVA where time (t_0 and t_{max}) is taken as one factor and surgery (non-CPB and CPB) is taken as the other factor. The interaction examines if the effect depends on the two factors combined.

Significant difference is shown in bold type. Asterisks above the figures indicate P values from the Bonferroni post-tests: $P<0.05$, 0.01 and 0.001 was denoted with one, two and three asterisks, respectively. IL-4, IL-12 and IFN-γ are not included in this figure because they did not show significant difference in two-way ANOVA.

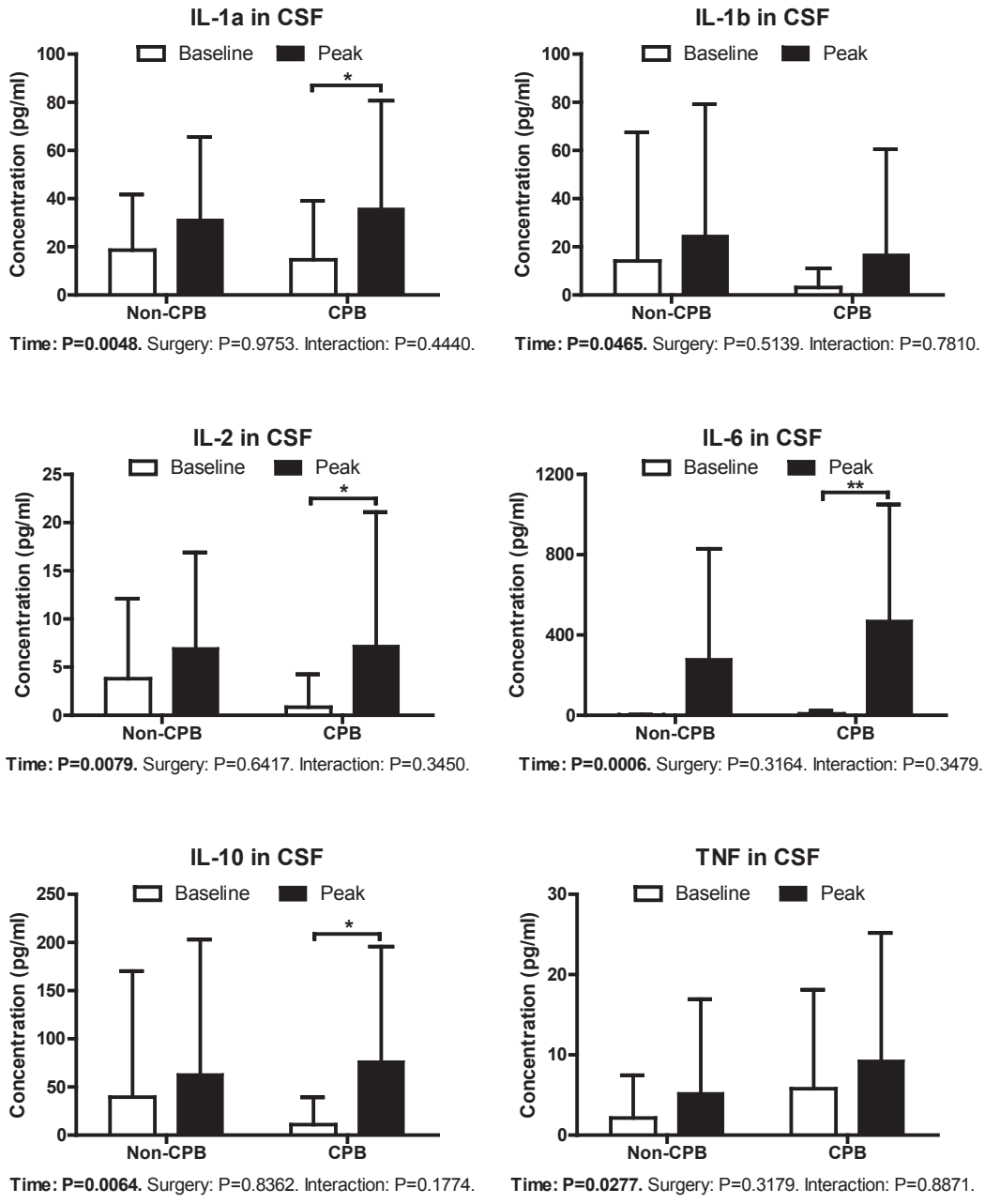


Figure 9. CSF baseline and peak concentrations of each cytokine

Data are shown as Mean ± SD. In the non-CPB group, n=18; in the CPB group, n=16. P values below figures are calculated using a two-way repeated ANOVA where time (t_0 and t_{max}) is taken as one factor and surgery (non-CPB and CPB) is taken as the other

factor. The interaction examines if the effect depends on the two factors combined. Significant difference is shown in bold type. Asterisks above the figures indicate P values from the Bonferroni post-tests: $P<0.05$, 0.01 and 0.001 was denoted with one, two and three asterisks, respectively. IL-4, IL-12 and IFN-γ are not included in this figure because they did not show significant difference in two-way ANOVA.

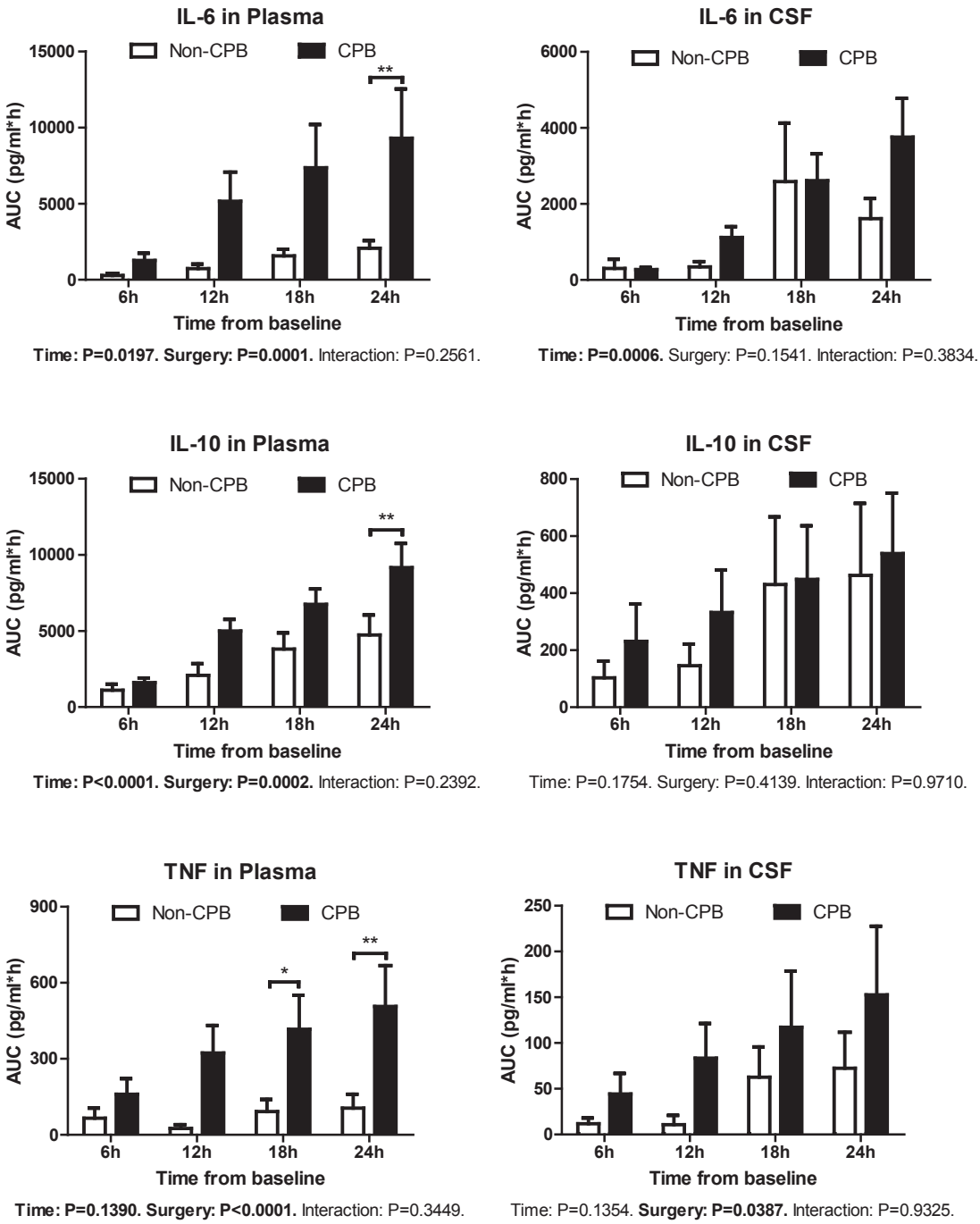
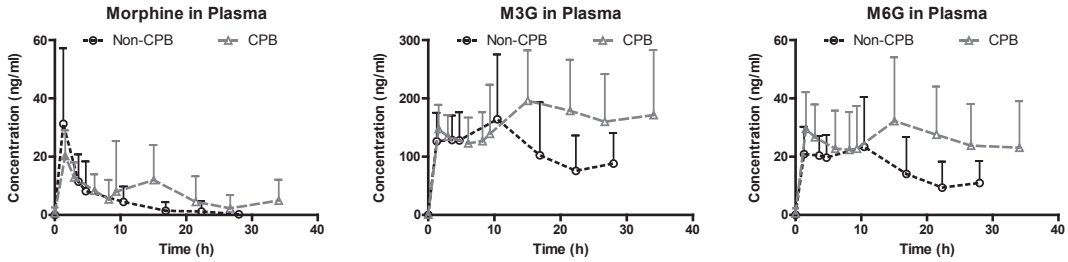


Figure 10. Plasma and CSF area under the curve (AUC) of IL-6, IL-10 and TNF

Data are shown as Mean ± SD. For plasma data at 6, 12, 18 and 24 h, n=18, 15, 17, 17 in the non-CPB group and n=17, 16, 16, 16 in the CPB group. For CSF data at 6, 12, 18 and

24 h, n=18, 15, 17, 17 in the non-CPB group and n=17, 14, 16, 16 in the CPB group. P values below figures are calculated in two-way repeated ANOVA where time (6, 12, 18 and 24 hours) is taken as one factor and surgery (non-CPB and CPB) is taken as the other factor. The interaction examines if the effect on AUC depends on the two factors combined. Significant difference is shown in bold type. Asterisks above the figures indicate P values from the Bonferroni post-tests: $P<0.05$, 0.01 and 0.001 was denoted with one, two and three asterisks, respectively. Other cytokines are not included in this figure because they did not show significant difference in post-tests.

A. Plasma



B. CSF

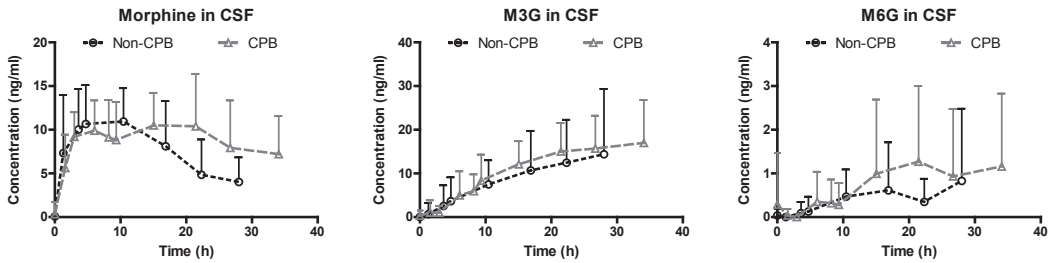
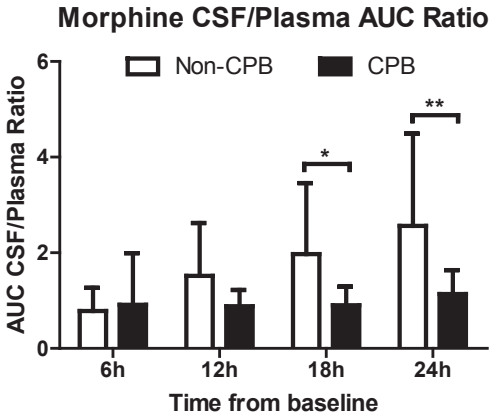
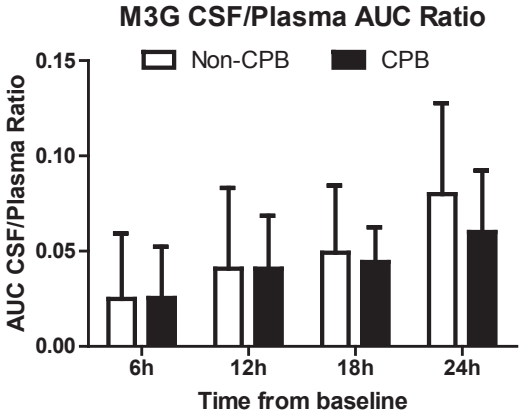


Figure 11. Mean concentration versus time curves for morphine, M3G and M6G

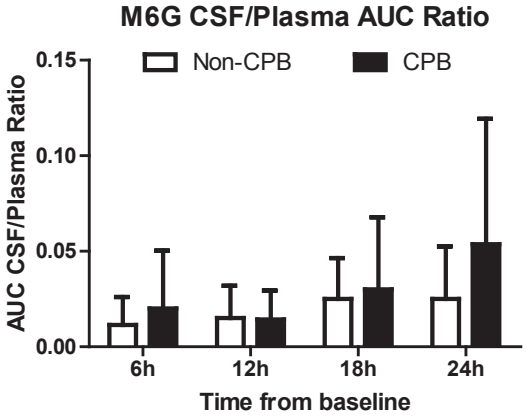
Each point represents mean concentration of morphine, M3G or M6G. Error bar represents the SD. In the non-CPB group, n=16; in the CPB group, n=18. Time points shown for non-CPB group are 1) baseline, 2) pre-incision, 3) skin closure, 4) ICU arrival, 5) post-surgery 6h, 6) post-surgery 12h, 7) post-surgery 18h and 8) post-surgery 24h; for CPB group are 1) baseline, 2) pre-incision, 3) pre-CPB, 4) post-CPB, 5) skin closure, 6) ICU arrival, 7) post-surgery 6h, 8) post-surgery 12h, 9) post-surgery 18h and 10) post-surgery 24h.



Time: P=0.0038. Surgery: P=0.0003. Interation: P=0.0329.



Time: P<0.0001. Surgery: P=0.3528. Interaction: P=0.6410.



Time: P=0.1481. Surgery: P=0.2570. Interaction: P=0.6189.

Figure 12. AUC CSF/Plasma Ratios of Morphine, M3G and M6G

Data are shown as Mean ± SD. P values below figures are calculated using a two-way ANOVA where time (6, 12, 18 and 24 hours) is taken as one factor and surgery (non-CPB and CPB) is taken as the other factor. The interaction examines if the effect on the AUC ratio depends on the two factors combined. Significant difference is shown in bold type. Asterisks above the figures indicate P values from the Bonferroni post-tests: $P<0.05$ was denoted with one asterisk; $P<0.01$ was denoted with two asterisks. For morphine, at 6, 12, 18 and 24 hours, n =16, 13, 14, 16 in the non-CPB group and n=16, 13, 14, 15 in the CPB group. For M3G, at 6, 12, 18 and 24 hours, n =14, 12, 13, 16 in the non-CPB group and n=13, 13, 14, 15 in the CPB group. For M6G, at 6, 12, 18 and 24 hours, n =7, 8, 8, 10 in the non-CPB group and n=6, 7, 7, 11 in the CPB group.

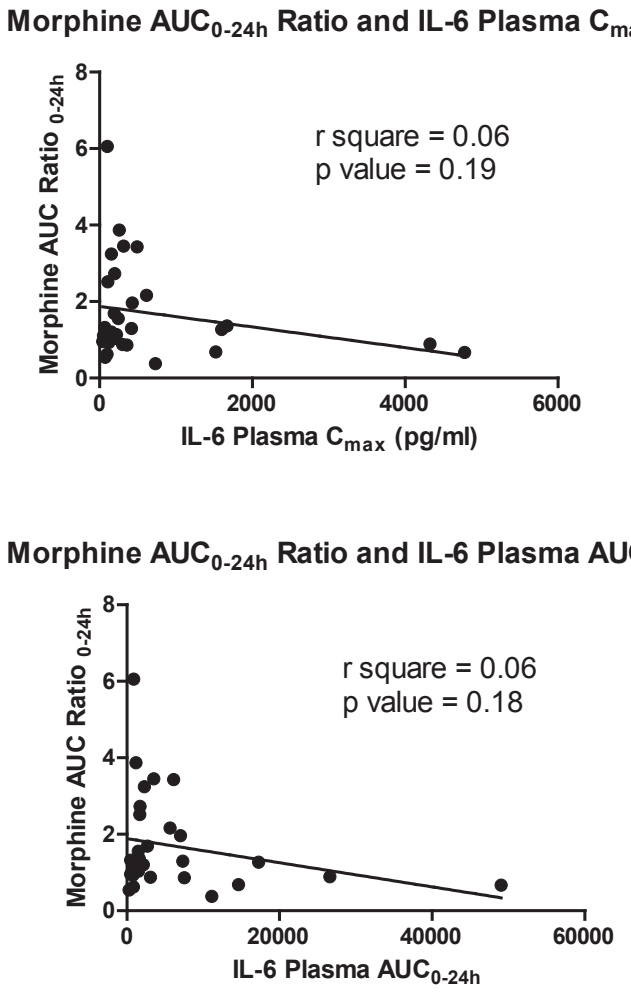


Figure 13. Correlations between morphine CSF/plasma $AUC_{0\text{-}24\ h}$ ratio and plasma IL6 C_{max} and $AUC_{0\text{-}24h}$

Linear regression was performed and the r square and P values are shown on the figures.

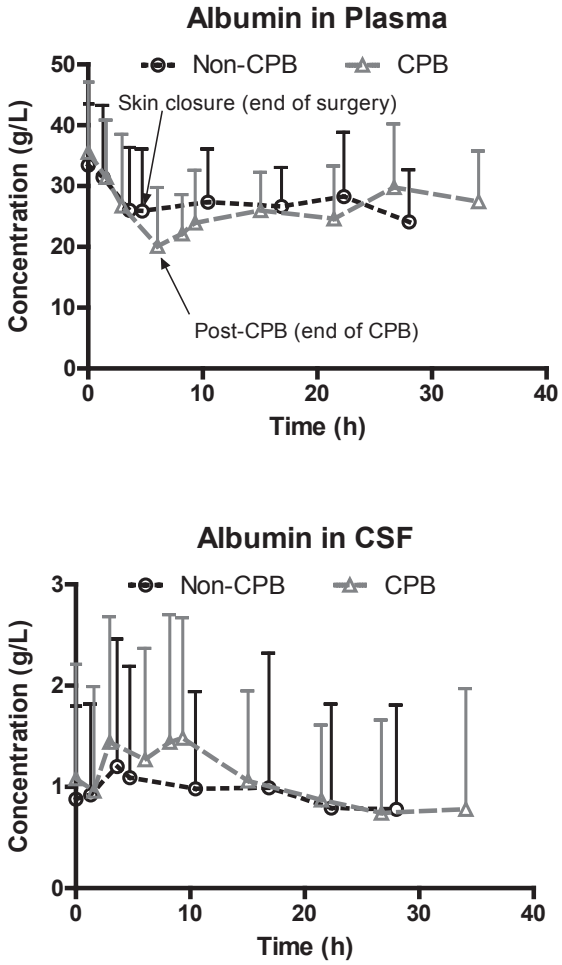


Figure 14. Albumin concentration versus time curves

Each point represents mean concentration of albumin. The error bars represents the SD. Arrows indicate significantly lower concentrations compared with baselines. In both the non-CPB and CPB groups, n=18. The time points shown for the non-CPB group are 1) baseline, 2) pre-incision, 3) skin closure, 4) ICU arrival, 5) post-surgery 6h, 6) post-surgery 12h, 7) post-surgery 18h and 8) post-surgery 24h; for CPB group are 1) baseline, 2) pre-incision, 3) pre-CPB, 4) post-CPB, 5) skin closure, 6) ICU arrival, 7) post-surgery 6h, 8) post-surgery 12h, 9) post-surgery 18h and 10) post-surgery 24h.

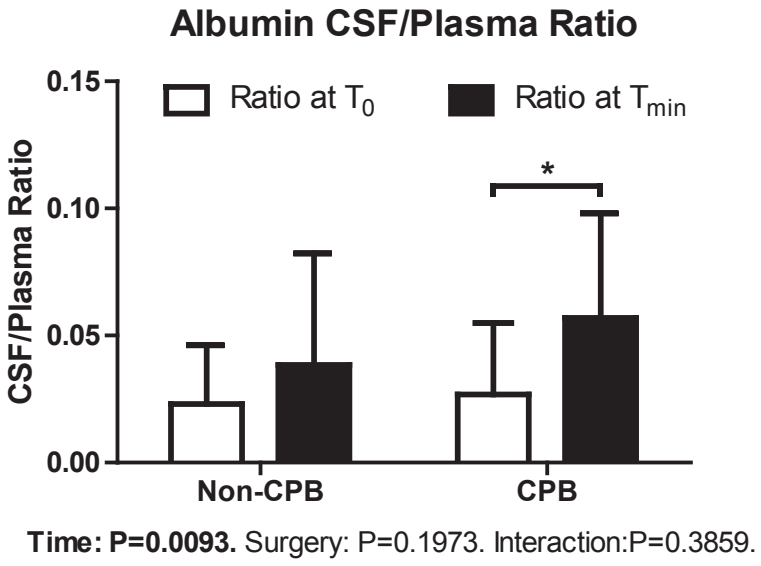


Figure 15. Albumin CSF to plasma concentration ratio

Data are shown as Mean ± SD. T_0 is the time at baseline. T_{min} is the time when minimum concentration was reached. At T_0, n=18 in both groups. At T_{min}, n=17 in non-CPB group, n=14 in CPB group. P values below figures are calculated in two-way repeated ANOVA where time (T_0 and T_{min}) is taken as one factor and surgery (non-CPB and CPB) is taken as the other factor. The interaction examines if the effect depends on the two factors combined. Significant difference is shown in bold type. Asterisks above the figures indicate P values from the Bonferroni post-tests: $P<0.05$ was denoted with one asterisk.

APPENDIX II. TABLES

Table 1. P-glycoprotein basic information

Transporter /alias (Gene)	**Selected substrates**	**Selected inhibitors**	**Organs/cells**	**Comments**
PGP/ MDR1 (ABCB1)	Digoxin, morphine, loperamide, berberine, irinotecan, doxorubicin, vinblastine, paclitaxel, fexofenadine	Cyclosporine, quinidine, tariquidar, verapamil	Intestinal enterocytes, kidney proximal tubule, hepatocytes (canalicular), brain endothelia	• Has a role in absorption, disposition and excretion • Has a role in clinical drug–drug interactions

Contents of this table are cited from reference1.

Table 2. Quantification limits (pg/mL) and standards range (pg/mL) of Q-plex cytokine ELISA

	Upper limit	Lower limit	Standards Range
IL-1α	7600	4.59	7600 – 4.59
IL-1β	4700	0.10	4700 – 0.10
IL-2	2700	1.35	2700 – 1.35
IL-4	415	0.22	415 – 0.22
IL-6	3700	1.89	3700 – 1.89
IL-10	2500	0.10	2500 – 0.10
IL-12	7100	7.08	7100 -7.08
IFN-γ	13000	3.06	13000 – 3.06
TNF	4500	3.27	4500 – 3.27

Information is provided by the manufacturer of Q-plex ELISA kits.

Table 3. Basic information of individual patients

Patient No.	Age	Gender	Height (cm)	Weight (kg)	Morphine Dose (mg)	Morphine Duration (h)	Morphine Dosing Rate (mg/h)	ICU Duration (h)
Non CPB Patients								
1	79	M	170	69	25	57.17	0.44	120.50
5	81	M	180	98	10	Single Dose	NA	12.25
6	75	M	178	94	11	11.68	0.94	23.42
7	63	M	173	76.4	7	9.00	0.78	21.50
8	86	F	155	76.2	24	15.80	1.52	20.75
9	87	M	180	94.5	10	Single Dose	NA	20.33
12	71	M	186	65.4	12	7.12	1.69	99.50
16	84	F	153	70.4	11.5	7.83	1.47	3.83
17	69	M	165	66	15	19.72	0.76	18.00
20	88	M	165	63.9	15	10.28	1.46	23.50
24	74	F	163	58	16	20.80	0.77	44.25
25	74	F	160	81.8	24.5	15.83	1.55	94.33
26	66	M	174	89	14	5.55	2.52	22.75
27	42	M	180	85	12	18.50	0.65	113.50

31	77	M	170	68	26	36.92	0.70	70.42
32	81	M	183	92	16	6.25	2.56	18.42
33	74	M	160	91	12	8.42	1.43	29.13
36	77	F	168	68	39	45.25	0.86	74.50
CPB Patients								
2	78	M	166	66	16	11.58	1.38	8.50
3	75	M	190	91	21	14.50	1.45	51.75
4	69	M	179	88.6	26	23.93	1.09	20.50
10	65	M	188	119	30	20.73	1.45	18.17
11*	41	M	175	68	49	48.75	1.01	354.73
13	80	F	154	73	16	12.82	1.25	120.50
14	34	M	206	91.3	18	23.95	0.75	22.08
15	77	M	178	75	NA	NA	NA	NA
18	72	M	173	103.8	30	31.35	0.96	35.25
19	33	M	180	72.6	49	45.68	1.07	66.33
21	69	F	168	86	18	11.45	1.57	209.33
22	55	M	187	100.7	15	12.87	1.17	19.58
23	52	F	157	69	24	50.22	0.48	46.33
28	49	M	183	103	NA	NA	NA	NA
29*	75	F	172	54.5	56	150.62	0.37	1142.42
30	70	M	158	67	42.5	32.85	1.29	116.83
34	60	M	178	80	22	37.58	0.59	44.50
35*	77	F	157	59	20	34.67	0.58	64.58

Most adverse events were resolved with medications or surgical procedures. Patients with treated but continuing adverse events are marked with *. Patient 11 was paralyzed; his continuing adverse outcomes were spinal cord infarction, respiratory failure, thoracotomy and bacteremia. Patient 29 died from renal and hepatic failure. Patient 35 died from ischemic bowel.

Table 4. Summary of patients' demographics, morphine dose and duration

	Total	Non-CPB	CPB
Sample Size	36	18	18
Age	69 ± 14	75 ± 11	63 ± 15 *
Gender	Male 26 Female 10	Male 13 Female 5	Male 13 Female 5
Height (cm)	173 ± 12	170 ± 10	175 ± 14
Weight (kg)	80 ± 15	79 ± 13	82 ± 18
Morphine Dose (mg)	22 ± 12	17 ± 8	28 ± 13*
Morphine Duration (h)	25.3 ± 26.9	16.5 ± 15.4	35.2 ± 33.5
Morphine Dosing Rate (mg/h)	1.14 ± 0.53	1.26 ± 0.64	1.03 ± 0.38
ICU Duration (h)	93.3 ± 198	46.2 ± 38.3	146 ± 280
Adverse outcomes	Hospitalized 6 (including 1 paralyzed) Death 2	Hospitalized 3: patient 1, 20 & 36	Hospitalized 3: patient 2, 3 and patient 11 (paralyzed) Death 2: patient 29, 35

Data are shown as Mean ± SD. A significant difference ($P < 0.05$) between CPB and non-CPB group is denoted with * as determined by an unpaired t-test, $P = 0.02$ for age, $P = 0.01$ for morphine dose.

Table 5. Concomitant Medications

	Non-CPB	CPB
PGP Inhibitors		
Amiodarone[165]	Patient 1*, 24	Patient 13
Nifedipine[165]	Patient 12	
Pantoprazole[166]	Patient 1*, 27, 31	Patient 29, 35
Other Commonly-Prescribed Medications		
Ranitidine (taken by 30 patients), Cefazolin (29 patients), Potassium Chloride (23 patients), Norepinephrine (16 patients), Human Recombinant Insulin (16 patients), Magnesium Sulfate (15 patients), Metoprolol (11 patients), Hydromorphone (10 patients), Calcium Chloride (10 patients)		

Patient 1* was excluded from morphine data analysis because of earlier administration of codeine. In addition to patient 1, 4 patients in non-CPB group took PGP inhibitors and 3 patients in CPB group took PGP inhibitors.

Table 6. Comparison of the mean M3G/morphine and M6G/morphine ratios in the CPB versus non-CPB group

At 6 hour	Plasma		CSF	
	Non-CPB (N=13)	CPB (N=13)	Non-CPB (N=14)	CPB (N=14)
M3G/Morphine	22.2 ± 8.95	17.2 ± 5.96	0.50 ± 0.34	0.78 ± 0.78
M6G/Morphine	3.24 ± 1.27	3.21 ± 1.43	0.04 ± 0.05	0.06 ± 0.12
At 12 hour	Plasma		CSF	
	Non-CPB (N=7)	CPB (N=10)	Non-CPB (N=11)	CPB (N=13)
M3G/Morphine	32.2 ± 9.85	27.5 ± 19.34	0.86 ± 0.51	1.02 ± 0.38
M6G/Morphine	4.57 ± 1.41	4.92 ± 4.00	0.05 ± 0.08	0.05 ±0.07
At 18 hour	Plasma		CSF	
	Non-CPB (N=4)	CPB (N=11)	Non-CPB (N=11)	CPB (N=13)
M3G/Morphine	89.6 ± 46.0	32.0 ± 24.2	2.00 ± 1.24	1.38 ± 0.60
M6G/Morphine	11.6 ± 7.12	5.18 ± 4.50	0.15 ± 0.20	0.10 ± 0.15
At 24 hour	Plasma		CSF	
	Non-CPB (N=2)	CPB (N=6)	Non-CPB (N=9)	CPB (N=14)
M3G/Morphine	40.6 ± 45.6	44.8 ± 27.1	2.92 ± 1.57	2.06 ± 0.89
M6G/Morphine	6.21 ± 7.13	6.57 ± 4.08	0.13 ± 0.17	0.19 ± 0.20

Data are shown as Mean ± SD. Unpaired t-test was performed to compare the difference between CPB and non-CPB group at each time, however, no significant difference was found.

Table 7. Comparison of the plasma clearance and CSF uptake and elimination half-lives of morphine and M3G and M6G in CPB versus non-CPB groups

		Non-CPB	CPB
Plasma Clearance$_{0\text{-}24h}$ (L/h)	Morphine	178 ± 100	125 ± 59.8
	M3G	11.4 ± 5.11	9.73 ± 3.85
	M6G	87.9 ± 54.5	64.2 ± 33.6
CSF Elimination Half-life (h)	Morphine	12.6 ± 6.13	20.8 ± 18.0
	M3G	NA	NA
	M6G	NA	NA
CSF Uptake Half-time (h)	Morphine	4.50 ± 5.58	18.2 ± 57.6*
	M3G	186 ± 149	136 ± 73.4
	M6G	123 ± 46.7	156 ± 75.5

Data are shown as Mean ± SD. The large SD of CSF uptake half-time in CPB group is due to patient 3 whose CSF uptake half-time is extremely high (217.91h).

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