Johanna Herajärvi

REMOTE ISCHEMIC PRECONDITIONING IN AORTIC SURGERY

EXPERIMENTAL STUDIES WITH A PORCINE MODEL
Abstract

During cardiac and aortic surgery, disturbance of the blood supply in the central nervous system occurs when the repair of aortic pathologies is performed or a bloodless operation field is needed in complex cardiac surgery. To enable the suitable operation environment, the technique named hypothermic circulatory arrest (HCA) has been utilized via heart-lung machine. In this method, the core temperature is lowered to the target temperature, after which blood circulation is halted for a certain period of time.

A challenge is that the successful usage of HCA, however still involves the risks of postoperative neurological complications and mortality. In cardiac and aortic arch surgery, the brain is at the highest risk for deficits, whereas in the repair of thoracoabdominal aortic aneurysms (TAAAs), spinal cord injury remains the most severe adverse outcome. Adjunctive protective strategies are required to reduce ischemic injury in these settings.

In this thesis, Studies I and II focused on the spinal cord and the Study III on the brain. The studies were performed using acute (II, III) or subacute (I) experimental porcine models, primarily aiming to assess the effectiveness of remote ischemic preconditioning (RIPC) in spinal cord protection along with the aim of studying the underlying mechanisms of RIPC in neuroprotection.

Studies I and II demonstrated enhanced motor evoked potential (MEP) responses in both hind limbs, indicating spinal cord protection by RIPC. The faster recovery of brain damage marker S100B along with higher cardiac index and lower systemic lactate levels confirmed the cardio- and neuroprotective properties of RIPC in Study III. The protective mechanism of RIPC was associated with increased antioxidant response (II, III).

Keywords: aortic surgery, central nervous system protection, remote ischemic preconditioning
Sydän- ja aorttakirurgiassa, keskushermoston verenkiertoa joudutaan häiritsemään toteutettaessa aortan korjausleikkausia tai vaikeissa sydänkirurgisissa toimenpiteissä verettömän leikkausalueen saavuttamiseksi. Sydän-kehukokoneen avulla toteutettava täydellinen verenkierron pysäytys mahdollistaa vaaditut olosuhteet. Tässä menetelmässä ydinlämpötilaa lasketaan ja verenkiervon pysäytys toteutetaan tavoiteltussa kohdelämpötilassa tietystä aikaikunnassa.


Väitöskirjan osatöissä I ja II keskittyivät selkäytimeen takajaloissa osoittaen esialtistavan perifeerisen raajaiskemian suojaavan selkäytimeen simuloidussa rinta-aorton korjaustoimenpiteessä. Osatyö III keskittyi alhaisessa lämpötilassa toteutettavaan täydelliseen verenkierron pysäytykseen. Tässä tutkimuksessa todetut aivotauhot ja alhaisemmat laktaatti tasot varmisti raajaiskinen sydän- ja verenkiervon suojaus

\textit{Asiasanat:} aorttakirurgia, esialtistava iskemia, keskushermoston suojaus
"It was the best of times,
  it was the worst of times,

it was the age of wisdom,
  it was the age of foolishness,

it was the epoch of belief,
  it was the epoch of incredulity,

it was the season of Light,
  it was the season of Darkness,

it was the spring of hope,
  it was the winter of despair,"

*Charles Dickens*
*A Tale of Two Cities (1859)*
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Janne Liisanantti, MD, PhD, you were the one introducing me the opportunity to prepare the thesis during the medical school. Along with the scientific world, you have mentored me throughout medical school and work life, teaching the principles of being a doctor. I give my warm thanks to you, Janne.

I am grateful for being surrounded by such amazing people. I warmly thank my close friends from the medical school; you have been the ones with whom I grew up and established my identity as a doctor. Ahti, Kaisa, Terhi along with Johanna and Tuukka are just a few to be mentioned. Siria, my partner in crime when it comes to PhD projects: You have been there for my ups and downs, and you have always understood my struggles throughout this project and life in general. My dear friends Anna, Elina, Elisa, Jenni, Maria, and Miia are thanked for their understanding throughout this project.

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mother Marja and father Ilkka, for the support you have given me throughout my life. I am extremely stubborn and persistent in achieving my goals, though I have not always shown how important and caring you are or thanked you enough.

Oulu, May 2017

Johanna Herajärvi
## List of abbreviations

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<tr>
<td>α-stat</td>
<td>Alpha-stat acid-base management strategy</td>
</tr>
<tr>
<td>αKG</td>
<td>Alpha keto glutarate</td>
</tr>
<tr>
<td>ACP</td>
<td>Antegrade cerebral perfusion</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AmBic</td>
<td>Ammonium bicarbonate</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>A1R</td>
<td>Adenosine A1 receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>ASA</td>
<td>Anterior spinal artery</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BCSFB</td>
<td>Blood-cerebrospinal fluid barrier</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass grafting</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CBP</td>
<td>Cardiopulmonary bypass</td>
</tr>
<tr>
<td>CB1R</td>
<td>Cannabinoid 1 receptor</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>Ch/Fr</td>
<td>The size of a catheter (Charrière)</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP (CCAAT/enhancer binding protein) homologous protein</td>
</tr>
<tr>
<td>CI</td>
<td>Cardiac Index</td>
</tr>
<tr>
<td>CK-MBm</td>
<td>Creatine kinase, myocardial specific</td>
</tr>
<tr>
<td>CMRO(^{2})</td>
<td>Cerebral metabolic rate of oxygen</td>
</tr>
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<td>CNP</td>
<td>Collateral network pressure</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclo-oxygenase 2</td>
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<tr>
<td>CPP</td>
<td>Cerebral perfusion pressure</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CSFD</td>
<td>Cerebrospinal fluid drainage</td>
</tr>
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<td>CSFP</td>
<td>Cerebrospinal fluid pressure</td>
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<tr>
<td>CTRL</td>
<td>Control</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CuSOD</td>
<td>Copper superoxide dismutase</td>
</tr>
<tr>
<td>CVP</td>
<td>Central venous pressure</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>Cx43</td>
<td>Connexin 43</td>
</tr>
<tr>
<td>DHCA</td>
<td>Deep hypothermic circulatory arrest</td>
</tr>
<tr>
<td>EGLN</td>
<td>Egl-nine</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EKG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>EtCO₂</td>
<td>End-tidal carbon dioxide</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>H⁺</td>
<td>Hydrogen ion</td>
</tr>
<tr>
<td>HCA</td>
<td>Hypothermic circulatory arrest</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin-eosin</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia-inducible factor 1 alpha</td>
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<td>HIF-1β</td>
<td>Hypoxia-inducible factor 1 beta</td>
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<td>HIF-P4Hs</td>
<td>Hypoxia-inducible factor prolyl-4-hydroxylases</td>
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<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>IL1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>K₁ATP</td>
<td>ATP-sensitive potassium channel</td>
</tr>
<tr>
<td>KAT</td>
<td>Kynurenine aminotransferase</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>KYN</td>
<td>Kynurenine</td>
</tr>
<tr>
<td>KYNA</td>
<td>Kynurenic acid</td>
</tr>
<tr>
<td>LCX</td>
<td>Left circumflex artery</td>
</tr>
<tr>
<td>LHB</td>
<td>Left heart bypass</td>
</tr>
<tr>
<td>MAF</td>
<td>V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
</tbody>
</table>
MDA  Malondialdehyde
MEP  Motor evoked potential
mgf  Mascot generic format
miR-144  MicroRNA 144
miR-1  MicroRNA 1
MMP  Mitochondrial membrane permeabilization
MnSOD  Manganese superoxide dismutase
MPTP  Mitochondrial permeability transition pore
MSA  Median sacral artery
Na⁺  Sodium ion
Na⁺/K⁺-ATP  Sodium-potassium pump
Na⁺/Ca²⁺-ch  Sodium-calcium channel
Na⁺/H⁺-ch  Sodium-hydrogen channel
Na⁺/Ca²⁺-ex  Sodium-calcium exchanger
NCX  Sodium-calcium exchanger
NAD⁺  Oxidized form of nicotinamide adenine dinucleotide
NADH  Nicotinamide adenine dinucleotide hydrogen
NADPH  Nicotinamide adenine dinucleotide phosphate hydrogen
LNAME  L-N⁴-G-Nitroarginine methyl ester
NF-kB  Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA  N-methyl-D-aspartate
NO  Nitric oxide
eNOS  Endothelial nitric oxide synthase
iNOS  Inducible nitric oxide synthase
nNOS  Neuronal nitric oxide synthase
NOX  NADPH oxidase
Nrf2  Nuclear factor erythroid 2-related factor 2
O₂  Oxygen molecule
O₂⁻  Superoxide anion
⁰ OH  Hydroxyl radical
ONOO⁻  Peroxynitrite
p53  Tumor protein 53
paCO₂  Arterial partial pressure of carbon dioxide
paO₂  Arterial partial pressure of oxygen
pCO₂  Partial pressure of carbon dioxide
PGI₂  Prostaglandin I₂
pH-stat  pH-stat acid-base management
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PHD</td>
<td>Prolyl hydroxylase domain</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA</td>
<td>Phospholipase A</td>
</tr>
<tr>
<td>pO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PRDX1</td>
<td>Peroxiredoxin 1</td>
</tr>
<tr>
<td>RCP</td>
<td>Retrograde cerebral perfusion</td>
</tr>
<tr>
<td>RIPC</td>
<td>Remote ischemic preconditioning</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S100B</td>
<td>Glial-specific S100 calcium binding protein B</td>
</tr>
<tr>
<td>SCBF</td>
<td>Spinal cord blood flow</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SCII</td>
<td>Spinal cord ischemic injury</td>
</tr>
<tr>
<td>SCPP</td>
<td>Spinal cord perfusion pressure</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SFP</td>
<td>Spinal fluid pressure</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SS</td>
<td>Sinus sagittalis (superior)</td>
</tr>
<tr>
<td>SEP</td>
<td>Somatosensory evoked potential</td>
</tr>
<tr>
<td>SvO₂</td>
<td>Venous saturation of oxygen</td>
</tr>
<tr>
<td>TAAA</td>
<td>Thoracoabdominal aortic aneurysm</td>
</tr>
<tr>
<td>TEVAR</td>
<td>Thoracic endovascular aortic repair</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TnI</td>
<td>Cardiac troponin I</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>TXN</td>
<td>Thioreredoxin</td>
</tr>
<tr>
<td>VC</td>
<td>Vena cava</td>
</tr>
<tr>
<td>VTN</td>
<td>Vitronectin</td>
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List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


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## Tiivistelmä

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1 Introduction

The central nervous system (CNS), consisting the brain and the spinal cord, is extremely dependent on constant energy and oxygen sources, and therefore notably vulnerable when an interruption in the supply occurs. In surgery, disturbance of the blood supply in these organs takes place when the repair of aortic pathologies is performed or a bloodless operation field is needed in the settings of complex cardiac surgery.

The breakthrough in the history of cardiac and aortic surgery dates back to the 1950s. The invention of cardiopulmonary bypass (CBP) by Dr. John Gibbon enabled the surgical treatment of complex cardiac and aortic dilemmas e.g. congenital heart defects, traumas and other cardiovascular deficits requiring the substitution of the functions of the heart and the lungs during the operations (Gibbon 1954). The neuroprotective effects of hypothermia were quickly combined with the CPB, and the technique of hypothermic circulatory arrest (HCA) was established (Niazi & Lewis 1957). In the 1970s, Dr. Randall Griepp introduced the concept of deep hypothermic circulatory arrest (DHCA) in the repair of aortic arch in humans, with beneficial results (Griepp et al. 1975). As the result of experimental and clinical data, the safe operation period using DHCA was approximated as the critical time frame of 36–40 minutes, after which radical loss of hippocampal neurons and irreversible cerebral damage would occur (Treasure 1984).

A persistent challenge is that the successful usage of HCA, for instance in the repair of acute aortic dissections, congenital heart defects, and thoracoabdominal aortic aneurysms (TAAAs), still involves the risks for postoperative mortality and neurological complications (Kouchoukos et al. 2013, Ziganshin et al. 2014, Kouchoukos et al. 2015). In cardiac and aortic arch surgery, the brain is at the highest risk for deficits, whereas in the repair of TAAAs, spinal cord injury (SCI), including postoperative paraplegia and paraparesis, remains one of the most severe adverse outcomes. Taken together, the adjunctive protective strategies are still required to reduce ischemic injury in these settings, especially when prolonged operation periods are needed.

The protective effects of the method of ischemic preconditioning were first reported in 1986 using a canine model. Murry and colleagues found that intermittent occlusion of the left coronary artery prior to prolonged occlusion of the same artery reduces myocardial infarct size remarkably, defining ischemic preconditioning as sublethal stimuli before the onset of a subsequent more severe
ischemic insult. (Murry et al. 1986). Thereafter, the protective actions of ischemic preconditioning were confirmed in other organs, e.g. kidney, brain, skeletal muscle, and spinal cord, as well (Zager et al. 1984, Kitagawa et al. 1990, Mounsey et al. 1992, Matsuyama et al. 1997).

In 1993, Przyklenk and coworkers extended this protective concept to comprise also other regions in the same organ (Przyklenk et al. 1993). Thereafter, it was discovered that the ischemic stimuli could be applied at a distance, as well, and the protective impacts were still detected in the heart, resulting in the concept of remote ischemic preconditioning (RIPC) (Gho et al. 1996). To date, different neuroprotective studies of RIPC have been performed widely both in experimental and clinical settings (Vlasov et al. 2005, Dave et al. 2006, Hu et al. 2010, Jensen et al. 2011, Koch et al. 2011, Meng et al. 2012, Cherry-Allen et al. 2015).

Some, but not all, human clinical trials of RIPC have reported improved outcomes after cardiac and aortic surgery (Ali et al. 2007, Thielmann et al. 2013, Hausenloy et al. 2015, Meybohm et al. 2015). To date, three decades have been surveyed to find the exact underlying mechanisms behind this protective phenomenon. Current consensus is based on the combinations of humoral factors, intact neural pathways, and different biochemical messengers (Gill et al. 2015, Meller & Simon 2015).

Studies I and II focused on simulating the repair of thoracic aortic aneurysms, and studying the methods to reduce spinal cord ischemia in a porcine model. Spinal cord ischemia was carried out with systemical, permanent closure of the left subclavian artery and segmental arteries to the level of diaphragm. RIPC was performed via two different methods: in the left hind limb with a blood pressure cuff (I) or with an occlusion of the left iliac artery (II). The beneficial effects were detected as enhanced motor evoked potential (MEP) responses indicating better resistance against subsequent spinal cord ischemia in both studies.

Study III concentrated on the mechanism underlying RIPC in a setting of DHCA. The pigs were cooled to 18°C with CPB, and the arrest lasted for 60 minutes. The cannulation of the main head vein, sinus sagittalis (SS), was performed to assess the metabolites and proteomics behind RIPC. Remote ischemic preconditioning was conducted in the right hind limb with a blood pressure cuff. In all studies, the ischemia cycle of RIPC lasted for 5 minutes and was followed by a 5-minute reperfusion period. The ischemia-reperfusion cycles were repeated four times.
2 Review of literature

The human nervous system is divided into the central and peripheral nervous systems. The spinal cord and the brain comprise the central nervous system (CNS). In contrast, the autonomic nervous system and the spinal nerves form the peripheral nervous system. Additionally, the cranial nerves are functionally counted as part of the peripheral component due to their wide range of motor, sensor, and autonomic actions.

The most cranial parts of the CNS include the two hemispheres of the cerebrum, attached to the brainstem via the interbrain. The brainstem itself consists of three components: midbrain, metencephalon and bulbus. The bulbus, also known as the medulla oblongata, integrates into the spinal cord, medulla spinalis, while orienting caudally. The human spinal cord is an approximately 50 cm column starting from the foramen magnum and extending to the level of L1 vertebra, after which the bunch of nerves forms the cauda equine. (MacDonald et al. 2013, Silva et al. 2014).

The basic unit in the nervous system is the nerve cell, the neuron. Neurons and the surrounding supporting cells, neuroglia, form the neural tissue. To convey the information from the starting point to the reception site, neurons typically form physically attached action sites between each other, enabling accurate regulation both locally and in a timely manner. Macrosopically, CNS tissue is divided into grey and white matter.

2.1 The human spinal cord

In the spinal cord, grey matter is present throughout the cord, forming butterfly shaped columns in cross sections (Lazorthes et al. 1971). These three columns are named anterior, posterior and lateral according to their positions. The connection link of the grey matter to the other side is made through grey commisure. Consequently, the edge of the spinal cord is formed by white matter, including myelinated axon tracts. (MacDonald et al. 2013, Silva et al. 2014).
2.1.1 Cellular structure

The structure of a neuron always consists of soma, dendrites, and axon regardless of the size and the shape of the cell. The different parts of the cell function coherently; the dendrites receive information from other neural cells and transport it to the soma, the cell body containing the nucleus: thereafter, the action potential stimulus is conducted to the next receiving cells via axons. (Mattson et al. 2000). The different roles of the supporting cells are described in the human brain section.

The grey matter

Generally, the grey matter includes interneurons, cell bodies, dendrites of efferent neurons, and the entering fibers of afferent neurons and glial cells (Silva et al. 2014). Next, more detailed descriptions of the cells included in the three columns are provided to clarify the roles of these columns.

In the anterior horns are situated the lower motor neurons, also known as alpha motoneurons. The main action of these motor neurons is to innervate the skeletal muscle. The cells synapse with interneurons and the axons of the cells from the pyramidal tract. (García-Alías et al. 2006, MacDonald et al. 2013).

The posterior horns include the central branches of the sensor neurons of the posterior root ganglion. Sensory information including fine touch, proprioception,
and vibration is gathered all around the body. The project neurons of the posterior horns transmit the sensor impulses to the brain via spinal tracts. (García-Alías et al. 2006, Kaas et al. 2008).

Together with these neurons, there are multiple interneurons in the anterior and posterior horns involving the regulation of sensor and motor impulses in each segment (García-Alías et al. 2006, Kaas et al. 2008, MacDonald et al. 2013). In addition, the interneurons are responsible for processing the information between different segments (García-Alías et al. 2006).

The lateral horn, located in between the anterior and posterior horns, includes the preganglionic neurons of the sympathetic nervous system as a part of autonomic nervous system.

The white matter

The white matter, composed of myelinated axons, forms the transition route for various longitudinal fiber tracts or pathways. The afferent tracts convey the information stimulus to the brain, whereas the efferent tracts execute the actions. (Bareyre & Schwab 2003, Silva et al. 2014). The motor functions are implemented by combinations of various tracts (Kaas et al. 2008).

Approximately 85% of the pyramidal tract, starting from the cerebral cortex, crosses over in the medulla oblongata, and the axons continue downward in the tractus corticospinalis anterior (Bareyre & Schwab 2003). In each segment, some of the axon fibers diverge to the anterior horn. The remaining 15% of the pyramidal tract, without crossing over into the brainstem, continues downward as a tractus corticospinalis lateralis, and in each segment, some of the axons shift on the opposite anterior horn. Most of these axons synapse with the interneurons, which are attached to the alpha motoneurons. (García-Alías et al. 2006, MacDonald et al. 2013).

The extrapyramidal tracts, starting from the brainstem, control the involuntary movements. Thereafter, tractus reticulospinalis controls the reflexes via gammadotoneurons of the skeletal muscle, whereas tractus rubrospinalis mediates the information from cerebellum and nucleus to the lower motor neurons. Tractus vestibulospinalis controls the balance along with tractus tectospinalis, controlling vision and possibly hearing, as well. (MacDonald et al. 2013).

In addition to the extrapyramidal and pyramidal tracts, there are several somatosensory tracts interacting in an afferent manner with e.g. thalamus and cerebellum (Kaas et al. 2008).
The spinal nerves

The spinal cord serves in communication between the brain and the peripheral nerves. Afferent sensory fibers from the periphery enter to the spinal cord on the posterior side via posterior roots. In contrast, on the anterior side, the efferent motor neurons leave the spinal cord via anterior roots. These posterior and anterior roots are combined at the same level, forming 31 spinal nerves in humans: 8 cervical nerves, 12 thoracic nerves, 5 lumbar nerves, and 5 sacral nerves along with one coccygeal nerve. (Silva et al. 2014).

Blood-cerebrospinal fluid barrier (BCSFB)

The spinal cord is protected by three layers, which are called meninges (dura mater, arachnoid mater, and pia mater). The subarachnoid space, between arachnoid and pia, filled with cerebrospinal fluid, and the epidural space, between dura and periosteum, form additive protection to the spinal cord. (Silva et al. 2014).

The cerebrospinal fluid (CSF) is constantly produced from the choroid plexus, ependymal cells, and extracellular space (Stenudd et al. 2015, Ueno et al. 2016). The distribution of CSF occurs between cerebral ventricles, subarachnoid space of the brain, and spinal canal (Brightman et al. 1970, Ueno et al. 2016).

In addition to the aforementioned protective strategies of the spinal cord, the blood-cerebrospinal fluid barrier (BCSFB) is a structure involving mainly one layer of epithelial cells of the choroid plexus, and therefore separating the blood-borne matter from the cerebrospinal fluid (Brightman & Reese 1969, Brightman et al. 1970, Tietz & Engelhardt 2015). In the choroid plexus, the endothelial cells of the capillaries include fenestrae, and thus control is needed via the BCSFB system (Ueno et al. 2016). Together with the blood-brain barrier (BBB), discussed in the human brain section, BCSFB forms and maintains the desired homeostatic microenvironment in the central nervous system (Tietz & Engelhardt 2015, Ueno et al. 2016).

2.1.2 Blood flow & metabolism

Spinal cord blood flow

Interest in the spinal cord blood supply dates back to the anatomical studies of Adamkievicz in 1881. He concluded that a single artery named after him, the artery
of Adamkievicz, arising from the level T7-T12/T8-L1 and typically on the left posterior intercostal artery, is crucial in maintaining the mid-cord blood supply. (Adams & van Geertruyden 1954, Lazorthes et al. 1971, Etz et al. 2011, Wynn & Acher 2014). During the past decades, the understanding of the spinal cord blood supply has evolved new insights.

Longitudinally, the blood supply of the spinal cord is divided between three arteries: the anterior spinal artery (ASA) and two posterior spinal arteries (Lazorthes et al. 1971). The ASA, formed from the subclavian arteries via vertebral arteries, supplies the blood to the anterior side of the spinal cord including the motor areas. In contrast, the two posterior spinal arteries, originating similarly from the subclavian arteries, are responsible for the blood supply of the posterior sensory areas of the spinal cord. Radicular arteries mediate the blood supply to the ASA from the thoracic and lumbar segmental arteries arising and coming in pairs from the aorta (Wynn & Acher 2014). Besides these interconnections the collateral circulation is involved in the spinal cord blood supply, and is discussed in the next section.

The autoregulation of the spinal cord functions between pressures of 50–150 mmHg and the blood flow is maintained via vasodilatation and –constriction (MacDonald et al. 2013). The most important factor determining blood flow is the spinal cord perfusion pressure (SCPP), defined as the difference between mean arterial pressure (MAP) and cerebrospinal fluid pressure (CSFP) (Marini et al. 1998). Moreover, to be described in detail, SCPP is the balance between inflow and outflow pressures, where inflow is mainly dependent on the aforementioned MAP and cardiac output, whereas outflow is determined according to the CSFP and central venous pressure (CVP), which have effects on inflow pressure, as well (Etz et al. 2008).

Generally, spinal cord blood flow (SCBF) is distributed depending on metabolic need. At rest, the grey matter flow is four times greater than the white matter flow based on the fact that dendrites and cell bodies have higher metabolic rate compared with axons (MacDonald et al. 2013). The experimental primate studies of SCBF have estimated the white matter blood flow to be constant throughout the areas with a mean value of 10 ml/100g/min. In contrast, the grey matter has been shown to have characteristics dependent upon varying grades of flow, with lower values in the dorsal horns and higher values in the central and anterior horn areas, resulting in a mean value of 58 ml/100g/min. The highest flow values have been detected in the dorsal root entry zone, and in the region of the anterior spinal artery and its branches (Sandler & Tator 1976).
The collateral network

In 1971, the first experimental studies concluded the presence of arterial collateral circulation with multiple anastomoses in the spinal canal, perivertebral tissues, and paraspinous muscles along with anterior and posterior spinal arteries (Lazorthes et al. 1971, Wynn & Acher 2014). Subsequently, the sources of the blood inputs were determined to involve segmental arteries, subclavian arteries, and mammary and hypogastric arteries (Lazorthes et al. 1971, Giglia et al. 1994, Wynn & Acher 2014).

Extensive experimental studies by Etz, Griep, and associates have revealed the detailed anatomy of this arterial circulation, named the collateral network. First, the network has paraspinous and intraspinal compartments. The segmental arteries feed the paraspinous network, and the multiple connections between different sides of the network function via ASA and epidural arteries (Etz et al. 2011, Wynn & Acher 2014). The identification of anterior radiculo-medullary arteries, in the intraspinal compartment, functioning towards the anterior spinal artery has raised questions concerning their position along the spinal cord, and their role in predicting the development of spinal cord ischemia (Etz et al. 2011, Kari & Beyersdorf 2015). Moreover, the role of arterial circles of the intraspinal system has been questioned as an immediate spinal cord flow backup (Kari & Beyersdorf 2015, Kari et al. 2015).

Furthermore, there are two distinguishing characteristics in this collateral network. The steal phenomenon occurs if cord blood flow is reduced due to an opened low-resistance pathway. The beneficial effect of the network can be detected when blood supply is restricted from one source: then another source can increase cord blood flow, working dynamically and maintaining resilience of the spinal cord perfusion, for instance during segmental artery sacrificing in thoracoabdominal aortic aneurysm (TAAA) repairs (Etz et al. 2011, Wynn & Acher 2014).

Spinal cord metabolism

Delivery of oxygen to the spinal cord is mainly controlled by the blood supply. The spinal cord is a highly oxygen-dependent organ, and thus vulnerable to the aforementioned steal phenomenon along with restricted blood flow and oxygen (O2) supply (Etz et al. 2011, Wynn & Acher 2014). Especially, myelinated axons are highly dependent on continuous energy supply produced via oxidative phosphorylation (Stys 1998, Silva et al. 2014). Moreover, the alpha motoneurons
of the anterior horn are 2 to 3 times more sensitive to the altered blood supply than is the white matter (Jacobs & Mess 2003).

2.2 The human brain

The human brain, weighing 1,200–1,400g, consists of over 10 billion neurons in close connection with a 10-fold number of supporting cells, neuroglia. The same distribution of the grey and white matter exists in the brain, as well as in the spinal cord.

2.2.1 Cellular structure

The cellular structure and functions of the parts of the neurons, including the soma, dendrites, and axons, are described in detail in the spinal cord section. The supporting cells, neuroglia, consist of different cell types, for instance astrocytes, oligodendrocytes, microglia and ependymal cells, with various forms and functions targeting to maintain CNS homeostasis (Parpura & Verkhratsky 2012).

Astrocytes, which form the greatest part of the neuroglial cells, work in a dynamic relationship with the neurons, taking part in many tasks, and thus resulting in maintaining the homeostatic balance of the neural microenvironment (Parpura et al. 2012). Their role is especially noted in supporting neurons metabolically via changes in their intracellular Na⁺ and Ca²⁺ dynamics and in detecting synaptic activity at the synapse. Together with these actions, their function is to modulate neuronal activity via synaptic transmission and plasticity modifications and to regulate immune reactions. One of the main tasks involves maintaining the integrity of the blood-brain barrier, as well (Parpura & Verkhratsky 2012, Parpura et al. 2012, Xu et al. 2014, Keaney & Campbell 2015).

S100B is a calcium binding glial-specific protein, and mainly expressed by specific astrocytes in the neuronal system functioning in an intracellular and extracellular fashion. High concentration of S100B has neurological toxicity. Moreover, elevated levels of S100B have prognostic importance in predicting neurological outcomes along with BBB permeability and the severity of brain damage both in traumatic and global ischemic cerebral injury. Furthermore, the elevated level of S100B is associated with ischemia-reperfusion injury of the CNS and especially its systemic inflammatory response (Sun et al. 2013).

Oligodendrocytes, such as the Schwann cells, are responsible for the production of myelin throughout the central nervous system via several signaling
pathways. The myelin sheaths speed up the transmission of action potentials in the axons (Gaesser & Fyffe-Maricich 2016). The formation of the myelin is a highly energy-consuming procedure. During neuronal injury, there is requirement for rapid remyelination, and thus these processes are vulnerable to damage (Silva et al. 2014, Stenudd et al. 2015, Gaesser & Fyffe-Maricich 2016).

Microglia, formed in the pia mater, play vital roles in defence mechanisms along with participation in innate immune responses of the CNS (Ginhoux et al. 2010). Microglia function via two different pathways: M1 and M2. M1, classically activated, induces the release of proinflammatory and cytotoxic mediators. As an alternative, the differently activated M2 pathway participates in tissue repair, phagocytosis, and chemokine release together with neurotrophic pathway activation (Aguzzi et al. 2013, Keaney & Campbell 2015).

Ependymal cells cover up the edges of the ventricles and the spinal canal, forming a special epithelium structure and reservoir. Recent studies have shown that these cells serve as neural stem cells in association with spinal cord injury (SCI) and repair, restricting the enlargement of the lesion and supporting the neuronal survival via the production of neurotrophic factors (Stenudd et al. 2015).

The grey and white matter

The grey matter of the human brain is formed by cerebral cortex, and cerebellar cortex together with nuclei situated in the cerebellum, cerebrum, and brainstem. The grey matter consists of neuronal cell bodies, dendrites, myelinated and unmyelinated axons, glial cells, synapses, and capillaries. Around these structures, the white matter, containing the myelinated axon tracts, is located.

Blood-brain barrier (BBB)

The majority of the brain capillaries are surrounded by a structure named the blood-brain barrier. This system actively functions to protect and restrict the influx of the blood-borne substances into the brain tissue and efflux from the cerebral tissue to maintain a balanced microenvironment in the brain (Reese & Karnovsky 1967, Brightman & Reese 1969, Keaney & Campbell 2015). The BBB structure consists of four cell membranes formed by the end-feet of the astrocytes, podocytes, the endothelium of the capillaries, pericytes and basement membrane. Generally, these structures are referred to as the neurovascular unit (Bauer et al. 2014, Ueno et al. 2016). The fat-soluble agents are more easily passed through the several cell
membranes compared with the water-soluble matter. Together with multilayer characteristics, the passage and maintenance of the barrier are based on the BBB-specific transporters and receptor proteins, tight junctions, adherens junctions, low levels of transcytotic vesicles, and absence of fenestrae (Tietz & Engelhardt 2015, Keaney & Campbell 2015).

**2.2.2 Blood flow & metabolism**

*Cerebral blood flow*

At rest, the brain receives approximately 20% of the cardiac output. Constant energy supply, involving oxygen (O₂) and glucose, via cerebral blood flow enables the fluent functioning of the brain. Due to high energy demand of the neurons, this supplement needs to be secured and stable regardless of the variations in systemic mean arterial pressure. Therefore, to fill this role, a system called autoregulation operates when MAP is ranging approximately between 60–170 mmHg, involving especially metabolic and myogenic mechanisms (Carlyle & Grayson 1956, Harper 1966).

The average cerebral blood flow (CBF) is around 50 ml/100g/min, divided into the grey and white matter, 75 ml/100g/min and 20 ml/100g/min, respectively (McHenry et al. 1978). The CBF depends on cerebral perfusion pressure (CPP), defined as the difference between MAP and intracranial pressure (ICP). The main regulators of CBF are then the blood pressure along with partial pressure of arterial carbon dioxide (paCO₂), oxygen (paO₂), and pH (Harper 1966).

*Cerebral metabolism*

The metabolism of the brain is highly oxygen-dependent, aerobic. Moreover, the neurons of the brain are not able to store or produce glucose, and thus are dependent on an adequate supply being available (Maran et al. 1994, Parpura et al. 2012). Under aerobic, normal circumstances, in glycolysis glucose is converted first to pyruvate and then to acetyl coenzyme A, which leads to the citric acid cycle. Ultimately, 38 moles of ATP are produced from one mole of glucose mainly via electron transport chain of the mitochondria (Valko et al. 2007, Niizuma et al. 2009, Klaunig et al. 2011). When energy supply is restricted, due to inadequate CBF and thus ischemia, the shift towards anaerobic metabolism is required, resulting in 2
moles of ATP, a hydrogen ion (H\(^+\)) and lactate accumulation (Boumezbeur \textit{et al.} 2010). Alternative energy sources of the brain have been studied (Parpura \textit{et al.} 2012). Interestingly, the role of lactate as an energy source instead of glucose in the brain has been reported when elevated in the blood and during hypoglycaemia associated with cerebral function protection (Maran \textit{et al.} 1994, Smith \textit{et al.} 2003, van Hall \textit{et al.} 2009, Boumezbeur \textit{et al.} 2010).

The produced ATP is used to maintain ionic homeostasis of the cells. The sodium-potassium pump (Na\(^+\)/K\(^-\)-ATPase), which maintains the resting potential of the cells and conduction of the nerve impulses, is the main user of the ATP sources (Mattson \textit{et al.} 2000, Kovac \textit{et al.} 2015). The energy is also utilized in the metabolism of neurotransmitters and synthesis of proteins and lipids. Hence, the reduction of the ATP production results in various consequences.

Moreover, the brain is susceptible to oxidative damage since there is a high rate of ROS production due to high oxygen consumption and limited endogenous antioxidants (Kovac \textit{et al.} 2015).

\textit{Ischemic tolerance}

The brain can endure an approximately 50\% reduction in the CBF due to physiological compensation mechanisms before the occurrence of permanent neuronal cell damage. When the ischemia period is prolonged and the reduction of the CBF is more than 50\% the risk for selective neuronal death increases (del Zoppo \textit{et al.} 2011).

The CA1 and CA3 sectors of the hippocampus are the most vulnerable to ischemia (Araki \textit{et al.} 1992). In addition to the pyramidal cells of the hippocampus, the striatum, the thalamus, and the cerebral cortex along with the Purkinje cells of the cerebellum are vulnerable to ischemia and the occurrence of selective neuronal death (Brightman \textit{et al.} 1970, Araki \textit{et al.} 1992, Shinnou \textit{et al.} 1998, Kimura \textit{et al.} 2002, Dietrich \textit{et al.} 2009). In contrast, the brainstem, the cerebellum, and the white matter of the cerebrum are more resistant to ischemic conditions (Shin’oka \textit{et al.} 1996).

\textbf{2.3 Ischemic injury}

The occurrence of ischemic injury is highly organ-specific. Some organs can endure the deprivation of energy sources for much longer periods than others. In
contrast, the highly oxygen- and glucose-dependent organs are extremely vulnerable to supplement disruptions.

2.3.1 The spinal cord

There are several causes for spinal cord injury, for instance penetrating forces, compression, or infarction due to a vascular insult. This so-called primary injury is the first step in the neurological damage process of the spinal cord followed by secondary injury, involving a cascade of biochemical and cellular events within minutes to weeks, resulting in further neurological damage. Thereafter, the final, chronic phase occurs over the course of days to years (Bareyre & Schwab 2003, Cramer et al. 2005, Yiu & He 2006, Silva et al. 2014).

Biological cascades of spinal cord injury

Several biochemical actions occur in secondary injury, worsening the damage to the spinal cord and leading to massive cell death and disruption of the BCSFB (Stenudd et al. 2015). The following mechanisms have been reported to be involved in the process.


Free radical formation and lipid peroxidation. Edema and inflammatory responses are aggravated due to the oxidative death of spinal cord neurons and reduced blood flow of the spinal cord (Sandler & Tator 1976, Goodman et al. 1979, Toborek et al. 1999, Silva et al. 2014). Free radicals act mainly in the disruption of the lipid-based cell membranes along with interrupting Na⁺/K⁺-ATPase, resulting in the loss of resting membrane potentials and neuronal function, and thus terminal tissue damage (Jamme et al. 1995, Hall & Braughler 1982, Savas et al. 2002, Silva et al. 2014). The role of free radicals is described in detail in the next section.

Imbalance of K⁺, Na⁺, Ca²⁺ ions. Ionic homeostasis is crucial for cell survival, and thus impractical depolarization of cell membranes, malfunctioning ATPases due to the disruption of ionic balance, leads to increased axoplasmic Na⁺, severe
K^+ depletion, and increased axonal Ca^{2+}, following cell death via several pathways (Stys 1998, Toborek et al. 1999, Bareyre & Schwab 2003, Silva et al. 2014). Increased intracellular Ca^{2+} has effects on reactive oxygen species (ROS) formation; activation of cellular proteases: calpain, phospholipases, and protein kinase C (PKC); disruption of cytoskeleton: and initiation of necrosis or apoptosis (Stys 1998, Toborek et al. 1999, Silva et al. 2014).

Glutamate excitotoxicity. Glutamate is the main excitatory neurotransmitter in the CNS (Nishizawa et al. 2001). Following spinal cord injury, the levels of extracellular glutamate increase, becoming neurotoxic, and stimulating glutamate receptors, ending in neuronal cell death (Farouque et al. 1996, Xu et al. 1998, Liu et al. 1999, Hermann et al. 2001, Silva et al. 2014). In the spinal cord, especially oligodendrocytes and the myelin sheath are affected by glutamate (Stys 1998, Silva et al. 2014).

Inflammatory response. The role of the inflammatory response is pivotal between neurodegeneration, axonal plasticity, and regeneration. The beneficial effects are associated with neural tissue repair (Donnelly & Popovich 2008). On the other hand, microglia are activated, infiltrating leukocytes to produce proinflammatory factors and ROS resulting in extravasation of leukocytes and tissue damage (Means & Anderson 1983, Popovich et al. 1997, Taoka et al. 1997, Mabon et al. 2000, Hermann et al. 2001, Bareyre & Schwab 2003, Silva et al. 2014). It is worth noting that the number of recruited and activated neutrophils, macrophages, and lymphocytes in spinal cord neuroinflammation is higher compared with such inflammation in the brain. Moreover, the actions of cytokines and chemokines differ between these organs during injury (Donnelly & Popovich 2008). In ischemic spinal cord injury, inflammatory cells cause a direct phagocytic effect or the secretion of cytokines and further activation of signaling cascades, resulting in caspase upregulation (Tompoulis et al. 2003). The activated neutrophils interact with the endothelial cells via P-selectin, developing endothelial cell injury and further spinal cord injury (Taoka et al. 1997). The effects of neuroinflammation on the BCSFB junctions are limited (Tietz & Engelhardt 2015).

Necrosis & Apoptosis. The involvement of passive cell death, necrosis, in the secondary injury of the spinal cord has been shown in several studies (Crowe et al. 1997, Beattie et al. 2000, Silva et al. 2014). Programmed active cell death, apoptosis, has been demonstrated to occur in end-stage SCI, especially in the white matter, along with facilitating damage after injury (Crowe et al. 1997, Schuman et al. 1997, Emery et al. 1998, Beattie et al. 2000, Silva et al. 2014). Moreover, the loss of large motor neurons following ischemia has been shown to be caused

**Reactive oxygen species (ROS) & redox regulatory mechanisms**

In addition to the above discussed ischemic biochemical cascade, ROS play a role both the ischemia and reperfusion periods. Under physiological conditions, mainly mitochondria produce endogenous ROS via aerobic respiration together with small amounts produced by inflammatory cells and peroxisomes (Karihtala & Soini 2007, Klaunig et al. 2011). The term ROS includes different types of reactive molecules or molecular fragments, for instance free radicals with one or more unpaired electrons enabling the unstable and reactive features of these molecules (Karihtala & Soini 2007). Generally, the extended term of ROS includes, for example the aforementioned free radicals such as superoxide (O$_2^-$) and hydroxyl radical (·OH), non-radicals such as hydrogen peroxide (H$_2$O$_2$), and nitric oxide-derived reactive molecules (Valko et al. 2004, Karihtala & Soini 2007). The term oxidative stress is defined as an imbalance between ROS production, ROS suppression and repair systems resulting in increased intracellular levels of ROS (Valko et al. 2006, Karihtala & Soini 2007, Klaunig et al. 2011, Schieber & Chandel 2014). The defence mechanisms against ROS include, for instance, antioxidant enzyme systems (Karihtala & Soini 2007).

In unphysiological conditions, the interactions between endothelial cells and leukocytes produce oxygen free radicals, thereafter forming even more reactive agents. The destructive characteristics of ROS include, for instance, inhibiting and shutting down mitochondrial function, cellular injury of the nucleus, and oxidizing lipids and proteins, along with DNA damage and epigenetic alterations (Valko et al. 2004, Karihtala & Soini 2007, Schieber & Chandel 2014).

The beneficial effects of ROS, especially H$_2$O$_2$, involve enhancing normal immune system function and cellular signaling pathways, and inducting mitogenic response (Valko et al. 2007, Schieber & Chandel 2014). At low levels of ROS, immune system cells consisting of macrophages and neutrophils utilize oxidative bursts as a defence mechanism against infectious agents, increasing oxygen consumption, and consequently leading to increased ROS formation during the
inflammatory process (DeCoursey & Ligeti 2005, Valko et al. 2006 & 2007, Dunn et al. 2015). In the intracellular signaling process, low levels of ROS, for instance, stimulate proliferation and enhance survival. ROS contributes to modulation of the activities of protein kinase C, mitogen-activated protein kinases (MAPK), and transcription factors such as activator protein-1 (AP-1), nuclear factor erythroid 2-related factor 2 (Nrf2), nuclear factor kappa B (NF-kB), and hypoxia-inducible transcription factor 1α (HIF-1α) (Dröge 2002, Chen et al. 2011, Klaunig et al. 2011, Prentice et al. 2015). Multiple cytokine receptors such as tumor necrosis factor (TNF) and interleukin 1 beta (IL1β) can generate ROS serving in cell signaling pathways and redox control (Thanickal & Fanburg 2000, Valko et al. 2006 & 2007, Schieber & Chandel 2014).

Antioxidants can be divided into enzymatic and non-enzymatic antioxidants (Valko et al. 2006). The functions of non-enzymatic antioxidants, e.g. carotenoids, selenium, and vitamins A, C, and E, involve protecting cells from free radicals and maintaining cellular redox state. Additionally, antioxidants can decrease ROS levels and further stimulate the survival of damaged cells (Valko et al. 2004, 2006 & 2007, Jomova & Valko 2013). Peroxiredoxin-1 (PRDX1), thioredoxin (TXN), and glutathione peroxidase (GPX) are enzymatic antioxidants regulated by cytoprotective transcription factor Nrf2. Additionally, the effects of Nrf2 involve gene transcription of proteins in restoration of cell homeostasis, inflammatory response, detoxification, and barrier permeability together with cell growth (Shibata et al. 2008, Sun et al. 2011, Ganán-Gómez et al. 2013, Zhan et al. 2013).

Normally, Nrf2 is coupled to the Kelch-like ECH-associated protein 1 (Keap1) in cytoplasm. However, due to the oxidative stress, this adherence is modified, and Nrf2 translocates to the nucleus and activates its target genes via v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (MAF) proteins and antioxidant response element (ARE) bindings (van der Wijst et al. 2014, Schieber & Chandel 2014). Independent movement of Keap1 between cytoplasm and nucleus can regulate Nrf2 translocation back to the cytoplasmic degradation, and thus modulate Nrf2 signaling (Sun et al. 2011).

Ischemia-reperfusion injury in the spinal cord

During the open repair of thoracoabdominal aortic aneurysms (TAAAs), the clamping and unclamping of the aorta occur, resulting in a reperfusion period after ischemia. This ischemia-reperfusion cycle causes damage in neuronal tissue in a biphasic manner: acute and delayed (Papakostas et al. 2006, Zhu et al. 2013). The
acute ischemia phase, due to the restriction in oxygen and energy sources, ends in different biological cascades as discussed above (Nishizawa 2001, Hermann et al. 2001, Papakostas et al. 2006).

The reperfusion period accounts for the delayed insult including reoxygenation, hyperemia, and an inflammatory response (Reece et al. 2004, Papakostas et al. 2006, Zhu et al. 2013). The damage of reoxygenation is explained via severe mitochondrial Ca²⁺ overload resulting in secondary failure of respiration (Stys 1998, Silva et al. 2014). Macrophages, neutrophils, microglia and astrocytes are the inflammatory cells involved in the reperfusion period (Zhu et al. 2013, Yamanaka et al. 2015). The main effectors in the delayed insult are excessive amounts of ROS, proinflammatory cytokines, adhesion molecules, chemokines, neutrophil proteases partly produced by activated neutrophils, and activated phospholipase A (PLA) (Taoka et al. 1997, Savas et al. 2002, Valko et al. 2007, Oyar et al. 2008, Zhu et al. 2013, Yamanaka et al. 2015). Moreover, the delayed reperfusion is thought to be caused by up-regulated secondary neurotoxic mechanisms presenting neuronal loss and strong inflammation in the grey matter (Papakostas et al. 2006). Neurotoxicity, endothelial damage, hypoperfusion in association with increased oxygen demand, and apoptosis are also events associated with the reperfusion period (Savas et al. 2002, Acher & Wynn 2009, Zhu et al. 2013).

Infarction in the spinal cord occurs, if the spinal cord ischemic tolerance is exceeded during aortic clamping. The reperfusion inflammation worsens significantly the ischemic insult when the permanently altered spinal cord blood supply due to aortic replacement cannot respond to the modified conditions of the circulation (Papakostas et al. 2006, Wynn & Acher 2014).

**Scar formation**

When irreversible actions take place, the chronic phase of SCI results. Scar formation occurs in this phase, involving fibrotic core and surrounding glial components, which function as a physical barrier and prevent axons to grow through it (Silva et al. 2014, Stenudd et al. 2015). The aforementioned components have both detrimental and beneficial effects on recovery (Stenudd et al. 2015).

Prior to the formation, white matter demyelination, grey matter dissolution, connective tissue deposition, and reactive gliosis take place (Silva et al. 2014). The effectors in this process include reactive astrocytes, microglia, macrophages, and extracellular matrix molecules (Gallo et al. 1987, Katoh-Semba et al. 1995, Jones et al. 2003, Yiu & He 2006, Aguzzi et al. 2013, Silva et al. 2014).
Neuronal plasticity after SCI

In normal physiological circumstances, the CNS is noted for its capability to alter its functions as a result of growth. After spinal cord injury, neuronal reorganization, synaptic rearrangements, and neuronal activation changes along with collateral sprouting of intact or lesioned axons occur. Incomplete SCI comprises adaptive actions in sprouting of spinal interneurons and of caudal motor neurons over time (Courtine et al. 2008, Boulenguez et al. 2010).

Experimental and clinical studies have shown cortical, thalamic, cerebellar, basal gangliar and brainstem modifications associated with SCI (Bruehlmeier et al. 1998, Qi et al. 2000, Kaas et al. 2008, Silva et al. 2014). Additionally, spinal cord tissue plasticity and exploiting existing neuronal pathways have been reported in SCI and its therapeutic targets (Wernig et al. 1995, de Leon et al. 1998, Silva et al. 2014). The underlying mechanisms of neuronal plasticity are incompletely understood. The possible mechanisms include increased levels of cytokines and growth factors. The strongest body of evidence lies in GABA, an inhibitory neurotransmitter in the brain, and its modifications (Hendry & Jones 1986, Jacobs & Donoghue 1991, Jones 1993, Boulenguez et al. 2010, Roy et al. 2011, Silva et al. 2014).

It needs to be mentioned that together with the beneficial effects of plasticity, adverse actions affecting the quality of life occur notably as well after SCI, for instance the appearance of central pain, spasticity, and autonomic dysreflexia. The central sensitization of neurons in the dorsal horn has been suggested to be the mechanism of chronic pain, and abnormal glutamatergic signaling is believed to cause spasticity and autonomic dysreflexia after SCI (Christensen & Hulsebosch 1997, Rabchevsky & Kitzman 2011, Silva et al. 2014).

2.3.2 The brain

In the brain, the type of ischemia is either global or focal. Focal brain ischemia, resulting from occlusion of the cerebral artery, damages the brain, causing local cortical or subcortical brain infarction (del Zoppo et al. 2011). In contrast, global brain ischemia accounts for cardiac arrest or low perfusion pressure of the brain, for instance, and leads to hypoxic-ischemic encephalopathy or death. HCA settings, including ischemia-reperfusion periods, represent mainly the global form of ischemic brain injury. The surgical interventions involving manipulation of great
atheromatous arteries, along with embolism risk due to the usage of CPB, account for the possible focal ischemia component associated with HCA.

**Pathogenesis of ischemic brain injury**

The brain tissue resists global ischemia for approximately 5–6 minutes in normothermia before irreversible neuronal damage occurs. The electrical activation of neurons is suppressed when the CBF is reduced below 16–18 ml/100g/min, and further reduction below 12 ml/100g/min results in the occurrence of brain infarction.

The steps from single cell necrosis leading to the infarction involve different functional impairments with biochemical alterations and suppression of single cell activity along with membrane failure. The infarction area, the ischemic core, is surrounded by penumbra involving tissue with salvage properties (del Zoppo et al. 2011). The several stages of global brain ischemia are discussed next.

**Alterations in cellular functions**

The decreased cerebral blood flow, and consequently the depletion of energy sources, initiates the anaerobic metabolism in the brain, increasing intracellular H+, phosphate, and lactate levels, and causing acidic conditions in the cells (Chen et al. 2011, González-Ibarra et al. 2011). ATP production decreases starkly during this metabolism switch (Lobner & Lipton 1993). The energy-consuming processes maintaining cellular electrical and ionic homeostasis are altered, ending in ischemic depolarization of neurons (Mattson et al. 2000). Ischemic depolarization of neurons results in the imbalance of intracellular ions. Na+ overload compounds water accumulation intracellularly, causing swelling of the cells and endoplasmic reticulum dilatation, and ending in cytotoxic edema (Lobner & Lipton 1993).

**Blood-brain barrier interruption**

Brain ischemia causes interruption of the BBB, ending in free passage of blood-borne molecules and immune system cells. Cytotoxic oxygen free radicals and proteases produced by neutrophils cause fragmentation of the endothelial cellular walls, and thus breakdown of the BBB (Merrill & Benveniste 1996). Moreover, the proinflammatory cytokines such as interleukin-1β (IL-1β) contribute to the BBB dysfunction during neuroinflammation (Tietz & Engelhardt 2015). The breakdown
of the barrier and ischemic-cytotoxic edema of the brain tissue increase brain edema and haemorrhage (Dietrich et al. 2009, Chen et al. 2011). The early phase of acute stroke shows initial effects on the BBB permeability according to magnetic resonance imaging, and shown by elevated systemic levels of ischemia-modified albumin produced by ROS (Abboud et al. 2007, Gunduz et al. 2008, Ueno et al. 2016).

Ischemic biochemical cascade

There are several factors mediating the ischemic biochemical cascade occurring in global cerebral ischemia (Araki et al. 1992).

Calcium ($\text{Ca}^{2+}$). Ischemic depolarization and malfunctioning of active and interrelated transporters in the cell membrane including Na$^+$/K$^+$-ATPase, Ca$^{2+}$-ATPase, voltage-gated Na$^+$ and Ca$^{2+}$ channels, Na$^+$/H$^+$- and Na$^+$/Ca$^{2+}$-exchangers result in massive Ca$^{2+}$ influx into the cell, with insufficient Ca$^{2+}$ removal (Lobner & Lipton 1993, Carini et al. 1994). Moreover, ROS are known to increase intracellular calcium and vice versa (Valko et al. 2006 & 2007). The Ca$^{2+}$ overload of the cells is the key initiator of cell death, activating proteolytic enzymes in cell membranes including endonucleases, phospholipases, and activation of ATPases, leading to accelerated ATP depletion. Additionally, ion gradients of Na$^+$, K$^+$, and Cl$^-$ are imbalanced due to reduced energy production during ischemic conditions (Nishizawa et al. 2001).

Glutamate. Glutamate is an excitatory neurotransmitter securing normal functions of the brain when present at reasonable levels. The ischemic neurons depolarize, releasing significant amounts of glutamate to the synaptic cleft, binding to the N-methyl-D-aspartate (NMDA) and alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptors of the postsynaptic neurons (Prentice et al. 2015). The release of glutamate occurs via calcium-dependent and -independent pathways (Nishizawa et al. 2001). Excitotoxicity is characterized by massive Ca$^{2+}$ influx, especially through NMDA receptors, leading to mitochondrial dysfunction (Stanika et al. 2012). Moreover, these actions compound both Na$^+$ and Ca$^{2+}$ overload of the neurons, since the energy-demanding transporters function and remove these agents insufficiently (Nishizawa et al. 2001). In conclusion, this worsens the cytotoxic edema, as well.

Nitric oxide (NO). The role of nitric oxide (NO) is pivotal in ischemic brain injury. The cellular effects of NO are dependent on its concentration, site of release, and duration of action (Peng et al. 2012). Nitric oxide is produced from L-arginine
via enzyme nitric oxide synthase (NOS). There are four isoforms of NOS: mitochondrial-specific nitric oxide synthase (mtNOS), neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS) (Davis et al. 2001, De Sanctis et al. 2014). Endotoxins or proinflammatory cytokines regulate iNOS expression, producing high levels of NO, and thus bacterial cytotoxic features of these cells (Davis et al. 2001). Instead, mtNOS, nNOS, and eNOS are regulated by intracellular calcium concentrations (Davis et al. 2001, De Sanctis et al. 2014). In neural tissue, NO is produced during the oxidative bursts triggered by inflammatory processes via ischemic leukocytes and neurons together with inflammatory cells conveyed to the brain tissue. Nitric oxide is itself a free radical, but it can potentiate the formation of even more reactive free radicals, for instance peroxynitrite (ONOO⁻), which causes protein, lipid, and nucleic acid modifications, DNA reactions, and mitochondrial respiratory chain disturbances (Davis et al. 2001, Valko et al. 2006, De Sanctis et al. 2014). The harmful effects of NO are also associated with the mediation of glutamate neurotoxicity and endoplasmic reticulum (ER) calcium homeostasis during ischemia-reperfusion injury (Paschen & Mengesdorf 2005). The beneficial side of the NO is related to the eNOS, which functions in vasodilatative and antiadherent manners in the microcirculation (Vlasov et al. 2005, Peng et al. 2012).

Mitochondrial permeability. Mitochondria produce large amounts of ATP through oxidative phosphorylation to satisfy metabolic demands (Perez-Pinzon et al. 2012). Mitochondrial deficits, involving complexes I-IV of the electron transport chain, result in various contributors such as a deficiency in energy supply, excitotoxicity by glutamate, and excessive generation of ROS (Prentice et al. 2015). Severe stress stimulus, e.g. ischemia, causes an irreversible collapse of mitochondrial membrane potential, leading to the opening of inner mitochondrial permeability transition pore (MPTP). In pathological ischemic conditions, MPTP opening occurs due to elevated mitochondrial matrix Ca²⁺, ROS, inorganic phosphate, and intracellular acidification and leads to dysregulation of the aforementioned inner mitochondrial membrane, uncoupling of oxidative phosphorylation, and cell death processes (Szabo et al. 1992, Perez-Pinzon et al. 2012, Giorgio et al. 2013, Prentice et al. 2015, Mnatsakanyan et al. 2016). The further release of cytochrome C (cyt C) and extra calcium from mitochondria promotes cell death (Mnatsakanyan et al. 2016).

Endoplasmic reticulum (ER) stress. ER functions in the processing and folding of new protein synthesis, requiring, for instance, Ca²⁺ and cellular Ca²⁺ storage (Kuznetsov et al. 1992, Lodish et al. 1992, Paschen & Mengesdorf 2005).
Moreover, the role of ER is recognized in the maintenance of cellular homeostasis and in the regulation of neuronal survival and death (Wang et al. 2013). Under impaired conditions, malfunctioning occurs and unfolded proteins accumulate in the ER, causing severe stress that leads to irreversible actions (Paschen & Mengesdorf 2005, Wang et al. 2013). ER stress is associated with ischemia-induced cell damage (Kumar et al. 2001, DeGracia & Montie 2004, Paschen & Mengesdorf 2005, Azfer et al. 2006, Wang et al. 2013). The impairment is due to the depletion of ER calcium stores, oxygen and glucose deprivation, oxidative stress, or genetically modified proteins, for instance. ER stress activates the unfolded protein response, involving several molecular pathways, balancing between the re-establishment of homeostasis and cell apoptosis (Yoneda et al. 2001, DeGracia & Montie 2004, Paschen & Mengesdorf 2005, Mercado et al. 2013). Under severe and prolonged ER stress, apoptosis is induced and processed via several proapoptotic and prodeath signals involving, for instance, C/EBP homologous protein (CHOP) and caspase 12, ending in caspase 3 activation (Yoneda et al. 2001, Oyadomari & Mori 2004, Paschen & Mengesdorf 2005). Consequently, crosstalk between ER and mitochondria has been reported during apoptosis induced by ER stress (Oyadomari & Mori 2004, Paschen & Mendesdorf 2005).

Apoptosis & Necrosis. The culminating processes in cell death are apoptosis and necrosis, both of them occurring in brain ischemia. Apoptosis, a programmed cell death, occurs in an interaction between proapoptotic and antiapoptotic proteins via intracellular enzyme pathways (Mattson et al. 2000). The caspase pathway, divided into intrinsic and extrinsic pathways, is one of the most crucial processes in apoptosis, the final resulting pathway. Cellular stress, e.g. hypoxia and substrate deprivation, activates the intrinsic pathway forming mitochondrial membrane permeabilization (MMP) acting in the release of cyt C from mitochondria (González-Ibarra et al. 2011). Cytochrome C then interacts with apoptotic protease activating factor-1 (Apaf-1), resulting in the activation of caspases 9 and 3, and thereafter cell death (Yoshida et al. 1998, Kuida 2000, Chen et al. 2011, Prentice et al. 2015). The activated extrinsic pathway ends in the activation of caspases 8, 3, 6, and 7, and thereafter apoptosis (Muzio et al. 1998, González-Ibarra et al. 2011). The changed genetic expression in ischemic penumbra cells directs them to the energy-consuming, immunologically silent, programmed cell death. The silence is due to quick phagocytosis of the dead cells. The necrosis occurs mainly in the ischemic core of the damaged neural tissue involving chaotic and uncontrolled cell death with inflammatory and immunological reactions in the surrounding tissue (Emery et al. 1998).
Reperfusion injury

The restoration of the blood, and thus reoxygenation of the cells, causes, secondary brain injury due to the altered interplay between endothelial cells and blood components exposed to ischemia (Schmitt et al. 2007). The ischemic insult generates inflammatory actions both in protective and harmful properties (Merrill & Benveniste 1996). Additionally, during neuroinflammation and reperfusion, the endothelial cells convert their antithrombotic and antiadherent features into prothrombotic and proadherent characteristics, producing intercellular adhesion molecule (ICAM-1), complement, and cytokines, and thus catching activated platelets and neutrophils (Merrill & Benveniste 1996, Shin’oka et al. 1996, Valko et al. 2005, Schmitt et al. 2007). Consequently, increased vasospasm, cellular aggregates, and thrombus affect endothelial dysfunction in reperfusion (Loukogeorgakis et al. 2005).

The cells, having properties to be salvaged in penumbra, are affected due to the intensified inflammatory response of the reperfusion injury. The cytotoxic proteolytic enzymes, metalloproteinases, and elastases induced by neutrophils intensify inflammatory reactions in the penumbra. The inhibition of neutrophil adhesion to the endothelium has beneficial effects of attenuating reperfusion injury (Dröge 2002). During the early reperfusion period, the neuronal protein synthesis has been shown to be inhibited (Kumar et al. 2001, DeGracia & Montie 2004). The role of PKC and its isoenzymes has been linked to ischemia-reperfusion injury modulating signaling pathways in a pivotal manner (Bright et al. 2004). However, the levels of extracellular glutamate quickly recover to the basal level during reperfusion (Nishizawa et al. 2001).

The cell membrane fragmentation occurs due to phospholipase A and oxygen free radicals. During the reperfusion, the key responsible for ROS formation is NADH oxidase (NOX) (Chen et al. 2011, Prentice et al. 2015). Additionally, the production of oxygen free radicals is accelerated via xanthin oxidase in the reperfusion period (Dröge 2002, Chen et al. 2011). The effect of increasingly more toxic and reactive free radicals occurs in ischemic tissue, resulting in the destruction of cell membranes (Bright et al. 2004, Chen et al. 2011). The membrane peroxidation in the reperfusion increases and enables the fragmentation by activated PLA. The activation of cyclo-oxygenase (COX-2) and lipo-oxygenase forms inflammatory and vasoactive eicosanoids from membrane lipids, including thromboxane, prostaglandins, and leukotrienes (Merrill & Benveniste 1996). These
actions culminate in increased thrombogenicity, permeability of microvessels, edema of the perivascular glia, and endothelial cells (Vlasov et al. 2005).

The role of oxidative stress during the reperfusion is associated with cell survival signaling, for instance, protein kinase B Akt/PKB, and cell death pathways, for instance, tumor protein p53, resulting in balancing between these two outcomes (Niizuma et al. 2009). The neuroprotective effects of antioxidant regulator and redox sensor Nrf2 have been reported in the penumbra, both in neurons and astrocytes, in association with neuronal ischemia-reperfusion injury (Takagi et al. 2014). The protective features of Nrf2 could be applied in different stages and to various targets in brain injury (Zhang et al. 2013). Additionally, the roles of epigenetic modifications during brain ischemia should be considered as therapeutic targets (Hu et al. 2016).

Fig. 2. The participants of the ischemia-reperfusion cascade. ER = endoplasmic reticulum, NOS = nitric oxide synthase, PKC = protein kinase C. Modified after Mattson et al. 2000.
2.4 Central nervous system protection in cardiac and aortic surgery

In the surgical repair of aortic pathologies and complex cardiac surgery, the risks for neurological deficit and postoperative mortality exist despite several protective strategies. To reduce the adverse events associated with these operations, different neuroprotective means have been studied. Clinically relevant experimental models match the demand to develop protective strategies and assess their effectiveness and underlying mechanisms in these settings.

2.4.1 Clinical considerations

During the repair of complex cardiac and aortic pathologies, a bloodless operation field is required, and to meet to this demand, the cessation of the blood flow needs to be performed. Halting the blood supply results in the depletion of the oxygen and nutrient flow into the most vulnerable organs of the body. Different operative and therapeutic techniques, balancing between these two tasks, have been widely studied to deliver acceptable postoperative results.

Recent data involving a three-decade span of open TAAA surgeries has shown the rate of permanent spinal cord deficit, including paraplegia and paraparesis, to be 5.4% (Coselli et al. 2016). In contrast, the early mortality and paralysis rate in the open surgical repairs of TAAA in association with cardiopulmonary bypass and hypothermic circulatory arrest are reported to be 7.8% and 5.3%, respectively (Kouchoukos et al. 2013). The incidence of neurological complications associated with paediatric complex cardiac surgery varies between 2% to 25%, whereas the mortality rate is reported to be below 5% (Ferry 1990, Schmitt et al. 2007, Sakamoto 2016). Additionally, up to 20–30% of adult patients, who underwent surgical repair using CPB have reported experiencing temporary neurological dysfunction or neuropsychological deficits due to inadequate cerebral protection (Ergin et al. 1999, Sakamoto 2016). In summary, both improved surgical and adjunctive protective strategies need to be further studied.

Thoracoabdominal aortic aneurysm (TAAA)

The classification of TAAAs is based on the studies of 605 patients performed by Crawford and associates in 1986. This classification takes into account the
anatomic extent of aneurysm correlating to the patient outcome (Crawford et al. 1986, Safi & Miller 1999).

Type I consists of aneurysms with involvement of most of the descending thoracic and upper abdominal aorta, in between the left subclavian and the suprarenal abdominal aorta. In contrast, type II involves most of the descending thoracic aorta and most or all of the abdominal aortic aneurysms, being the most extensive type. Type III aneurysms involve the distal descending thoracic aorta and varying segments of abdominal aorta. Most or all of the abdominal aorta, including the segment from which the visceral vessels arise, are classified as type IV aneurysms (Crawford et al. 1986, Frederick & Woo 2012).

Subsequently, this classification was modified and the type V aneurysms are now included, as well. Type V is classified as aneurysms extending from the distal thoracic aorta including the celiac and superior mesenteric origins without the renal arteries (Safi & Miller 1999, Frederick & Woo 2012). Moreover, to predict the risk of paraplegia, more accurate analysis of aneurysms, involving the number and location of segmental arteries to be sacrificed, has been introduced to be utilized in surgical and endovascular repairs (Zoli et al. 2010).

In the repair of thoracoabdominal aortic aneurysms in postoperative paraplegia or paraparesis exists one of the most devastating complications both in open and endovascular procedures involving modifications in the blood supply, and thus leading to SCI (Etz et al. 2008, Etz et al. 2011). The anterior motor areas of the spinal cord, supplied by ASA, are the most vulnerable to injury in these procedures (Wynn & Acher 2014). Additionally, types I and II, being the most extensive aneurysms and thus interrupting the segmental arteries the most, involve the highest risk for postoperative neurological deficit (Zoli et al. 2010, Wynn & Acher 2014). Along with the extent of aneurysm, the presence of dissection and emergent presentation are factors especially associated with the risk of paralysis in reported series (Crawford et al. 1986, LeMaire et al. 2003, Zoli et al. 2010, Wynn & Acher 2014).

Paraplegia or paraparesis can occur in acute or delayed fashion (Etz et al. 2008, Etz et al. 2011). In the open repair of TAAAs proximal aortic clamping decreases the spinal cord blood supply, consequently increases CVP and CSFP, and thus leads to inadequate tissue oxygen delivery and ischemia (Marini et al. 1998, Wynn & Acher 2014). In delayed paraplegia, the occurrence results from inadequate adaptation to changed anatomical and physiological features or inadequate postoperative haemodynamic management (Etz et al. 2008).
The maintenance of SCPP above 50–60 mmHg results in the avoidance of the adverse event of spinal cord injury. The difficulty of direct SCPP measurements has established approaches in assessing SCPP and resulted in experimental settings, and findings of positive correlation between spinal cord blood flow, SCPP, and mean systemic blood pressure (Kise et al. 2015).

In summary, the duration and degree of ischemia, and the failure to achieve adequate blood flow postoperatively, along with reperfusion injury, are the components associated with spinal cord injury in TAAA surgery (Oyar et al. 2008). The critical time frame of 30 minutes is associated with increased risk for neurological complications, and thus spinal cord ischemia due to cross-clamping during the TAAA repairs (Safi & Miller 1999, Gloviczki 2002).

2.4.2 Hypothermia

Therapeutic hypothermia is considered one of the most important neuroprotective methods. The beneficial usage of hypothermia is due to its various blocking effects in the ischemia cascade, including metabolic pathways, inflammation reactions, and apoptotic events, together with many other pathways (Ji et al. 2007, Dietrich et al. 2009, González-Ibarra et al. 2011). Moreover, the stability of the BBB and inhibiting glutamate release are the beneficial actions of hypothermia (Zhao et al. 2004). However, the glutamate effects remain controversial in association with hypothermia (Dietrich et al. 2009). In spite of the definite advantages of hypothermia in the future, the combined therapies of both hypothermia and synergistic methods need to be studied in reducing adverse neurological outcomes (González-Ibarra et al. 2011). Next, the clinical applications of hypothermia are described in association with aortic surgery.

Protection of the spinal cord

In the surgical repair of thoracoabdominal aortic aneurysms, therapeutic hypothermia can be achieved and delivered in both a systemic and a local manner. The most widely used method, mild to moderate systemic hypothermia (30–34°C), achieved mainly via cardiopulmonary bypass during distal perfusion, has shown beneficial results with reduced paralysis rate (Safi et al. 2003, Jacobs et al. 2006, Acher et al. 2008, Dietrich et al. 2009, Etz et al. 2010b, Conrad et al. 2011, Wong et al. 2011, Lima et al. 2012, Wynn & Acher 2014). The other way to achieve systemic hypothermia and target even lower temperatures is via CPB and the
hypothermic circulatory arrest technique utilized in open TAAA repairs (Etz et al. 2010b, Kouchoukos et al. 2013). Moreover, local spinal cord cooling performed with 4°C saline infusion to the epidural space before aortic clamping, has shown reduced paralysis rates and improved safety in reported series (Cambria et al. 2000, Conrad et al. 2007, Dietrich et al. 2009, Tabayashi et al. 2010).

Protection of the brain

In the repair of aortic arch pathologies and complex congenital heart defects, the usage of therapeutic hypothermia is widely applied due to its cardio- and neuroprotective properties (Schmitt et al. 2007). It is applied systemically while using HCA together with combined surgical strategies. Moreover, topical head cooling with ice packs has been reported to have beneficial effects in brain protection, and has consequently been applied in clinical settings (Etz et al. 2010b).

2.4.3 Surgical strategies

Several operation techniques are involved in the repair of thoracic and thoracoabdominal aortic pathologies together with cardiac operations. The choice of the technique results in the need to account for different aspects; in some cases, there are clearly centre-preferred techniques along with surgeon-related preferences. The choice of the technique highly depends on the nature of the treated condition, aiming to have the surgical strategy be individually tailored to suit the optimal treatment in each situation.

The shift towards mini-invasive techniques including different endovascular procedures has also widened the broad field. Moreover, the combination of both surgical and endovascular techniques, also known as a hybrid technique, has doubled the variation, especially in high-risk patients.

Protection of the spinal cord

Several surgical strategies can be applied in the repair of TAAAs. The paralysis risk is quite similar in all surgical techniques when adjunctive strategies, focusing on increasing oxygen delivery and prolonging ischemic tolerance, are used (Wynn & Acher 2014).

Cross-clamp. In the cross-clamping technique, the aorta is clamped at both proximal and distal sites of the aneurysm. This approach results in the distal aortic
pressure decreasing, consequently leading to the reduction in spinal artery perfusion pressure and to the increase in cerebrospinal fluid pressure. These actions result in diminished spinal cord blood flow (Svensson et al. 1993b, Safi & Miller 1999). In TAAA repairs, the era of this technique lasted during the 1980s and was associated with risk factors such as aortic cross-clamp time and extent I TAAA (Safi et al. 2003).

Assisted circulation. To increase distal aortic pressure and thus to produce adequate blood flow during aortic cross-clamping, the strategy of assisted circulation was established via left atrial or left pulmonary veins (outflow) to left femoral artery or distal descending aorta (inflow) bypass (Safi & Miller 1999, Gloviczki 2002, Wong et al. 2011). In addition to distal perfusion the preload and afterload can be reduced in patients with poor left ventricular function along with the minimization of renal and visceral ischemia (Wong et al. 2011, Conrad et al. 2011, Schepens 2016). The indications for left heart bypass (LHB) usage involve open repairs of descending thoracic and thoracoabdominal aorta, when the total clamping time is assumed to exceed 20 minutes (Schepens 2016). In reported series, mortality and morbidity rates have been reduced with the usage of LHB (Schepens et al. 1999 & 2009). Moreover, the increased aortic cross-clamp time during LHB has no adverse effects on the incidence of paralysis (Schepens et al. 1999).

In TAAA repairs, the hypothermic circulatory arrest (HCA) technique is applied in particular when the extent of aneurysms involves the distal aortic arch and the placement of proximal clamp is not optimal or the problems related to right lung ventilation require the cessation of the blood supply (Schepens et al. 2009, Lima et al. 2012, Wynn & Acher 2014). The usage of HCA is restricted since its application is associated with increased risks for mortality and major pulmonary, renal and bleeding complications, in open TAAA surgery (Safi et al. 1998, Tabayashi et al. 2009, Conrad et al. 2011, Lima et al. 2012). However, some high-volume centres apply HCA in all TAAA repairs, with acceptable results (Kouchoukos et al. 2013). The HCA technique is discussed in detail in the next section.

The shift towards the mini-invasive direction and the demand to treat high-risk patients have introduced the thoracic endovascular aneurysm repair (TEVAR) technique. However, the paralysis risk still exists in this technique covering intercostal arteries, and consequently leading to inadequate collateral circulation (Acher & Wynn 2009, Etz et al. 2010b, Rossi et al. 2015). The hybrid technique represents the newest technique in the field of TAAA repairs using both open and endovascular approaches (Conrad et al. 2007, Coselli et al. 2007, Frederick & Woo
Cerebral neuroprotection

In the 1950s, the invention of cardiopulmonary bypass by Dr. John Gibbon enabled the performance of complex cardiac and aortic repairs requiring the substitution of the functions of the heart and the lungs during the operations (Gibbon 1954, Cohn 2003). In addition to this, during the following years, the improved technique employing a bubble oxygenator and roller pump enhanced the usage of CPB (Black & Bolman 2006). Next, the neuroprotective effects of hypothermia were quickly combined with the CPB, and the technique of hypothermic circulatory arrest was first established in experimental studies (Niazi & Lewis 1957).

Generally, the extracorporeal machine CPB is utilized via cannulas. The venous cannula receives and directs the deoxygenated blood from the body to the reservoir, from where the blood is directed to oxygenation via the roller pump. The pump functions as a heart, directing the oxygenated blood to the circulation via aortic cannula. The heat exchanger is required during the cooling and warming periods to achieve the target temperatures. Under the target temperature, the heart and the circulation are halted to achieve the hypothermic circulatory arrest.

Currently, total hypothermic circulatory arrest alone or in combination with adjunctive selective cerebral perfusion strategies including antegrade cerebral perfusion (ACP) and retrograde cerebral perfusion (RCP) form the selection of the surgical strategies in cerebral protection in aortic arch and cardiac surgery (Di Eusanio et al. 2003, Gega et al. 2007, Habertheuer et al. 2015). Moreover, in different surgical situations, the selection can be extended, for instance regarding the low-flow perfusion in the congenital heart defect repairs (Sakamoto 2016). Each cerebral protection technique has its advantages and limitations.

In the mid-1970s, HCA was refined and introduced into clinical settings, in the prosthetic replacement of the aortic arch, by Griep and associates. It was shown that total body hypothermia and circulatory arrest could be carried out with an acceptable mortality rate. (Griep et al. 1975). Since then, HCA has established its usage alone or in a combination of other techniques while managing different

HCA can be achieved in different temperature settings. Recently, in order to assess the impacts of temperature on operative outcomes, and to facilitate better reporting and therefore more thorough understanding of HCA, as well as developing more effective surgical procedures together with guiding patient selection and good clinical practice, a clear consensus of various categories of hypothermia has been reached (Yan et al. 2013). The classification of hypothermia is based on the belief that brain metabolism is the key determinant of beneficial arrest temperature (Kamiya et al. 2007, Yan et al. 2013). Hypothermic temperatures are classified as mild (28–34°C), moderate (20–28°C), deep (14–20°C), and profound (< 14°C) in aortic arch surgery (Yan et al. 2013).

The main debate concerning the HCA has always been the safe duration within it should be performed. McCullough and associates predicted the safety duration of HCA to be 31 minutes at 15°C based on the cerebral metabolic rate for oxygen (CMRO₂) via the calculations utilizing the clinical data of cerebral blood flow and metabolism (McCullough et al. 1999). Their results were in line with the first findings of combined experimental and clinical data supporting the evidence: approximately 36–40 minutes of DHCA to be safe, after which exponential neuron loss would occur (Treasure et al. 1984). A more strict time window of the safety period of DHCA, of 20–25 minutes, has also been suggested, as longer periods have been shown to result in postoperative adverse outcomes and poor quality of life (Ergin et al. 1999, Di Eusanio et al. 2003, Immer et al. 2004). In contrast, a recently published study from an experienced centre has suggested the safety period of DHCA to be extended to 50 minutes instead of the approved 40 minutes, or at least giving a 10-minute extra period in complex procedures utilizing DHCA (Ziganshin et al. 2014). All in all, the general consensus lies in the period of 30–40 minutes of DHCA to be safe, after which adjunctive protective actions need to be considered. Preoperatively, when the circulatory arrest period is assumed to exceed 30 minutes, ACP is usually involved in the operations (Angeloni et al. 2015).
Table 1. Calculated safe duration of HCA (McCullough et al. 1999).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>CMRO₂ (% of baseline, 37°C)</th>
<th>Safe HCA (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>56 (52–60)</td>
<td>9 (8–10)</td>
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<tr>
<td>25</td>
<td>37 (33–42)</td>
<td>14 (12–15)</td>
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<tr>
<td>20</td>
<td>24 (21–29)</td>
<td>21 (17–24)</td>
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<td>15</td>
<td>16 (13–20)</td>
<td>31 (25–38)</td>
</tr>
<tr>
<td>10</td>
<td>11 (8–14)</td>
<td>45 (36–62)</td>
</tr>
</tbody>
</table>

CMRO₂ = cerebral metabolic rate, HCA = hypothermic circulatory arrest. Values are presented as median and 95% confidence intervals.

The main benefits of and evidence for the use of DHCA alone are explained by a clear, bloodless operation field without extra perfusion cannulas and clamps, and thus simple and quick establishment of the setting along with neither manipulation of the head vessels nor occurrence of brain edema. Consequently, DHCA has established its position especially in the acute and straightforward surgical repairs of aortic arch pathologies (Di Eusanio et al. 2003, Ziganshin et al. 2014). The most significant limitations of this technique include the limited safety period as discussed above together with inflammation and coagulation problems associated with low temperatures at which the total HCA needs to be performed (Kamiya et al. 2007). The coagulation problems, requiring extra blood and plasma transfusions, might, however, be related more to the prolonged total CPB time, i.e. extra cooling and rewarming needed to achieve the target temperatures instead of the depth of the hypothermia.

The adjunctive cerebral perfusion strategies antegrade cerebral perfusion and retrograde cerebral perfusion, needed in more complex procedures of the aortic arch, are discussed next. The knowledge of selective brain cooling has been established via experimental models (Misko 1965). ACP is a technique in which the blood supply to the brain is secured throughout the experiment. This setup can be performed via various sites, e.g. axillary arteries, femoral arteries, or innominate arteries. In addition, this strategy can be achieved both unilaterally and bilaterally with similar outcomes in both setups. However, when the arrest time is prolonged, the bilateral ACP is considered to be safe. Recent results of the meta-analysis support the combined usage of ACP and hypothermic circulatory arrest in ascending aorta or aortic arch surgery and along with moderate hypothermia (> 24°C) in association with best outcomes. (Angeloni et al. 2015, Leshnower et al. 2015). The advantages of this technique involve longer safety periods of lower body circulatory arrest in combination with possible reduced adverse neurological effects and better quality

RCP utilizes the venous system, directing the blood to the brain through the veins during the operations, and thus being to some extent unphysiological. The advantages of RCP consist of the flush of toxic metabolic products and embolic debris from the cerebral vascular system (Juvonen et al. 1998). The benefits are associated with improved brain cooling, as well (Anttila et al. 2000). The dilemma lies in the oxygen and nutrient amount that actually reaches the neural tissue being low and therefore partially insufficient; consequently, the usage of this technique is less popular (Reich et al. 2001, Gega et al. 2007, Ziganshin et al. 2014). Moreover, worse neurological outcome and cerebral edema are actions associated with RCP (Reich et al. 2001, Gega et al. 2007).

2.4.4 Evoked potential monitoring

Intraoperative evoked potential monitoring provides precise measurements of transmission of both motor-evoked (MEP) and somatosensory-evoked (SEP) potentials together with reflex responses and muscle activation. The measurements are based on the stimulation of a specific point, transmission of the evoked potential through the injured area, and impulse recording on the other point. The combined usage of monitoring both potentials provides information about injured and regenerating neuronal pathways (Silva et al. 2014, Shils & Sloan 2015).

Electrophysiology monitoring has been shown to be reliable in assessing spinal cord function, determining the integrity of the motor and sensory pathways, detecting intraoperative stress or damage, and consequently identifying evolving iatrogenic spinal cord injury during complex surgery (Jacobs et al. 2006, Costa et al. 2007, Silva et al. 2014, Stoiccia et al. 2016). In open TAAA repairs, evoked potential monitoring is involved, especially in type II aneurysms (Frederick & Woo 2012). Additionally, evoked potential monitoring is useful in assessing experimental therapies for spinal cord injuries (Gaviria et al. 2000, Imaizumi et al. 2000, Bradbury et al. 2002, Cramer et al. 2005, Silva et al. 2014).

The usage of this technique is limited due to the need for specialized devices and due to its invasiveness and painfullness often requiring general anaesthesia (Jacobs & Mess 2003, Silva et al. 2014). Besides, the anaesthesia conditions need to be carefully planned, since evoked potentials are susceptible to various anaesthesia agents including many volatile anaesthetics (MacDonald et al. 2013, Shils & Sloan 2015). Propofol-based total intravenous anaesthesia and opioid-
based anaesthesia are considered as some of the optimal anaesthetics (Agarwal et al. 1998, Stoicea et al. 2016). Additionally, the beneficial effects of ketamine on evoked potential monitoring are known, though the adverse side effects need to be considered (Stoicea et al. 2016).

**Motor-evoked potentials (MEPs)**

Motor-evoked potentials (MEPs) can be recorded intraspinally or at lower extremity muscles after receiving stimulus from the sensorimotor cortex (Zileli & Schramm 1991, Costa et al. 2007, Silva et al. 2014). The combined usage of epidural and muscle MEPs is thought to be the most reliable way to assess the spinal cord (Costa et al. 2007). MEPs focus on the descending pathways, for instance, reticulospinal and corticospinal tracts (García-Alías et al. 2006).

MEP is performed via repetitive stimulation to achieve reliable recordings. The potentials are named as direct D-waves, with origin from stimulated pyramidal cells of the motor cortex, and as indirect I-waves, with origin from directly activated motor tract or involvement of activated interneurons, and compounded as motor-evoked potential at the level of the alpha motoneuron in the spinal cord when stimulated cortically (Jacobs & Mess 2003). In conclusion, MEP responses require summation at the motoneuron pool (Roy et al. 2011). The amplitude height of MEP reflects the number of intact alpha motoneurons. Mean arterial distal aortic pressure of 60 mmHg is a limit for 75% of the patients to achieve adequate MEP levels, whereas the remaining 25% of patients require pressures of above 70 mmHg (Jacobs & Mess 2003).

In clinical settings, MEPs are useful in assessing critical intercostal arteries causing spinal cord ischemia in TAAA repairs and assessing intraoperative spinal cord function to guide surgical and haemodynamic strategies during operations (Jacobs & Mess 2003, Safi et al. 2003, Etz et al. 2006, Jacobs et al. 2006, Estrera et al. 2010, Greiner et al. 2012, Boezeman et al. 2015). The reductions of amplitude and changes of latency of motor cortical potentials are associated with spinal cord injury (Cramer et al. 2005). In incomplete spinal cord injury, MEPs are reduced, shown by amplitude decreases of the descending I waves (Roy et al. 2011). The decrease of > 50–75% of MEP baseline amplitude values is thought to signify critical spinal cord ischemia and require further actions to avoid irreversible loss of motor function (Etz et al. 2006, Jacobs et al. 2006). Additionally, a change of less than 10% in latency is considered to reflect stable MEP recordings (Park & Hyun 2015).
Somatosensory-evoked potentials (SEPs)

Somatosensory-evoked potentials (SEPs) focus on the ascending sensory spinal cord tracts in the posterior column (Etz et al. 2006, Costa et al. 2007). These potentials are measured after stimulation of large peripheral nerves mediated over the sensory cortex or the spinal cord (Zileli & Schramm 1991, Silva et al. 2014, Park & Hyun 2015). Generally, a decrease of 50% in cortical SEP amplitude and an increase of 10% in latencies are considered as warning criteria (Etz et al. 2006, Costa et al. 2007, Park & Hyun 2015).

The disadvantage of SEP monitoring is its sensitivity to sources of error and delayed information, and thus the MEPs have instead been established to have more extensive usage in clinical settings (Jacobs & Mess 2003, Costa et al. 2007). However, SEP enables postoperative recordings in detecting spinal cord dysfunction, while MEP monitoring is not suitable (Etz et al. 2006). The motor pathways can be damaged independently of sensory tracts, and since initially the spinal cord ischemia harms mainly the alpha motoneurons of the anterior horn, the usage of MEP recordings is justified (Jacobs & Mess 2003, Costa et al. 2007, Wynn & Acher 2014).

2.4.5 Intraoperative & adjunctive strategies

Several adjunctive protection strategies have been studied both in spinal cord and in brain protection over the years. Some strategies are widely recognized and have established their usage in surgical repairs. However, centre-preferred choices still exist, as well.

Protection of the spinal cord

In reducing paralysis risk several adjunctive protection strategies have been studied and utilized. In general, spinal cord protection focuses on the physiology, indeed on the pathophysiology, of spinal cord ischemia and infarction, in changed anatomic conditions due to TAAAs repair. Oxygen saturation, haemoglobin concentration, and cardiac index (CI) are the physiologic factors determining tissue oxygen delivery, and thus careful optimization of these parameters together with protection against ischemia-reperfusion injury, prolonging ischemic tolerance and reducing spinal cord oxygen demand, are crucial in association with spinal cord protection intra- and postoperatively (Wynn & Acher 2014).
Cerebrospinal fluid drainage (CSFD) reduces the spinal fluid pressure, consequently improving spinal cord perfusion pressure and blood flow, leading to improved neurological outcome after aortic occlusion, which was first discovered in experimental models (Svensson et al. 1998, Safi & Miller 1999, Wynn & Acher 2014). Initially, clinical studies by Crawford did not establish its position during open TAAA repairs. Subsequently, this study has been criticized due to several factors concerning the drainage setup (Crawford et al. 1991, Svensson et al. 1998, Coselli et al. 2002, Wynn & Acher 2014). Thereafter, the clinical usage of CSFD in TAAA repairs was popularized by two different studies by Svensson and Coselli (Svensson et al. 1998, Coselli et al. 2002). Currently, the type I and II aneurysm repairs include routine drainage; the level below 10 mmHg is considered to be useful (Safi & Miller 1999, Frederick & Woo 2012). This adjunctive has been shown to be the most effective and reliable in reducing spinal cord ischemia over the years alone and in combination with other methods (Svensson et al. 1998, Acher & Wynn 2012). It is noteworthy that adverse complications are associated with this technique, intracranial bleeding being one of the most serious (Youngblood et al. 2013).

Maintaining and increasing mean arterial pressure in TAAA surgery determines the spinal cord perfusion (Acher et al. 2008). Extensive collateral network pressure studies by Etz and associates have revealed its behaviour and dependency in association with adequate MAP during and after TAAA surgery (Etz et al. 2010a). Postoperative MAP controlling is crucial to avoid hypotension, and thus the occurrence of delayed paralysis (Acher et al. 2008, Etz et al. 2010a). Moreover, the relationship between MAP fluctuations and intraoperative MEP recordings has been reported, as mentioned in the evoked potential section (Jacobs & Mess 2003). Sufficient CI also has effects on collateral network perfusion during and after surgery (Acher et al. 1998 & 2008).

Reimplantation of intercostal arteries is one method of spinal cord protection, since intercostal blood flow is estimated to account for 20% of paraplegia risk. In contrast, collateral blood flow and metabolism are suggested to determine 80% of this risk. The reimplantation can be performed selectively or nonselectively (Acher et al. 2008). Initially, the reimplantation of the artery of Adamkievicz was established to be critical. To study this critical intercostal artery, Etz, Griepp, and associates have performed sequential intercostal artery sacrifice, providing more evidence of collateral network circulation and network pressure instead of a single critical artery. They have also suggested that routine implantation of segmental arteries is not indicated (Etz et al. 2006). On the other hand, especially the most
extensive aneurysms have been shown to benefit and reduce paralysis with nonselective reimplantation of the intercostal arteries. These results support the general concept of a collateral network being a dynamically functioning structure while receiving blood from one source and redistributing it via interconnections to increase spinal cord blood flow to a sufficient level. However, it is necessary to mention the risks: the technical challenges due to small and atherosclerotic intercostal arteries, and increased aortic occlusion time and blood loss in association with reimplantation (Acher et al. 2008). In summary, the protection strategies that have been reviewed to this point all aim to increase spinal cord perfusion (Wynn & Acher 2014).

Additionally, several pharmacological therapies are used in TAAA repairs as adjuncts to other therapies. The evidence of the favourable effects results from experimental investigations, since randomized clinical trials are lacking. The beneficial effects of drug therapy are shown in increasing spinal cord blood flow, decreasing metabolic rate and oxygen demand, scavenging free radicals, decreasing inflammation, and the release of excitatory neurotransmitters, stabilizing cell membranes along with mitigating reperfusion injury (Hall & Braughler 1982, Mabon et al. 2000, Lima et al. 2012, Wynn & Acher 2014). The studied pharmacotherapies comprise intrathecal papaverine, barbiturates, corticosteroids, naloxone, and mannitol (Svensson et al. 1998, Lima et al. 2012, Wynn & Acher 2014).

**Cerebral protection**

Several adjunctive protection strategies are considered and involved when optimal cerebral protection is required and the usage of CPB is applied. Hematocrit and pH strategy represent the key elements to be handled during cardiopulmonary bypass.

**Hematocrit.** In experimental studies, the improved neurologic outcome after DHCA was associated with a high level of hematocrit by Shin’oka and associates, indicating inadequate oxygen delivery due to haemodilution, and thus anemia in lower hematocrit values (Shin’oka et al. 1996). Before this study, haemodilution during hypothermic CPB had been accepted for an extended period (Shin’oka et al. 1996, Sakamoto 2016).

To underline experimental setups in clinical studies, the improved developmental outcomes are associated with hematocrit higher than 25% (Jonas et al. 2003, Sakamoto et al. 2016). In contrast, in infants undergoing cardiac surgery, with hematocrit levels of 35% compared with those of 25%, they do not have major
benefits or risks (Newburger et al. 2008). In the aspect of neuroprotection, hematocrit less than 15–20% is considered to be deleterious, and thus increasing it sufficiently is beneficial to avoid haemodilutional anemia (Miura et al. 2007, Ranucci et al. 2015). To reach adequate hematocrit level, blood transfusions are needed. Consequently, these actions lead to a systemic inflammatory response (Sakamoto 2016). Overall, balancing between sufficient hematocrit and minimized blood transfusions requires careful decisions in clinical settings.

pH. In hypothermia, due to the interrelation between \( \text{paCO}_2 \) and pH, their values are dependent on the chosen strategy for the maintenance of acid-balance (Swan 1982 & 1984, White & Somero 1982, Erecinska et al. 2003). The acid-balance strategies consist of \( \alpha \)-stat and pH-stat, and are required when body temperature is lowered by more than 3°C to 5°C (Erecinska et al. 2003).

In the \( \alpha \)-stat strategy, the \( \text{paCO}_2 \) is balanced in a manner to reach arterial pH of 7.35 to 7.45 when measured at 37°C. The blood is then hypocapnic (\( \text{paCO}_2 < 5.92 \) kPa) and alkalotic (\( \text{pH} > 7.40 \)) during hypothermia (Kelman 1966, Severinghaus 1966, Thomas 1972, Priestley et al. 2001). In the pH-stat strategy, the arterial pH is maintained at 7.40 regardless of the hypothermic temperature. To reach this target, during cooling the addition of \( \text{CO}_2 \) to the inspired gas is used to achieve \( \text{paCO}_2 \) of 5.26 kPa corrected to the core temperature. In normothermia, the blood is acidic (\( \text{pH} < 7.35 \) to 7.40) and hypercapnic (\( \text{paCO}_2 > 5.26 \) kPa) (Swan 1982 & 1984, White & Somero 1982, Priestley et al. 2001, Erecinska et al. 2003).

During hypothermic CPB, the optimal choice of the acid-balance strategy has been controversial and debated for a long time (Priestley et al. 2001, Sakamoto 2016). Overall, and especially in paediatric cardiac surgery the trend is towards the pH-stat strategy with beneficial neurological and neuropsychological effects both in experimental and clinical studies (Pua & Bissonette 1998, Kurth et al. 1998, Priestley et al. 2001, Sakamoto 2016). The increase of \( \text{paCO}_2 \) in the pH-stat strategy results in better cerebral blood flow vasodilatating arteries and thus possible increasing oxygen delivery and brain cooling effect, which is protective in children (Kety & Schmidt 1948, Kurth et al. 1998, Priestley et al. 2001, Sakamoto 2016). However, there are risks for microembolism and free radical damage associated with the pH-stat strategy along with the loss of autoregulation (Stephan et al. 1992, Kurth et al. 1998, Priestley et al. 2001).

The advantages of the \( \alpha \)-stat strategy are preserved autoregulation and optimized cellular enzyme activity, but less metabolic suppression is achieved (Stephan et al. 1992, Priestley et al. 2001). This strategy maintains its position in adult cardiac surgery with improved neurological outcome (Stephan et al. 1992, Sakamoto 2016).
Pua & Bissonette 1998). The improved CBF associated with the pH-stat strategy may result in increased embolic load in adult patients due to atheromatous debris in their vascular system (Sakamoto 2016). Consequently, the combined usage of both strategies is used.

Over the years, the improved bypass circuit and oxygenator have decreased the inflammatory response. Moreover, different anti-inflammatory strategies including biocompatibility, leukocyte filter and minimal extracorporeal circulation have been studied in CPB settings (Sakamoto 2016).

2.5 Remote ischemic preconditioning

Ischemic preconditioning was first introduced by Murry et al. in 1986 in a canine model, with an intermittent occlusion of the circumflex branch of the left coronary artery (LCX) prior to the prolonged occlusion of the same artery, limiting myocardial infarct size to 25% of that detected in the control group (Murry et al. 1986).

This preconditioning method, exposing target tissue to ischemic stimuli and providing protection against subsequent more severe insult, was later found to be similarly effective when applied at a distance along with another nontarget tissue (Przyklenk et al. 1993, Gho et al. 1996). A year later, researchers performed studies that confirmed the protective effects of ischemic preconditioning at a distance combined with electrical stimulation in cardioprotection (Birnbaum et al. 1997). In 2002, Kharbanda et al. further confirmed the beneficial cardioprotective effects of remote ischemic preconditioning (RIPC) with the use of hindlimb skeletal muscle as the ischemic stimuli enhancing the clinical usage of this method (Kharbanda et al. 2002).

The methods to apply RIPC vary; the choices consist of bilateral or unilateral upper or lower limb ischemia performed by tourniquets or direct occlusion of the artery. The larger tissue volume of the lower limb has been thought to cause a greater biological effect induced by RIPC, and is additionally more practical. The optimal time period of the ischemia and repetition of the cycles remain unclear. The low-cost, safe, easily applicable and noninvasive features of remote ischemic preconditioning have enabled its transfer to clinical settings, as well (Koch et al. 2011).

The first clinical study of RIPC consisted of pediatric patients who underwent surgical repair of congenital heart defects with the usage of CPB, presenting reduced damage in the heart by lower cardiac enzyme release (Cheung et al. 2006).
Adult cardiac and aortic surgery, for instance in coronary revascularization and elective abdominal aortic aneurysm repairs along with angioplasty due to acute myocardial infarction, has been studied in association with RIPC with beneficial results (Ali et al. 2007, Hausenloy et al. 2007, Bøtker et al. 2010, Thielmann et al. 2013).

However, recently published results of two large, multicentre, randomized, controlled trials, ERICCA and RIPheart, failed to support these previous results suggesting that the detection of the underlying protective mechanism requires further actions and understanding together with the consideration of the suitable patients and clinical settings to be performed in both adult and paediatric cardiac surgery (McCrindle et al. 2014, Hausenloy et al. 2015, Meybohm et al. 2015). Moreover, the choice of anaesthetics in experimental and clinical settings needs to be carefully considered together with evaluating the results of studies with different anaesthetic protocols. For instance, propofol is known to attenuate the effects of RIPC, but it was nevertheless the anaesthetic employed in the aforementioned large controlled trials recently published reporting neutral effects of RIPC (Kottenberg et al. 2012, Kottenberg et al. 2014, Meybohm et al. 2015, Hausenloy et al. 2015).

2.5.1 RIPC in neuroprotection

In the CNS, ischemic tolerance was first found in the brain using a gerbil model by Kitagawa and colleagues (Kitagawa et al. 1990). Subsequently, the concept was extended to the spinal cord. In 1997, Matsuyama and associates directly preconditioned the spinal cord by cross-clamping the aorta prior to a prolonged 60-minute cross-clamping, with protective results against postoperative paraplegia in a canine model (Matsuyama et al. 1997). Thereafter, these results have been confirmed via other spinal cord studies with different species and different time windows of preconditioning and ischemic insult (Abraham et al. 2000, Tompoulis et al. 2003).

The neuroprotective effects of RIPC were first found by Dave and colleagues using a rat model. In these experiments, global brain ischemia was achieved via cardiac arrest. (Dave et al. 2006). A year prior to that study, Vlasov and associates demonstrated beneficial delayed effects of RIPC in reducing cerebral edema in their global brain ischemia model (Vlasov et al. 2005). At the same time, the protective role of RIPC was demonstrated in experimental spinal cord studies, as well (Gurcun et al. 2006). In clinical settings, for instance in cervical decompressive surgery, the beneficial effects of RIPC on CNS protection were demonstrated (Hu et al. 2010).
A recent report of enhanced motor learning by RIPC has suggested its beneficial role in neuroplasticity and rehabilitation, as well (Cherry-Allen et al. 2015). The following sections focus in detail on the potential underlying mechanisms behind RIPC to broaden the views of this multilevel phenomenon.

### 2.5.2 Mechanisms of RIPC

The protective effects of RIPC are well documented in cardioprotection. However, the underlying molecular mechanisms involved in this protection remain largely unclear. At present, there are three theories: the neural, the humoral, and the systemic (Gill et al. 2015, Meller & Simon 2015).

The ischemic tolerance in the brain occurs in rapid and delayed phases, and the mechanisms differ significantly, similar to two staged cardioprotective effects of RIPC (Meller 2009). In the rapid phase, lasting 30–60 minutes, the protection is mediated by intracellular signaling cascades along with post-translational modifications. In contrast, in the delayed phase, lasting 24–72 hours, the synthesis of new proteins is required. The stimulus for the synthesis and protective actions possible results quickly from the intracellular changes occurring in the rapid phase. (Barone et al. 1998, Meller et al. 2006, Meller 2009).
Fig. 3. Mechanisms involved in RI PC response. AKT = Protein kinase B, CGRP = Calcitonin gene-related peptide, ERK = Extracellular signal-regulated kinases, K\text{ATP} = ATP-sensitive potassium channel, MPTP = Mitochondrial permeability transition pore, NO = Nitric oxide, PKC = Protein kinase C, RI PC = Remote ischemic preconditioning, ROS = Reactive oxygen species. Modified after Hausenloy 2013.
2.5.3 Neural factors of RIPC

Already in 1996, Gho and associates had demonstrated the involvement of a neural component in RIPC. They showed that the cardioprotective effect of RIPC was abolished when a ganglion blocker, hexamethonium, was administered after mesenterial artery occlusion. (Gho et al. 1996). Moreover, the neuronally mediated protection occurs both in rapid and delayed phases (Loukogeorgakis et al. 2005). The involvement of neural mechanisms partially mediating RIPC has been reported in studies resecting different nerves and consequently preventing cardioprotective actions, suggesting, for instance, adenosine to stimulate this neural activation (Lim et al. 2010). Moreover, the role of the autonomic, especially the parasympathetic, nervous system has been reported to take part in RIPC along with the neural afferent pathway (Donato et al. 2013).

In brain protection, the neural component involved in RIPC has been reported in several studies; for instance, a blockade with capsaicin or hexamethonium on the spinal processing of sensory inputs reduced the beneficial effects of RIPC (Malhotra et al. 2011, Wei et al. 2012). The main brain structure mediating this event has not been concluded, and thus the research addressing this subject remains justified (Meller & Simon 2015). In contrast, spinal cord studies have suggested that the neural signal pathway might not be included in the induction of ischemic tolerance by RIPC (Dong et al. 2010).

2.5.4 Humoral factors in RIPC

Additionally, Gho and associates were one of the first to show that the RIPC stimulus requires a reperfusion period to be protective, and thus the research of humoral factor(s) has been established (Gho et al. 1996). Therefore, the preclinical data supports the concept of RIPC-induced factors entering into the bloodstream, which mediate the production from the transiently ischemic limb to the remote organs (Konstantinov et al. 2005, Shimizu et al. 2009, Hepponstall et al. 2012, Hibert et al. 2013 & 2014). Additionally, the preconditioning protection has been shown to be transferrable with the plasma, even in cases of cross-species (Dickson et al. 1999, Shimizu et al. 2009, Skyschally et al. 2015).

The protection appears to be mediated via small, unknown hydrophobic factors less than 15–30 kDa (Shimizu et al. 2009, Breivik et al. 2011, Jean-St-Michel et al. 2011, Hepponstall et al. 2012, Hibert et al. 2014). The suggested factor might be a protein, a micro-RNA or an exosome carrying either proteins or micro-RNAs. On
the other hand, there might be a resident cell or cellular compartment releasing a paracrine factor mediating the RIPC protection (Skyschally et al. 2015). In neuroprotection, the existence of the BBB has imposed its own limits on this research of the factors. The exact identity of the factors remains unclear.

### 2.5.5 Molecular mechanisms of RIPC

The underlying mechanisms of RIPC are widely studied, especially in cardioprotection. To some extent, the mechanisms are thought to be similar in neuroprotection, as well as in local and remote preconditioning. Next, the evidence of some key factors associated with RIPC and their role in neuroprotection is discussed.

**Adenosine**

The abundant presence of adenosine, due to the breakdown of intracellular ATP, in mediating preconditioning has been studied widely in different setups (Heurteaux et al. 1995, Reshef et al. 2000, Nakamura et al. 2002, Yoshida et al. 2004, Ordonez et al. 2010, Hu et al. 2012, Meller & Simon 2015). There are several cell membrane receptors, but adenosine A1 receptor (A1R) locates extensively in the neuronal synapse (Heurteaux et al. 1995, Hu et al. 2012). A1R antagonist has been shown to block the neuroprotective effects of preconditioning, whereas A1R agonist has been shown to mimic the beneficial actions both in cell and rat models (Reshef et al. 2000, Nakamura et al. 2002, Yoshida et al. 2004, Ordonez et al. 2010, Hu et al. 2012). Early phase opening of the K\textsubscript{ATP} channels has been shown to be one of the key elements mediating the adenosine-activated signal transduction pathway, and eventually controlling neurotransmitter release and Ca\textsuperscript{2+} (Heurteaux et al. 1995, Reshef et al. 2000, Nakamura et al. 2002, Yoshida et al. 2004). In addition to K\textsubscript{ATP} channels, MAPK- and PKC-activated pathways are induced via adenosine in neuroprotection (Reshef et al. 2000, Ordonez et al. 2010). Along with A1R, endocannabinoids acting through cannabinoid 1 receptors (CB1R) have established their role in RIPC spinal cord protection. The elevated endocannabinoids in association with RIPC might have been triggered due to ROS or endothelial progenitor cell mechanisms. Additionally, to speculate on the protection, CB1R might cause calcitonin gene-related peptide (CGRP) release and elevate the levels of prostaglandin I\textsubscript{2} (PGI\textsubscript{2}), inducing subsequent spinal cord ischemic tolerance. (Su et al. 2009).
ATP-activated potassium channel (K\textsubscript{ATP} channel)

In neuroprotection, adenosine triphosphate-sensitive potassium channels (K\textsubscript{ATP} channels), mainly locating in sarcolemma and mitochondria, have been detected in ischemic preconditioning due to interrelated actions between these channels and adenosine (Pérez-Pinzon & Born 1999, Reshef \textit{et al}. 2000, Horiguchi \textit{et al}. 2003). The strong evidence of K\textsubscript{ATP} channels in communicating ischemic tolerance has been reported in both rapid and delayed manners (Reshef \textit{et al}. 1998, Pérez-Pinzon & Born 1999, Horiguchi \textit{et al}. 2003, Ballanyi 2004, Huang \textit{et al}. 2005). In addition to adenosine, these actions have been shown to be mediated to some extent via PKC or p53, as well (Reshef \textit{et al}. 1998, Huang \textit{et al}. 2005). However, contradictory results of the involvement of PKC in preconditioning have been reported (Pérez-Pinzon & Born 1999). Interestingly, the studies of remote ischemic preconditioning and K\textsubscript{ATP} channels remain absent in large scale in neuroprotection (Meller & Simon 2015). Instead of this, remote postconditioning studies, applied after harmful ischemic stimulus, support the role of K\textsubscript{ATP} channels mediating the protection in an experimental cerebral model (Sun \textit{et al}. 2012). Moreover, in humans, remote ischemic pre- and postconditioning have been shown to share the same mechanisms involving K\textsubscript{ATP} channels (Loukogeorgakis \textit{et al}. 2007).

Hypoxia-inducible factor 1alpha (HIF-1\alpha)

Hypoxia-inducible factors, HIFs, are transcription factors consisting of two subunits, unstable HIFalpha (HIF\textalpha) and stable HIFbeta (HIF\textbeta) (Wang \textit{et al}. 1995). Overall, there are three HIF\textalpha subunits: HIF-1\textalpha, -2\textalpha, and -3\textalpha (Bergeron \textit{et al}. 2000). The amount of HIF accumulation depends on alpha subunit. The human Egl-nine (EGLN) 2, 1, and 3, also named as prolyl hydroxylase domain (PHD) enzymes 1–3 or hypoxia-inducible factor prolyl 4-hydroxylases (HIF-P4Hs) 1–3, sensor O\textsubscript{2} and coordinate cellular response in adaptation to hypoxia and ischemia (Metzen \textit{et al}. 2003, Myllyharju 2008). To function properly these enzymes require O\textsubscript{2}, 2-oxoglutarate, Fe\textsuperscript{2+} and ascorbate (Myllyharju 2008). EGLN1, which is specifically one of the \alpha-ketoglutarate (\alpha-KG)-dependent dioxygenases, is widely known for its role in regulating the HIF alpha subunit under normoxia (Olenchock \textit{et al}. 2016). In hypoxia, the alpha subunit is stabilized, resulting in upregulation of various hypoxia-responsive genes such as erythropoietin, glucose transporters, glycolytic enzymes, and vascular endothelial growth factor (Bergeron \textit{et al}. 2000, Klaunig \textit{et al}. 2011). The role of HIF-1\textalpha has been studied and established in hypoxic and
ischemic preconditioning of the brain, but in RIPC neuroprotection its role is partly unclear (Bergeron et al. 2000, Ruscher et al. 2002). In cardioprotection studies of rapid and delayed RIPC, the effects have been different, suggesting, that the protective role of HIF-1α lies in new protein synthesis required in delayed settings (Meller et al. 2006, Cai et al. 2013, Kalakech et al. 2013). The increased levels of interleukins such as IL-8, IL-1β, and IL-10 in possible association with HIF-1α after RIPC have demonstrated beneficial effects in cardioprotection (Albrecht et al. 2013, Cai et al. 2013). In contrast, IL-10 has shown its role in mediating neuroprotection, and thus the speculation about its possible role in RIPC-related neuroprotection is justified, as well (Cai et al. 2013).

Inflammatory factors

To study the inflammatory factors, Konstantinov and colleagues performed a clinical study suggesting the role of inflammatory signal suppression and white cell modifications associated with RIPC (Konstantinov et al. 2004). Generally, TNF-α is thought to serve as an inflammatory, cell death-promoting agent, whereas in preconditioning it possibly mediates the beneficial actions (Meller & Simon 2015). RIPC has been found to affect different pathways involving Toll-like receptor signaling, TNF-α receptor signaling, and TNF synthesis, supporting the role of TNF-α-mediated signaling in preconditioning. Moreover, in preconditioning TNF has been thought to be activated and to negatively autoregulate subsequent production of proinflammatory cytokines (Konstantinov et al. 2004). Additionally, in ischemic preconditioning of the brain, TNF-α has been shown to beneficially mediate the expression and function of different transporters and enzymes (Pradillo et al. 2005 & 2006, Meller & Simon 2015). In contrast, reduced levels of TNF-α have been observed in RIPC-treated rats possibly due to a preservation of antioxidants induced by preconditioning (Hu et al. 2012). Moreover, the interplay and complex formation between platelets and neutrophils has been attenuated by RIPC (Kharbanda et al. 2001). In brain protection, RIPC has been shown to reduce the number of adherent leukocytes in cerebral vessels after hypothermic circulatory arrest, underlining previous results (Yannopoulos et al. 2014). Moreover, spinal cord studies of RIPC have shown attenuated recruitment of leukocytes into the inflammatory site (Gurcun et al. 2006).
**Mammalian target of rapamycin (mTOR)**

Mammalian target of rapamycin (mTOR) is a kinase that is thought to serve as a key regulator of protein synthesis and a controller of cell growth and cell survival in the nervous system (Zare et al. 2013). Antiapoptotic properties having AKT, contributing to neuroprotection, have been shown to activate mTOR (Zare et al. 2013, Meller & Simon 2015). The protective effects of mTOR are associated with RIPC, since its inhibitor, rapamycin, has been shown to block beneficial actions including inhibited neuron apoptosis, and increased cell density, along with improved behavioural and memory function, supporting RIPC-induced hippocampal protection (Zare et al. 2013).

**Heat shock proteins (HSPs)**

Intracellular chaperons such as heat shock proteins (HSPs) act in cellular defence mechanisms against ischemic stress injuries, supporting cellular regeneration and repair along with stabilizing the structure of intracellular proteins (Selimoglu et al. 2008). The first ischemic preconditioning studies on spinal cord have shown detectable levels of HSP, suggesting their role in neuroprotection and tolerance from ischemia (Matsuyama et al. 1997, Abraham et al. 2000). In contrast, paraplegic controls have failed to show heat shock protein reactivity (Abraham et al. 2000). In ischemic preconditioning, heart shock proteins are associated with the delayed phase of protection. Additionally, spinal cord studies with RIPC have shown the induced expression of HSP, suggesting their role in this protection cascade (Selimoglu et al. 2008).

**Nitric oxide (NO) and survival kinase signaling**

The brain protective role of nitric oxide in RIPC has been confirmed in cell culture and experimental models. The protective effects have been reduced when L-NAME, nitric oxide synthase inhibitor nitro-L-arginine methyl ester, has been administered (Gonzalez-Zulueta et al. 2000, Vlasov et al. 2005, Zhao et al. 2007). NO has shown biphasic response representing the rapid and delayed phases of protection (Vlasov et al. 2005, Zhao et al. 2007). This has been speculated to occur due to different isoforms of NOS in different phases, and interrelated regulation of these isoforms (Zhao et al. 2007). In the late phase, an increase in eNOS and concurrently an increase in prosurvival factor AKT phosphorylation have been reported (Vlasov et al. 2005, Zhao et al. 2007).
The protective actions of nNOS and of eNOS together with extracellular signal-regulated protein kinase ERK and AKT have been reported in remote postconditioning models of focal and global cerebral ischemia, as well (Peng et al. 2012, Pignataro et al. 2013). Additionally, the further signaling of NO has been demonstrated to occur via other prosurvival factors such as MAPK signal pathways (Gonzalez-Zulueta et al. 2000, Sun et al. 2006). It is noteworthy that neutral results of NO actions have been reported in RIPC spinal cord studies (Gurcun et al. 2006). Taken together, there are several kinase signaling pathways, similar but also different, associated with NO mediating the protective actions of conditioning both in remote and direct, as well as pre- and postconditioning, along with focal and global ischemia models.

**Low levels of ROS**

As discussed earlier, large amounts of ROS are noxious, affecting different cell structures and eventually leading to apoptosis and necrosis. However, low amounts of oxygen free radicals might be sufficient to modify cellular activities and serve as part of the preconditioning mechanisms (Ambrosio et al. 1995). In the CNS, there is an antioxidant defense mechanism maintaining the redox balance (de Vries et al. 2008). The transcriptional induction via antioxidant gene regulator Nrf2 leads to activation of a number of endogenous antioxidant genes, as mentioned earlier (de Vries et al. 2008, Bell et al. 2011, Perez-Pinzon et al. 2012, Prentice et al. 2015). Moreover, the monofunctional inducers of the Nrf2-ARE pathway are able to cross the blood-brain barrier (de Vries et al. 2008). Exogenous activation, such as ischemic preconditioning, of the antioxidant response is thought to prepare and defend the cell against subsequent more severe insult. In ischemic preconditioning of the brain, mild oxidative stress may contribute to important and protective effects in the cell signaling associated with activation of beneficial pathways targeting, for instance, K<sub>ATP</sub>-channel opening in mitochondria or antioxidant response (Bell et al. 2011, Thompson et al. 2012). RIPC-induced neuroprotection has been shown to involve an antioxidant balance mechanism (Hu et al. 2012). The levels of superoxide dismutase (SOD), manganese SOD (MnSOD) have been reported to be increased after RIPC neuroprotection (Hu et al. 2012, Zare et al. 2013). In spinal cord studies, RIPC has shown reduced levels of lipid peroxidation end product malondialdehyde (MDA), indicating that free radical-mediated cellular damage was prevented (Gurcun et al. 2006). Additionally, the protective mechanism of
RIPC has been reported to be mediated via ROS-dependent pathway via increased cytoprotective antioxidant response, as well (Dong et al. 2010).

Table 2. Mechanisms involved in RIPC neuroprotection.

<table>
<thead>
<tr>
<th>Molecule/pathway</th>
<th>Presumed mechanisms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>Adenosine A1 receptor</td>
<td>Hu et al. 2012</td>
</tr>
<tr>
<td>Endocannabinoids</td>
<td>Cannabinoid 1 receptor</td>
<td>Su et al. 2009</td>
</tr>
<tr>
<td>Inflammatory agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>Decreased levels of TNF-alpha</td>
<td>Hu et al. 2012</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>Reduced leukocyte migration</td>
<td>Gurcun et al. 2006</td>
</tr>
<tr>
<td></td>
<td>Reduced adherent leukocytes</td>
<td>Yannopoulos et al. 2014</td>
</tr>
<tr>
<td>mTOR</td>
<td>Increased mTOR phosphorylation</td>
<td>Zare et al. 2013</td>
</tr>
<tr>
<td>HSP</td>
<td>Increased levels of HSP</td>
<td>Selimoglu et al. 2008</td>
</tr>
<tr>
<td>NO, NOS</td>
<td>Increased NO generation</td>
<td>Hu et al. 2012</td>
</tr>
<tr>
<td>Kinase signaling</td>
<td>Increased AKT phosphorylation</td>
<td>Vlasov et al. 2005</td>
</tr>
<tr>
<td>Low levels of ROS</td>
<td>Reduced MDA levels</td>
<td>Gurcun et al. 2006</td>
</tr>
<tr>
<td></td>
<td>Increased catalase activity</td>
<td>Dong et al. 2010</td>
</tr>
<tr>
<td></td>
<td>Increased MnSOD activity</td>
<td>Hu et al. 2012</td>
</tr>
<tr>
<td></td>
<td>Increased SOD activity</td>
<td>Dong et al. 2010, Zare et al. 2013</td>
</tr>
<tr>
<td></td>
<td>Better respiratory chain function</td>
<td>Yannopoulos et al. 2014</td>
</tr>
</tbody>
</table>

TNF-alpha = tumor necrosis factor alpha; mTOR = mammalian target of rapamycin; HSP = heat shock protein; NO = nitric oxide; NOS = nitric oxide synthase; ROS = reactive oxygen species; AKT = protein kinase B; MDA = malondialdehyde, end product of lipid peroxidation; MnSOD = manganese superoxide dismutase; SOD = superoxide dismutase.

### 2.5.6 New emerging mechanisms

In summary, the RIPC stimulus, consisting of triggers, mediators, and effectors, has evoked several studies to identify the key elements of this protective mechanism over the years. However, RIPC functions at several levels including extracellular, membrane, intracellular and in the cell components mitochondria and nucleus (Hausenloy 2013, Heusch et al. 2015). Moreover, the application of RIPC in the clinical setting has not been completely satisfying. The shift back to experimental studies focusing the research on the mechanisms is justified to deepen the understanding of this phenomenon. All in all, there is a wide cross-talk between different signaling pathways, and additionally these pathways function to some extent in different manners in different organs, challenging the understanding of the concept.
Lately, the following agents have been shown to have association with remote ischemic preconditioning or have expressed interesting characteristics and might partly explain or mediate the underlying mechanism of RIPC. In cardioprotection, connexin 43 (Cx43), extracellular vesicles, micro RNA-144 (miR-144), and micro RNA-1 (miR-1) have shown the aforementioned characteristics, and thus have been reported to have beneficial effects in association with RIPC (Brandenburger et al. 2014a & 2014b, Giricz et al. 2014, Li et al. 2014). However, it is noteworthy that controversial or pivotal reports do exist (Li et al. 2014, Gill et al. 2015).

Mitochondria

Mitochondria, critical cell organelles controlling the last phase of cell death pathways, have shown interesting features in ischemic preconditioning studies involving prevented mitochondrial swelling, preserved membrane integrity fluidity, and protected mitochondrial energy metabolism, along with the integrity of mitochondrial oxidative phosphorylation (Sisalli et al. 2015, Thompson et al. 2015). Additionally, RIPC studies have demonstrated better respiratory chain function during the recovery phase after global brain insult (Yannopoulos et al. 2014). Recently reported membrane Na⁺ - Ca²⁺ exchangers (NCXs) controlling mitochondrial calcium homeostasis have been shown to be expressed and involved in preconditioning-induced neuroprotection. Moreover, NCX1 and NCX3 have demonstrated interplay with endoplasmic reticulum in neuronal ischemic preconditioning, revealing new molecular targets in cerebroprotection (Sisalli et al. 2015).

Proteomics

Additionally, there are some proteomic studies in which RIPC has evoked a global proteomics response both in the early and late phase of RIPC (Hepponstall et al. 2012, Hibert et al. 2013 & 2014). Generally, anti- and proinflammatory apolipoproteins A-I, A-IV, and C-III; antioxidant and anti-inflammatory haptoglobin; anti-inflammatory transthyretin; and oxygen transporter haemoglobin beta chain; along with haemostatic and proinflammatory fibrinogen beta chain have been shown to be regulated via RIPC, and thus possibly contribute to the protective actions of RIPC (Hibert et al. 2013 & 2014). Moreover, the role of immune response components complement C3 and vitronectin have been speculated to be
involved in RIPC (Pang et al. 2013). However, the specific mediator of RIPC has not been identified.

**Alpha-ketoglutarate and kynurenic acid (αKG & KYNA)**

Kynurenic acid (KYNA), formed via the kynurenine pathway, has been reported to modulate excitotoxicity and neurodegeneration since it competitively inhibits the glycine-binding site of the N-methyl-D-aspartate receptor and blocks the α-7-nicotinic acetylcholine receptor noncompetitively in the brain. Glutamate, an NMDA receptor agonist, mediates the excitotoxicity and free radical formation in different neurodegenerative disorders (Zwilling et al. 2011). Reno- and neuroprotective effects of KYNA via attenuated ischemia-reperfusion injury, reduced oxidative stress, modulated excitotoxicity, neurotransmission, immune cell function, mitochondrial function, and neurodegeneration have been reported (Zwilling et al. 2011, Pundir et al. 2013).

Recently, Olenchock et al. have reported the inhibition of EGLN1, αKG-dependent dioxygenase, resulting in the systemic accumulation of its co-substrate αKG, inducing transamination of kynurenine (KYN) by kynurenine transaminases (KATs) to produce hepatic tryptophan metabolite KYNA, serving in remote induced cardioprotection also independently of HIF transcriptional activity (Olenchock et al. 2016). The roles of αKG and KYNA in association with RIPC neuroprotection form an interesting field of study.

### 2.6 Experimental models in cardiac and aortic surgery

Experimental models represent the aim to perform studies in which the precise mechanisms and the specific tissue changes need to be evaluated. Traditionally, a wide range of animal species has been used in cardiac and aortic surgery studies. Dating back to the development of the entire field of cardiac surgery, animals have played a crucial role (Niazi & Lewis 1957, Cohn 2003, Black & Bolman 2006).

#### 2.6.1 Animal species

Different species provide certain advantages and limitations. Starting from cell cultures, the results are first transferred into rodent models. Thereafter, large animal models are used providing the last step prior to clinical settings.
Rodent and rabbit models

Certain animal models, especially rabbit and rodent, including rats and gerbils, provide the opportunity to perform studies with a large number of experiments. In rodent models, the superiority of these species is due to the low cost, accessibility, and existence of analysis techniques along with the fact that one researcher can perform the studies, and thus the facilities can be maintained simply (Silva et al. 2014). However some anatomical limitations need to be considered while utilizing rodents in spinal cord injury studies. For instance, the position of the corticospinal tract fibers varies substantially between humans and rodents (Silva et al. 2014). Rabbits provide excellent cardiovascular correspondence, but some physiological characteristics including high heart rate complicate the experiments since a decrease in heart rate can modify ischemia-reperfusion injury, and thus pacing the heart is justified in some setups (Donato et al. 2013). In particular, rabbits have established their role in spinal cord studies associated with TAAAs (Savas et al. 2002, Oyar et al. 2008).

Large animal models

Large animal models in cardiac and aortic surgery involve monkeys, dogs, lambs, and pigs to mention a few. Canine models have provided beneficial usage over the decades (Black & Bolman 2006). The usage of monkeys has been limited due to ethical and economic reasons, but the studies are justified on a small scale in advance of human experiments to test safety and efficacy in SCI studies (Silva et al. 2014).

At present, pigs serve as the foundation of the large animal models used in experimental cardiac and aortic surgery. The superiority of using porcine models is due to the similar growth of the heart and cardiovascular system during the first 4–5 months together with sharing similar physiologic, anatomic, and histologic characteristics with humans. The cardiovascular models utilizing pigs involve myocardial infarction, transplantation, hypertrophy and cardiomyopathy setups, aneurysm repairs, and valvular and stent implantations, along with atherosclerosis-related therapies (Swindle et al. 2011).

The brain of the pigs is sufficiently large and its anatomical structures are similar to those of other mammals. In addition to this, the development peaks are similar to those of humans, forming features utilized in CNS studies. However, the
thickness of the skull and the bone structure of the vertebrae complicate the approach related to working with the CNS (Swindle et al. 2011).
3 Aims of the study

This thesis was completed to assess the neuroprotective effects of RIPC in simulated open thoracic aortic aneurysm repairs and HCA settings, along with the aim of studying the mechanisms underlying RIPC.

Aim I
To study whether RIPC preserves spinal cord function after the interruption of the spinal cord blood supply in a porcine model of spinal cord ischemia with intraoperative measurement of MEPs.

Aim II
To assess whether RIPC prior to spinal cord ischemia improves spinal cord protection along with the aim to study the possible underlying mechanisms of RIPC in an experimental porcine model.

Aim III
To assess the additive protective effects of RIPC and identify humoral circulating factors mediating the effects of RIPC in protection against neuronal damage caused by DHCA in a large animal porcine model.
4 Materials and methods

This thesis was based on three different studies, and this section summarizes the materials and methods used in these studies. The first two studies (I, II) focused on the simulated thoracic aortic aneurysm repair whereas the third study (III) utilized the DHCA settings. The design of the first study was subacute, while studies II and III involved acute experimental models.

4.1 Experimental porcine models

In all three studies, the animals were randomized into two groups, a remote ischemic preconditioning, RIPC, group and a control, CTRL, group receiving identical surgical interventions excluding the pretreatment. The same number of experimental animals was included in both groups.

4.1.1 Studies I, II

Studies I and II were inspired by experimental spinal cord studies by Doctors Christian Etz and Randall Griepp along with their coworkers at the Mount Sinai School of Medicine in New York. In advance of performing the spinal cord ischemia studies, critical improvement and development of the experimental model was made by the leader of the experimental surgery research group, Professor Tatu Juvonen in Oulu, Finland.

4.1.2 Study III

Study III utilized the model of DHCA. This porcine model, and especially its surviving features, originates from the laboratory of Doctor Randall Griep from the Mount Sinai School of Medicine, New York, as well. However, this study concentrated on the acute experimental setup.

4.2 Experimental animals and preoperative management

The experimental animals used in these studies originated from a native stock near Oulu, Finland. The age of the female pigs ranged from 7–8 weeks, and the mean weight was 20.4 kg (Interquartile range, IQR: 19.0–21.7 kg).
All animals received humane care in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Resource Council (Published by National Academy Press, revised 1996). The Research Animal Care and Use Committee of the University of Oulu approved the study protocols.

A minimum of three days before the experiments, pigs were delivered to the Laboratory Animal Centre to become acclimated to the surroundings and to permit the assessment of their suitability to the experiments.

4.3 Anaesthesia

All animals were sedated with intramuscular injection of ketamine (350 mg), midazolam (45 mg) and medetomidine (1.5 mg). Peripheral catheters were inserted into a vein of both ears. Anaesthesia was induced with thiopental (25–125 µg) and fentanyl (0.5 mg) as required. The animals were intubated with a 6.0 mm cuffed endotracheal tube and ventilated with 55%/45% oxygen-air mixture ratio in the respirator to achieve an end-tidal carbon dioxide (EtCO₂) concentration in the expired air of 4.5–5.0%. After the induction cefuroxime (1.5 g) prophylaxis was administered preoperatively. Additionally, in Study II, intramuscular glycopyrrolate (0.2 mg) was given to reduce the excessive mucus secretion.

4.3.1 Studies I, II

The maintenance of anaesthesia varied between the study protocols due to the monitoring of the motor evoked potentials in Studies I and II. The susceptibility of MEPs to many anaesthesia agents, especially volatile anaesthetics, along with the known abolishing effect of RIPC by propofol, necessitated the careful consideration of anaesthesia conditions.

Anaesthesia was maintained by a continuous infusion of fentanyl (0.025 mg/(kg/h)) and ketamine (15 mg/(kg/h)), as well as inhalation anaesthesia of sevoflurane 1.0%, which was discontinued before measuring baseline values. A single intravenous dose of rocuronium (0.1 mg/kg) was used for surgical relaxation at the beginning of each experiment.
4.3.2 Study III

In Study III, the anaesthesia was maintained by a continuous infusion of fentanyl (0.025 mg/(kg/h)), midazolam (0.25 mg/(kg/h)), and rocuronium (1.0 mg/(kg/h)), as well as inhalation anaesthesia of 1.0% sevoflurane throughout the experiment excluding the HCA period. For surgical relaxation, a single intravenous dose of rocuronium (0.1 mg/kg) was used. The animals were kept under terminal anaesthesia, lasting 8 hours postoperatively.

4.4 Remote ischemic preconditioning

Remote ischemic preconditioning was performed in two different manners, in the hind limb with a blood pressure cuff (I, III) and with a direct occlusion of the iliac artery (II). The durations and repetitions of the ischemia-reperfusion cycles were performed similarly in all three studies.

4.4.1 Studies I, III

In RIPC, a children’s blood pressure cuff was placed around the hind limb, left (I) or right (III). The cuff was inflated to > 250 mmHg for 5 minutes followed by a 5-minute deflation. This inflation-deflation cycle was repeated four times, and the total time of RIPC stimulus lasted for 40 minutes. In the control group the blood pressure cuff was placed for 40 minutes around the hind limb without any inflation-deflation cycles.

In Study I, preconditioning was applied 15 minutes in advance of spinal cord ischemia. In contrast, in Study III, preconditioning was performed 65 minutes (IQR: 51–72 minutes) prior to CPB, and HCA was achieved 95 minutes from the last preconditioning cycle (III).

4.4.2 Study II

The left iliac artery was exposed through the incision over the left iliac crest. The clamp was placed around the artery for RIPC. The artery was occluded for 5 minutes followed by a 5-minute reperfusion period accomplished by releasing the clamp. This intermittent ischemia-reperfusion cycle was repeated four times. In the control group, the incision was made and the left iliac artery exposed for 40 minutes.
without RIPC. Preconditioning was performed 15 minutes prior to spinal cord ischemia.

4.5 Haemodynamic monitoring

An arterial line for pressure monitoring and blood sampling was placed on the right (I, II) or left (III) femoral artery. A 7Fr pulmonary artery thermodilution, Swan-Ganz, catheter (CritiCath; Ohmeda GmbH & Co, Erlangen, Germany) was inserted through the right (I, II) or left (III) femoral vein for invasive hemodynamic monitoring, and for blood sampling. Blood temperature, pulmonary pressures, cardiac output, and pulmonary capillary wedge pressure, along with central venous pressure could be measured through the venous catheter. Electrocardiogram (EKG) and rectal temperature monitoring were carried out throughout the entire operations. Additionally, to monitor urine output and fluid balance, an 8 Ch catheter was introduced to the urinary bladder.

4.6 Cranial procedures

Cranial procedures were performed in all three studies as described below.

4.6.1 Studies I, II

The skull was exposed by a 7-centimetre midline longitudinal incision. A total of four wire leads for MEP stimulation were placed and secured over the parietal cortex: two of the leads were attached on the right side and two of them on the left side. The placement was based on sagittal and coronal sutures: 8 mm anterior and 8 mm posterior to the coronal suture and 10 mm lateral to the sagittal suture. Thereafter, two of the leads were connected to an electrical stimulator (Cadwell, TCS-1) and MEP stimulation was tested with different electrode selections.

4.6.2 Study III

Animals were positioned on their ventricular side. A cranial window (35 x 35 mm) was performed on top of the scalp using a 14/11 mm disposable cranial perforator (200–253 DGR-II, Acra Cut Inc, Acton MA, USA). A peripheral venous cannula (22 G x25 mm BD Venflon™ Peripheral IV Catheter with Injection port) for blood
sampling was inserted through dura mater to the sagittal sinus and secured thoroughly.

4.7 Motor evoked potential monitoring

Stainless steel needle electrodes were placed on both hind limbs to measure cortical motor nerve stimuli. Intraoperative neuromonitoring was performed by a Cadwell Cascade Elite system (Cadwell Inc., Kennewick, WA, USA) with Cadwell Cascade software (v. 2.6.). A Cadwell TCS-1 constant voltage electrical stimulator was used for eliciting transcranial electrical MEPs. The multi-pulse-stimulus characteristics were the following: train length 4, interstimulus interval 2 milliseconds, and stimulus pulse width 75 microseconds.

MEP baseline measurements were recorded under stable anaesthetic and haemodynamic conditions. After baseline, MEPs were monitored at the end of RIPC or sham treatment, at closure procedures with 1-minute intervals, at 30-minute postoperative period with 5-minute intervals, and every 30 minutes throughout the 90-minute (II) or the 240-minute (I) follow-up.

The analysis focused on the peak-to-peak amplitude, peak and onset latencies and difference between peak and onset latency (I, II). Additionally, baseline-to-peak amplitude and duration were analysed in Study II. A 50% decrease of the motor evoked potential amplitudes compared to baseline values was considered indicative of critical ischemia.
4.8 Spinal cord ischemia

Left anterolateral thoracotomies were performed through the fourth, seventh and eleventh intercostal spaces with two separate incisions to expose the left subclavian artery, as well as segmental arteries to the level of the diaphragm. The left azygous vein was ligated and cut.

Following the RIPC, within 15 minutes, the permanent closure of the prepared left subclavian artery as well as a sequential permanent closure of the prepared segmental arteries were performed with 5-minute intervals simulating the thoracic aortic aneurysm repair (I, II).
4.9 Cardiopulmonary bypass

Prior to the cardiopulmonary bypass and hypothermic circulatory arrest procedures, blood transfusion was performed in all experiments.

4.9.1 Blood transfusion

Preoperatively, a membrane oxygenator (D905 Eos, Dideco, Mirandola, Italy) was primed with 800–1,000 ml of Ringer acetate solution, heparin (15,000 IU), and fresh whole blood from a donor pig. Blood donation was required to maintain the hematocrit of all animals above 20% after the operation.

The donor pigs were under anaesthesia during the donation procedure. An aortic cannula 6 Fr was placed on the left femoral artery prior to this the pigs being heparinized (1,000 IU/kg). Fresh whole blood 1,000–1,700 ml was drawn and transfused into the prime solution of the heart-lung machine until arrhythmias could be detected in EKG as a sign of terminal situation or an EtCO₂ concentration in the
expired air was below 3.5%. The aortic cannula was then removed and intravenous injection of pentobarbital (90 mg/kg) was given to euthanize the donor animal.

4.9.2 CPB and HCA

A right anterolateral thoracotomy was performed through the fourth intercostal space, the right internal thoracic artery and vein were ligated and cut, and the pericardium was opened. The right atrium was exposed for cardiopulmonary bypass (CPB) and after systemic heparinization (500 IU/kg), the right atrial appendage was cannulated with a 24 Fr venous cannula and the ascending aorta was cannulated with a 14 Fr arterial cannula.

CPB was initiated and the flow was adjusted to maintain MAP of 50 to 70 mmHg. A 30-minute cooling period was carried out to attain a temperature of 18°C; a heat-exchanger was used to achieve the target temperature. Acid-base management was the pH-stat strategy throughout the perfusion, maintaining the pH value of blood stable by increasing the CO₂ content in the blood, despite the decrease in temperature. After the cooling, a 60-minute period of HCA at 18°C was initiated and potassium chloride (40 mmol) was injected towards the heart to achieve cardiac arrest. Topical ice water for cardiac cooling was applied throughout HCA. Pulmonary and rectal temperatures were monitored continuously and the temperature was maintained at 18°C with topical cooling, using ice packs placed over the body and head.

The rewarming was initiated with a 5-minute cold perfusion period. At the beginning of rewarming, furosemide (40 mg), lidocaine (40 mg), methylprednisolone (80 mg), mannitol (150 g), and calcium bioglyconate (1375 mg, 2.25 mmol Ca²⁺) were administered into the circulation via the heart-lung machine. During a 45-minute period, the pigs were warmed to normothermia. The heart was defibrillated at 27 to 30°C, if necessary. Ventilation was restored 10 minutes before weaning from CPB. Protamine sulfate was administered after decannulation to reverse the anticoagulant effects of heparin. Throughout rewarming and after weaning from CPB, animal temperature was regulated using heating lamps, paracetamol infusions (1 to 2 g intravenously), and ice packs as required (III).
Fig. 6. The simplified protocol of Study III. In advance of RIPC, baseline measurements were carried out after anaesthesia induction, thoracotomy, and haemodynamic monitoring were performed. CPB = cardiopulmonary bypass, HCA = hypothermic circulatory arrest, RIPC = remote ischemic preconditioning.

4.10 Intraoperative measurements

In all studies, haemodynamic variables including pulse, systemic and pulmonary blood pressures, CVP, pulmonary capillary wedge pressure, and cardiac output along with diuresis and fluid balance were monitored at several time points throughout the experiments.

Additionally, systemic arterial and venous blood samples were collected including blood gas values, oxygen saturation, pH, electrolytes, glucose, plasma ionized calcium, plasma lactate levels, hematocrit, and haemoglobin levels (iSTAT Analyzer; iSTAT Corporation, East Windsor, NJ). Troponin I, creatine kinase (CK-MBm) were analysed, as well.

In Studies I and II, the time points at which the aforementioned data was collected were as follows: at baseline (after the thoracotomies and venous catheter and arterial line had been applied), at the end of remote ischemic preconditioning...
or sham treatment, at the end of the closure procedure, at 60 minutes, at 120 minutes, and at 240 minutes from the last segmental artery ligation (I). The postoperative period was different in Study II, and the time points were at 60 minutes and at 90 minutes from the last segmental artery ligation. In Study III, the time points were at baseline, at the end of remote ischemic preconditioning, after 20 minutes of cooling, and at 5 minutes and 1, 2, 4, and 8 hours from the end of the HCA.

### 4.11 Postoperative management

#### 4.11.1 Studies I, II

Surgical incisions were closed, and after a 90-minute (II) or a 240-minute (I) follow-up period the animals were weaned from mechanical ventilation, extubated, and transferred to a recovery room. Postoperative analgesia was maintained with intramuscular injections of buprenorphine (6 µg/kg) after extubation until euthanasia.

Postoperatively, neurological assessment was carried out 6 (II) or 24 (I) hours after the onset of spinal cord ischemia using Tarlov score 0 to 4 (Tarlov 1957). The quantified assessment of motor function is summarized in Table 3. The maximum score of 4 indicates normal motor function and lower values reflect varying grades of spinal cord damage.

<table>
<thead>
<tr>
<th>The explained Tarlov score</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spastic paraplegia, no movements</td>
<td>0</td>
</tr>
<tr>
<td>Paraparesis, slight movements</td>
<td>1</td>
</tr>
<tr>
<td>Paraparesis, powerful movements in hind limbs, but not able to stand</td>
<td>2</td>
</tr>
<tr>
<td>Able to stand but unable to walk</td>
<td>3</td>
</tr>
<tr>
<td>Full recovery, normal walking function</td>
<td>4</td>
</tr>
</tbody>
</table>

#### 4.11.2 Study III

Intravenous noradrenaline infusion was used postoperatively if necessary to maintain mean arterial pressure over 60 mmHg. Animals were under terminal anaesthesia, which together with mechanical ventilation were maintained for 8 hours after the hypothermic circulatory arrest followed by euthanization using pentobarbital (90 mg/kg).
4.12 Histopathology

In Studies I and II, after the neurological assessment, animals were euthanized using intravenous pentobarbital (90 mg/kg). The thoracic and lumbar spinal cords were harvested and immersed in 10% neutral formalin for fixation. The spinal cord was divided into five sections based on nerve roots (Th1–3, Th4–6, Th7–9, Th10–13, L1–4).

In Study III, the tissue samples were collected from the brain, the right ventricle of the heart, and the right kidney. Cortex, thalamus, hippocampus, brainstem, and cerebellum were the selected brain areas for the total histopathological sum score of the central nervous system.

All study samples were sectioned with 6 μm thickness and stained with hematoxylin-eosin (HE). Thereafter, the tissue sections from each animal were screened and scored by an experienced neuropathologist unaware of the experimental design and fate of an individual animal. Scoring of the HE samples is summarized in Table 4 including the presence of edema, haemorrhages, neuron degeneration, and infarcted tissue. The histological sum for each section was calculated by summing the scores of each sign of injury. Thereafter, the total histopathological sum score was calculated by adding up the scores of each specific spinal cord (I, II) or brain area (III).

Table 4. The scoring of the hematoxylin-eosin (HE) stainings for signs of injury.

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edema</td>
<td>No</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>No</td>
<td>-</td>
<td>Yes</td>
<td>-</td>
</tr>
<tr>
<td>Neuron degeneration</td>
<td>No</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Infarcted tissue</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
</tbody>
</table>

4.13 Immunohistochemical analysis

In Studies II and III, immunohistochemical stainings were performed for the tissue samples mentioned in the histopathology section. Samples in 4.5 μm sections were cut from paraffin-embedded specimens and deparaffinized in xylene, after which they were rehydrated through graded alcohols. A microwave oven was used for antigen retrieval; the sections were pre-treated with either Tris-EDTA (pH 9) or with citrate buffer (pH 6). After concluding the neutralization of the endogenous peroxidase activity, the sections were incubated at room temperature with diluted
antibodies. EnVision FLEX (Dako, Agilent Technologies, Santa Clara, CA, USA) was used to detect bound antibodies. Diaminobenzidine served as the chromogen and hematoxylin as the counterstain.

The scoring system for stainings, including apoptotic marker caspase 3 and antioxidant response regulator Nrf2, was based on semiquantitative protocol and on numerical calculations described in detail in Tables 5 and 6.

Table 5. Immunohistochemical stainings of Nrf2 and caspase 3 (II).

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strongly positive</td>
<td></td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Very strongly positive</td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Caspase 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No stained motor neurons</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–10 stained motor neurons</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–20 stained motor neurons</td>
<td></td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>&gt; 20 stained motor neurons</td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
</tbody>
</table>

Nrf2 = nuclear factor erythroid 2-related factor.

Table 6. Immunohistochemical stainings of Nrf2 and caspase 3 (III).

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>The intensity of staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strongly positive</td>
<td></td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Very strongly positive</td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>The stained areas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No stained areas</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–25% stained areas</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25–50% stained areas</td>
<td></td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>&gt; 50% stained areas</td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
</tbody>
</table>

These two systems were added together with 6 indicating the maximum score in one area. Nrf2 = nuclear factor erythroid 2-related factor.

4.14 Biochemical analysis

In Study III, blood samples were collected from two different vessels, vena cava (VC) and sinus sagittalis (SS), at the aforementioned time points along with at the
end of each reperfusion cycle during the RIPC. The samples were allowed to clot undisturbed at room temperature and centrifuged 2,000 x g for 20 min at 4°C, after which the sera were collected and stored at -80°C. The sera from RIPC and control were pooled and sent for proteomics analysis to the Proteomics Karolinska core facility at the Karolinska Institute, Stockholm, Sweden. The detailed proteomics analyses are described in the following section.

Serum glial-specific S100 protein levels were analysed with ELISA kit following the manufacturer’s instructions. The metabolite analyses including kynurenic acid (KYNA) and alpha-ketoglutarate (αKG) levels in serum were analysed by our collaborator at the Division of Cardiovascular Medicine, Department of Medicine, The Brigham and Women’s Hospital, Harvard Medical School, Boston (Olenchock et al. 2016).

4.14.1 Proteomics analyses

Protein extraction and solubilization. The serum samples were not depleted from the major serum protein in order to avoid unintended removal of nontargeted proteins with the targeted ones. A quantity of 10 µg of plasma proteins from each sample was dissolved in a mixture of 50 mM ammonium bicarbonate (AmBic) in 10% acetonitrile (ACN) with 0.1% Protease MAXTM Surfactant Trypsin Enhancer (Promega). The sample mixtures were incubated for 15 min at 50°C, sonicated for 10 min, and centrifuged for 5 min to eliminate the undissolved debris.

In solution digestion. Proteins were reduced with 20mM dithiothreitol (Sigma) in 50mM AmBic and incubated at 56°C for 30 min. 66mM iodoacetamide (Sigma) in 50mM was added for alkylation at room temperature for 30 min. Then 0.3 µg of sequencing grade modified Trypsin (Promega) was added to each sample (1:33 trypsin:protein) and incubated for 16h at 37°C. The digestion was stopped by the addition of formic acid at final concentration of 5% and incubation of the solution for 20min at 37°C. Then the samples were cleaned by C18 Hypersep plate (Thermo Scientific), dried using a Speedvac, and resuspended in 25µl 0.1% formic acid and 2% ACN.

PRLC-MS/MS analysis. Chromatographic separation of peptides was achieved using a 30cm homemade C18 column connected to an Ultimate-LC system (Thermo Fisher Scientific). The peptides were loaded onto the column at a flow rate of 1,000 nL/min, and then eluted at a 300 nL/min flow rate for 120 min at a linear gradient from 4% to 26% ACN in 0.1% formic acid. An Orbitrap Q Exactive plus mass spectrometer (Thermo Fisher Scientific) analysed the eluted peptides that
were ionized with electrospray ionization. The survey MS spectrum was acquired at the resolution of 60,000 in the range of m/z 200–2,000. MS/MS data were obtained with higher-energy collisional dissociation for ions with charge z > 1 at a resolution of 15,000.

**Data processing.** The raw files were converted to Mascot Generic Format (mgf) using an in-house written Raw2mgf program. Proteins were identified by searching mgf files against the SwissProt database (Porcine) using the Mascot v. 2.4 (MatrixScience) database search engine.

**Protein quantitation.** Quantitative information was extracted using in-house developed label-free software Quanti v.2.4.3.1 (Lyutvinskiy et al. 2013). The program performs quantification of peptides found by Mascot based on peptides’ extracted ion chromatograms. Proteins are quantified based on peptide abundances. Only reliably identified (false discovery rate, FDR < 0.01), unmodified peptides with unique sequences were considered, and only proteins discovered with at least two such peptides were quantified. The results were reported as a set of relative protein abundances scaled such that the geometric mean of the abundance of each protein over all samples was 1.0.

**ELISA.** The ELISAs were performed using the commercially available ELISA kits for porcine apolipoprotein E (Cloud-Clone Corp, USA), complement C3 (CUSABIO, Wuhan, China), transthyretin (Cloud-Clone Corp, USA), and vitronectin (CUSABIO, Wuhan, China) according to the manufacturer’s instructions.

### 4.15 Statistical analysis

SPSS (version 22.0; SPSS Inc. Chicago, IL) and SAS (version 9.3; SAS Institute, Cary, NC) statistical software packages were used for statistical analysis. Continuous and ordinal variables are expressed as the median with interquartile range (IQR; 25th and 75th percentiles). In the figures, mean and standard error of mean (SEM) are used. Complete independence was assumed across all animals (by random statement). Distribution of the variable was tested using the Shapiro-Wilks test of normality. Either the Student $t$ test or Mann-Whitney U test was used to assess the $p$ values between the variables of the study groups. Two-tailed significance levels are reported. $P \leq 0.05$ was considered statistically significant. Moreover, in Study III, in the proteomics analysis of serum samples, non-parametric statistic tests, Friedman and Dunn’s multiple comparison tests, were used to detect differences across multiple sample populations.
The repeatedly measured data was analysed using a linear mixed model with animals fitted as random, and the covariance pattern was chosen according to Akaike’s information criteria. Reported $p$ values are as follows: $p$ between groups ($p_{g}$) indicates a level of difference between the groups; $p$ for time by group ($p_{t\times g}$), indicates behaviour between the groups with time.

Due to the individual conduction velocity of the spinal cord the motor evoked potentials are presented as relative changes compared to the baseline values. The purpose of the statistical analysis for MEP responses was to explore the remote ischemic preconditioning effects at different time points instead of an exact global assessment over time.
5 Results

In advance of experiments, pilot studies were performed to assess the motor evoked potential monitoring as well as to determine the suitable approach to perform spinal cord ischemia in Studies I and II. In Study III, the cranial procedures required careful consideration prior to experimental studies.

Unfortunately, some animals needed to be excluded from the analyses; the exclusion reasons are presented in the Table 7. In the final analyses, a total of 58 animals were included.

Table 7. The reasons for exclusion of animals.

<table>
<thead>
<tr>
<th>Indication for exclusion</th>
<th>Study I (SCII)</th>
<th>Study II (SCII)</th>
<th>Study III (HCA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RIPC CTRL</td>
<td>RIPC CTRL</td>
<td>RIPC CTRL</td>
</tr>
<tr>
<td>Pilot studies</td>
<td>3 1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Failure in SS cannulation</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraoperative bleeding</td>
<td></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Failure in A/V cannulation</td>
<td></td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Inadequate MEP responses</td>
<td></td>
<td>1*</td>
<td></td>
</tr>
<tr>
<td>POP respiratory failure</td>
<td>3 1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>POP acute heart failure</td>
<td>4 4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>No neurological status</td>
<td>11 13</td>
<td>27 27</td>
<td></td>
</tr>
<tr>
<td>Total number of animals excluded without pilots</td>
<td>3/10 3/10</td>
<td>1/9 1/8</td>
<td>3/11 3/11</td>
</tr>
<tr>
<td>Percentage of excluded animals without pilots (%)</td>
<td>30 30</td>
<td>11 13</td>
<td>27 27</td>
</tr>
</tbody>
</table>

A/V = aortic/venous, CTRL = control group, HCA = hypothermic circulatory arrest, MEP = motor evoked potential, POP = postoperative, RIPC = remote ischemic preconditioning group, SCII = spinal cord ischemic injury, SS = sinus sagittalis. In spinal cord studies, the postoperative exclusion involved the neurological assessment; other end-points were included in the analyses. In Study III, the exclusion comprised all of the studied end points. * = completely excluded from the analyses.

5.1 Studies I, II

Studies I and II focused on spinal cord ischemic injury with the same surgical approach. The performance of RIPC, along with the follow-up periods, varied between the studies. Next, the combined results of these two studies are presented.
5.1.1 Comparability of study groups

The weight of the pigs did not differ between the RIPC and control groups. The baseline haemodynamic and metabolic values were also similar in both groups.

Throughout the experiments blood temperatures were comparable in Study I \( p_g = 0.45 \) and in Study II \( p_g = 0.19 \). Moreover, the rectal temperatures \( p_g = 0.18 \) were similar in Study I. Haemoglobin did not differ between groups in Studies I \( p_g = 0.20 \) and II \( p_g = 0.91 \). No differences were found in mean arterial pressure throughout the entire experiment; this is true for both Study I \( p_g = 0.78 \) and Study II \( p_g = 0.51 \).

Postoperatively, the rectal temperatures \( p_g = 0.04 \) were higher in the control group in Study II. Additionally, partial pressure of oxygen \( p_g = 0.05 \) was higher and partial pressure of carbon dioxide \( p_g = 0.04 \) was lower in the RIPC group during the follow-up period in Study II.

5.1.2 Motor evoked potentials

The MEP amplitude change in the RIPC group demonstrated a tendency towards better function of the spinal cord after occlusion of the left subclavian artery and segmental arteries in both studies. In addition to this, the RIPC itself caused an increase in MEP before the onset of spinal cord ischemia.

The difference between groups reached statistical significance in the right hind limb recording at several time points; the left hind limb recordings showed a similar trend. The results are summarized in the following figures. The amplitude change started to diminish steadily after ligation of the left subclavian artery in the control group, whereas the amplitude was higher in the RIPC group after the intervention.
Fig. 7. Peak-to-peak amplitudes of the right hind leg (l). Measurements are compared to the baseline value of 0. The amplitude change begins to diminish steadily after ligation of the left subclavian artery in the control group, whereas the amplitude is higher in the RIPC group after remote ischemic preconditioning. Values represent medians; error bars represent the interquartile range. ASUB = the occlusion of the left subclavian artery, SSA = the occlusion of the 5th segmental artery, 9SA = the occlusion of the 9th segmental artery, CTRL = control, POP = postoperative time points with minutes, RIPC = remote ischemic preconditioning. *P < 0.05, **P < 0.01, ***P < 0.001.
Fig. 8. Peak-to-peak amplitudes of the left hind leg (I). Measurements are compared to a baseline value of 0. The difference is statistically significant after RIPC. Values represent medians; error bars represent the interquartile range. ASUB = the occlusion of the left subclavian artery, 5SA = the occlusion of the 5th segmental artery, 9SA = the occlusion of the 9th segmental artery, CTRL = control, POP = postoperative time points with minutes, RIPC = remote ischemic preconditioning. *P < 0.05; ▽P < 0.06 at single time point.

Study II underlined the findings of the first setup. The MEP peak-to-peak amplitude responses in both hind limbs in the RIPC group were higher at several time points than in the control group. A consistent group difference persisted until the end of the measurement series in both hind limbs, summarized in the following figures. The baseline-to-peak amplitude responses demonstrated the difference with statistically significant responses in both hind limbs: in the left (p = 0.021) and in the right (p = 0.012) hind limb at the post-intervention time point, and after occlusion of the left subclavian artery in the right (p = 0.046) hind limb.
Fig. 9. Peak-to-peak amplitude changes of the right hind leg (II) compared to the baseline values in the remote ischemic preconditioning (RIPC) group and the control (CTRL) group. The difference is statistically significant at the time point after remote ischemic preconditioning (PostRIPC) along with after occlusion of the left subclavian artery and 15 minutes postoperatively. ASUB indicates the closure of the left subclavian artery. 3SA, 6SA, and 9SA indicate the specific time points with the number of occluded segmental arteries. POP indicates postoperative time points in minutes. Values are shown as medians and 25th and 75th percentiles. *P < 0.05 at a single time point.
Fig. 10. Peak-to-peak amplitudes of the left hind leg (II) compared to the baseline values in the remote ischemic preconditioning (RIPC) group and the control (CTRL) group. The difference is statistically significant at the time point after remote ischemic preconditioning (PostRIPC). ASUB indicates the closure of the left subclavian artery. 3SA, 6SA, and 9SA represent the specific time points with the number of occluded segmental arteries. POP indicates postoperative time points in minutes. Values are shown as medians and 25th and 75th percentiles. *P < 0.05 at a single time point.

In Study I, in the RIPC group the amplitude in the right hind limb lasted throughout the 240-minute follow-up, whereas in the control group, the median time to a 50% amplitude decrease was 195 minutes (p = 0.044). The left hind limb recordings did not show differences between the groups (p = 0.271). In Study II, in both study groups the amplitudes were maintained throughout the 90-minute follow-up in both hind limbs.

Onset latency in the right hind limb was significantly shortened in the RIPC group after pretreatment and endured until the final segmental artery occlusion in Study I. In the left hind limb, no statistically significant differences were found. Additionally, the differences between onset and peak latency were not significantly different in either limb. Similarly, in Study II, peak latency, onset latency, and
difference between peak and onset latency or duration did not demonstrate statistically significant differences in the hind limbs at any time point.

Fig. 11. The onset latency of the right hind leg (I). Measurements are compared to a baseline value of 0. The onset latency is significantly reduced in the RIPC group after intervention until the last segmental artery occlusion. Values represent medians; error bars represent the interquartile range. ASUB = the occlusion of the left subclavian artery, 5SA = the occlusion of the 5th segmental artery, 9SA = the occlusion of the 9th segmental artery, CTRL = control, POP = postoperative time points with minutes, RIPC = remote ischemic preconditioning. *P < 0.05 at a single time point.

5.1.3 Neurological outcome

The neurological outcome was assessed after a 6-hour (II) or a 24-hour (I) follow-up period. The results are described in detail in Table 8, and the number of assessed animals in each group is presented, as well.

In Study I, the RIPC group had a better mean Tarlov score of 2.8 compared with that of the control group, having a value of 1.8, but a statistically significant difference was not reached (p = 0.169). One subject from the control group had a Tarlov score of 0; in contrast, the minimum Tarlov score in the RIPC group was 2.
The mean Tarlov score was 1.5 in the RIPC group compared with the control group, with its mean Tarlov score of 1.1 (p = 0.279) in Study II. One animal of the control group suffered from total paraplegia in this study, as well.

<table>
<thead>
<tr>
<th>Tarlov score</th>
<th>Score</th>
<th>Study I (24h)</th>
<th>Study II (6h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spastic paraplegia, no movements</td>
<td>0</td>
<td>1/6</td>
<td>1/7</td>
</tr>
<tr>
<td>Paraparesis, slight movements</td>
<td>1</td>
<td>2/6</td>
<td>4/8</td>
</tr>
<tr>
<td>Paraparesis, powerful movements</td>
<td>2</td>
<td>2/6</td>
<td>4/8</td>
</tr>
<tr>
<td>Able to stand, but unable to walk</td>
<td>3</td>
<td>3/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Full recovery, normal walking function</td>
<td>4</td>
<td>1/6</td>
<td>1/6</td>
</tr>
</tbody>
</table>

P-value between the study groups   0.169   0.279

CTRL = control group, RIPC = remote ischemic preconditioning.

5.1.4 Histopathology

Fourteen animals were included in the histopathologic analyses of Study I. The analyses demonstrated the median sum of the histopathologic scores in the RIPC group to be 9 (IQR: 7.5–10.5), and also 9 in the control group (IQR: 6.0–11.0) (p = 0.713). The main differences between the groups were edema (p = 0.410) and haemorrhage (p = 0.598); infarction (p = 1.0) or neuron degeneration (p = 1.0) scores were similar between the study groups.

In Study II, the mean sum of the histopathologic scores was 3.6 (IQR: 0.0–6.5) in the RIPC group, and that of the control group was 2.8 (IQR: 1.0–4.5) (p = 0.788). The main differences between the groups were edema (p = 0.868), neuron degeneration (p = 0.343), and infarction scores (p = 0.064). Haemorrhage score was similar between the groups (p = 1.0).

5.1.5 Immunohistochemical analyses

Immunohistochemical analyses of caspase 3 and Nrf2 were performed in Study II. The mean sum of Nrf2 total scores was higher in the RIPC group, with a value of 11.0 (IQR: 8.5–14.0), compared with the control group value of 5.2 (IQR: 1.0–9.0) with a statistically significant difference (p = 0.023). These scores were in favour of the RIPC group in all spinal cord sections shown in Table 9. The cytoplasmic staining reactions were detected in the anterior horns of the motor neurons. In the nucleus, the expression of Nrf2 was negative in all samples. The
immunohistochemical total scores with caspase 3 did not differ between the study groups (p = 0.713).

<table>
<thead>
<tr>
<th>Table 9. Immunohistochemistry.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td><strong>Nrf2 (cytoplasm)</strong></td>
</tr>
<tr>
<td>RIPC</td>
</tr>
<tr>
<td>CTRL</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
</tr>
<tr>
<td><strong>Caspase 3 (nucleus)</strong></td>
</tr>
<tr>
<td>RIPC</td>
</tr>
<tr>
<td>CTRL</td>
</tr>
<tr>
<td><strong>P-value</strong></td>
</tr>
</tbody>
</table>

Control = 8, RIPC n = 8. CTRL = control, L = lumbar, Nrf2 = nuclear erythroid 2-related factor 2, RIPC = remote ischemic preconditioning, Th = thoracic. Immunohistochemical stainings of caspase 3 and Nrf2. Score: mean (IQR; 25th and 75th percentiles). *P < 0.05.

5.2 Study III

5.2.1 Comparability of study groups

The mean weight of the pigs did not differ between the study groups (p = 0.316). The amount of transfused blood was 69.0 ml/kg (62.5–78.6) in the RIPC group and 68.7 ml/kg (62.3–74.4) in the control group (p = 0.767). Additionally, the study groups did not differ in number of defibrillations (p = 0.494), need for inotropes after the operation (p = 0.643), or fluid balance at the end of the follow-up (p = 0.987).

The baseline electrolyte contents were comparable, with no significant differences between the groups. Neither haemoglobin (p = 0.33) nor hematocrit (p = 0.31) differed between the groups. Blood (p = 0.85) and rectal (p = 0.91) temperatures were similar throughout the experiments.

Systemic concentration of venous lactate was lower in the intervention group after the operation, and the baseline level was more rapidly reached in the RIPC group.
5.2.2 Cardiac function

After weaning from the CPB, the RIPC group demonstrated a better cardiac index reaching the baseline values. The control group resulted in lower cardiac index compared with the baseline values. Troponin I ($p_g = 0.99$) and creatine kinase isoenzyme MB ($p_g = 0.95$) levels did not differ between the groups. Additionally, mean arterial pressure ($p_g = 0.39$) was similar in both groups.
5.2.3 Proteomics analyses

The proteomics analyses displayed 18 known porcine proteins that appeared differently expressed in response to the RIPC protocol compared with baseline and/or control values. In all samples, there were proteins with unknown functions according to the open-source bioinformatics gene analyses tool (PANTHER). The known proteins were related to variety of biological processes such as metabolic, cellular, development, immune system processes, and localization. The proteins included common blood circulating proteins, coagulation or complement factors, and transport proteins along with secreted and extracellular proteins.
Fig. 14. Changed recognized porcine proteins at different time points of HCA surgery. For analysis of the difference in expression, a change of > 20% was set as a threshold. BL = baseline, HCA = hypothermic circulatory arrest, PR = postintervention time point, SS = sagittal sinus, VC = vena cava, WS = rewarming phase after arrest time point.

Fig. 15. Pie chart representation of the biological functions of the known serum proteins using the PANTHER software. PR = postintervention time point, SS = sagittal sinus, VC = vena cava, WS = rewarming phase after arrest time point.
Particular interest was given to the sagittal sinus blood samples since these were taken behind the blood-brain barrier. Based on literature data related to protein functions and possible relevance in RIPC, four proteins were selected: apolipoprotein E (APOE), complement C3 (C3), transthyretin (TTR), and vitronectin (VTN). The levels of those four proteins were, however, not significantly changed between the study groups at any of the studied time points.

![Graph](image.png)

**Fig. 16.** The sagittal sinus serum concentration of vitronectin (VTN), transthyretin (TTR), complement C3 (C3), and apolipoprotein E (ApoE) at baseline (BL), postintervention (PR), and rewarming phase after HCA (W5). Data are reported as means +/- 1 SEM. HCA = hypothermic circulatory arrest, SEM = standard error of mean.

### 5.2.4 Metabolic analyses

The levels of αKG and KYNA decreased following the ischemia-reperfusion cycles in the vena cava samples (Figure 17). No significant changes in their levels were seen in the sagittal sinus samples following RIPC.
Fig. 17. The behavior of serum (vena cava, VC) and sagittal sinus (SS) levels of αKG and KYNA during ischemia-reperfusion cycles in the RIPC group. αKG = alpha-ketoglutarate, KYNA = kynurenic acid, RIPC = remote ischemic preconditioning, SS = sagittal sinus, TIC = total ion chromatogram.

5.2.5 Neurological marker

The serum levels of S100B protein changed in a similar manner during the surgery in both control and RIPC groups, peaking after HCA at the initial rewarming phase and slowly decreasing to baseline over the course of the follow-up period. Interestingly, the serum levels of S100B protein declined faster in the intervention group, reaching a significant difference compared with the control group after 4 hours of the arrest.
Fig. 18. Serum concentrations of S100B levels at different time points of the surgery. Mean +/- 1 SEM. BL = baseline time point, HCA = hypothermic circulatory arrest, PR = postintervention time point, RIPC = remote ischemic preconditioning, SEM = standard error of mean, S100B = glial-specific S100 calcium binding protein B, W5 = rewarming phase after arrest time point. *P < 0.05 at a single time point.

5.2.6 Histopathology

The total histopathologic sum score of the central nervous system did not differ between the study groups (p = 0.854). Regional differences were observed in the hippocampus and brainstem. The most defining finding was edema in both study groups (p = 0.764). Haemorrhages were observed only in the control group, but this difference did not reach a statistically significant difference between the groups (p = 0.317). There was no infarction detected in the study groups.

5.2.7 Immunohistochemical analyses

In the kidney, heart, or central nervous system the total immunohistochemical sum scores of caspase 3 (p = 0.622) or Nrf2 (p = 0.583) did not differ between the study groups. The regional CNS differences of the Nrf2 scores were detected in the hippocampus and in the cerebellum. In the cerebellum, the Nrf2 scores were higher in the RIPC group 1.1 (IQR: 0.0–2.5) compared with the control group 0 (IQR: 0.0–0.0), (p = 0.064). The CNS scores are summarized in Table 10.
Table 10. Immunohistochemistry.

<table>
<thead>
<tr>
<th>Immunostaining</th>
<th>Cortex score</th>
<th>Thalamus score</th>
<th>Hip.camp. score</th>
<th>Brainstem score</th>
<th>Cerebel. score</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2 (cytoplasm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIPC</td>
<td>0.6 (0.0–1.0)</td>
<td>0.0 (0.0–0.0)</td>
<td>3.4 (1.5–5.0)</td>
<td>1.9 (0.0–4.5)</td>
<td>1.1 (0.0–2.5)</td>
<td>6.9 (4.5–9.3)</td>
</tr>
<tr>
<td>CTRL</td>
<td>0.6 (0.0–1.0)</td>
<td>1.0 (0.0–2.0)</td>
<td>5.1 (4.5–6.0)</td>
<td>1.0 (0.0–2.0)</td>
<td>0.0 (0.0–0.0)</td>
<td>8.1 (5.0–10)</td>
</tr>
<tr>
<td>P-value</td>
<td>1.000</td>
<td>0.143</td>
<td>0.084</td>
<td>0.442</td>
<td>0.064</td>
<td>0.583</td>
</tr>
<tr>
<td>Caspase 3 (nucleus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIPC</td>
<td>5.6 (5.3–6.0)</td>
<td>4.4 (4.0–5.0)</td>
<td>5.5 (5.0–6.0)</td>
<td>5.4 (5.0–6.0)</td>
<td>4.6 (4.0–5.0)</td>
<td>25.5 (24.3–27)</td>
</tr>
<tr>
<td>CTRL</td>
<td>5.6 (5.3–6.0)</td>
<td>4.3 (3.5–5.0)</td>
<td>5.6 (5.5–6.0)</td>
<td>4.9 (4.0–6.0)</td>
<td>3.9 (3.5–4.5)</td>
<td>25.0 (23–26)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.727</td>
<td>0.883</td>
<td>0.593</td>
<td>0.281</td>
<td>0.138</td>
<td>0.622</td>
</tr>
</tbody>
</table>

RIPC n = 8, Control n = 8. Cerebel = cerebellum, CTRL = control, Hip.camp = hippocampus, Nrf2 = nuclear factor erythroid 2-related factor 2, RIPC = remote ischemic preconditioning. Immunohistochemical stainings of caspase 3 and Nrf2. Score: mean (IQR; 25th and 75th percentiles).

5.3 Summary of results

Table 11. Summary of the results.

<table>
<thead>
<tr>
<th>Study</th>
<th>Main results</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Enhanced MEP amplitude and onset latency responses.</td>
</tr>
<tr>
<td>II</td>
<td>Enhanced MEP amplitude responses. Better neuronal cell protection against oxidative stress indicated by Nrf2.</td>
</tr>
<tr>
<td>III</td>
<td>Faster recovery of serum S100B levels. Better regional neuronal cell protection against oxidative stress. Lower lactate levels along with cardioprotective effects.</td>
</tr>
</tbody>
</table>
6 Discussion

The operations of cardiac and aortic surgery involve the risks of postoperative neurological complications and mortality (Kouchoukos et al. 2013, Ziganshin et al. 2014, Kouchoukos et al. 2015). The brain and spinal cord are at the highest risk for deficits. Over the years, improved surgical techniques and adjuncts have reduced the incidence of adverse outcomes. The adjunctive protective strategies are still required to further reduce ischemic injury in these settings, especially when prolonged operation periods and bloodless operation fields are needed.

The research group of experimental surgery has been active for almost 20 years in Oulu, Finland. The main aim of this research group has always been to test adjunctive strategies and to test hypotheses in the field of complex cardiac and aortic surgery utilizing different experimental setups. The survival porcine models have formed the distinguished characteristics of the group. However, in these studies, the models were acute or subacute due to the newly introduced settings of spinal cord ischemia by our laboratory, and the focus of the studies was on the neuroprotective mechanisms underlying RIPC in the acute phase.

6.1 Experimental models

These studies were performed utilizing porcine models of spinal cord ischemia and DHCA. The pigs were healthy, young individuals compared with the heterogeneous human population involving medications, comorbidities, and some other confounding factors. It should be noted that these studies were experimental, limiting the exact correspondence to clinical cases, but still permitting hypotheses to be generated that are relevant for clinical settings.

6.1.1 Porcine models

Pigs provide excellent cardiovascular system correspondence with that of humans, excluding some exceptions. The usage of pigs required some characteristics to be considered in advance of the experiments. It should be noted that in humans the intercostal arteries arise from the aorta as two different branches, in contrast to the anatomy of pigs, with one branch dividing later into two branches. Additionally, in pigs the spinal cord receives the blood from the median sacral artery (MSA) in the lower parts, whereas in humans the hypogastric arteries feed the spinal cord.
Experimental limitations

These studies were performed mainly in acute models focusing on the underlying mechanisms of RIPC during the rapid phase (II, III), whereas Study I was subacute with a 24-hour follow-up period. The number of experiments was limited to ensure the ethical use of animals according to the three principles of reduction, refinement, and replacement along with economic reasons, as well.

6.2 Haemodynamic and metabolic data

The haemodynamic conditions were kept similar and stable throughout Studies I and II, and thus no interference with the spinal cord perfusion pressure, which is crucial for normal spinal cord blood supply, occurred. Study II demonstrated higher partial pressure of oxygen and lower partial pressure of carbon dioxide, suggesting better oxygenation systemically. More direct measurements on the spinal cord tissue might have been more reliable in assessing these findings.

In Study III, better cardiac index postoperatively demonstrated preserved cardiac function and the cardioprotective effects of RIPC. The surgical setting itself, including manipulation of the heart and DHCA, explains the release of the cardiac enzymes in both groups. The lower systemic lactate levels in the RIPC group indicate better surveillance under ischemic conditions and indirectly better cellular and mitochondrial functions.

6.3 Motor evoked potentials (MEPs)

The anaesthesia agents were carefully considered and controlled in the spinal cord studies (I, II), since the MEPs are extremely susceptible to different anaesthetics. Taking this into account, the results can be considered reliable, increasing the credibility of the studies.

6.3.1 Amplitudes

The motor evoked potential amplitude responses were coherent in Studies I and II; however, some points need to be discussed.

The left preconditioned hind limb responses were minor compared with the right leg in Study I. RIPC treatment might have caused a reversible peripheral nerve injury. Faster neurological recovery of the RIPC group rejects the irreversible injury.
Moreover, occluding the left subclavian artery caused interruption of left-side circulation and might have interfered with the MEP responses. However, the occlusion theory lacks support, since in Study II both hind limbs showed similar MEP amplitude responses, and the spinal cord ischemia was performed uniformly.

In Study II, after occlusion of the left subclavian artery, the ultimate peak in the peak-to-peak amplitudes was observed in both study groups bilaterally. The closure itself might serve as an ischemic preconditioning, and therefore the effect can be detected in control group, as well. In Study I, the control group did not demonstrate this same peak effect, which might be explained via differently selected time points while recording MEP responses.

In both studies, the effect was detected after RIPC but before any spinal cord ischemia. This induction could partly be explained via a response of the collateral network by increasing spinal cord perfusion from one source when another is reduced (Griepp & Griepp 2007). Moreover, the preconditioning method could be recognized as a cellular sensor preparing cells for upcoming ischemia.

### 6.3.2 Latencies

The MEP latency recordings differed between the two spinal cord studies. The shortened onset latency was detected only in Study I and in the right hind limb during the spinal cord ischemia procedure, indicating a considerably higher conductivity of motor responses in the spinal cord after RIPC. The lack of shortened latencies in Study II cannot be comprehensively explained since the study designs were kept similar.

Moreover, hyperthermia reactions in the control groups were detected in both studies postoperatively. Hyperthermia is known to reduce the latency and increase the conduction velocity of the MEPs, thus the differences in rectal temperature do not explain the result in favour of the RIPC group (Pajewski et al. 2007).

### 6.4 Remote ischemic preconditioning

In spinal cord studies, remote ischemic preconditioning was performed via two different methods, with a blood pressure cuff and a direct occlusion of a single iliac artery. Both methods provided protective actions against spinal cord ischemia as indicated by MEP responses. The beneficial effects were detected in both hind limbs, with minor variations between the studies.
The best method of delivering RIPC in spinal cord protection needs to be considered in clinical settings. The clinical application of RIPC in open TAAA surgery via iliac arteries provides an ideal application target, since the requirement to dissect and cross-clamp these arteries is included routinely as part of the operations and thus operative delays can be avoided (Ali et al. 2007). Moreover, the direct artery preconditioning could be transformed to endovascular procedures with balloon inflations and deflations.

On the other hand, RIPC by limb ischemia-reperfusion periods provides noninvasive, easily applicable, safe features benefiting especially cardiac and aortic arch surgery setups, which would be complicated due to direct artery occlusions.

6.5 Biochemical data

The study aims related to the underlying neuroprotective mechanisms behind RIPC focused on the analyses of different biochemical markers expressed systemically as well as behind the blood-brain barrier.

6.5.1 Proteomics analyses

Specific attention was given to apolipoprotein E, complement C3, transthyretin, and vitronectin since these proteins had crossed the blood-brain barrier. However, no significant differences were detected, suggesting that these proteins were unlikely to mediate neuroprotective effects of RIPC in this DHCA model, especially for this particular time frame.

The samples were pooled to ensure that all potential proteins were present and to reduce individual variations. Notably, the ability to statistically monitor differentially expressed proteins in response to the RIPC protocol was reduced due to the pooling. Moreover, the annotation for more than 50% of the detected proteins was impossible since there is a limited number of annotated porcine proteins in the public databases. To conclude, these uncharacterized proteins might have contributed to the remote ischemic preconditioning protection.

6.5.2 Metabolic analyses

The decline in $\alpha$KG and KYNA in vena cava samples following RIPC, opposite to their accumulation seen following pharmacologic or genetic inhibition of EGLN1 (Olenchock et al. 2016), could be explained due to a redox perspective, since
pseudohypoxia and ischemia are polar opposites. Moreover, the activity of HIF prolyl-4-hydroxylases differs during ischemia and reperfusion, which is opposed to conditional deletion.

6.5.3 S100B

The serum levels of S100B protein declined faster in the RIPC group, indicating better tolerance against ischemic insult of HCA. High concentration of S100B is associated with neurological toxicity. Moreover, S100B levels have importance in predicting neurological outcomes, blood-brain barrier permeability, and the severity of brain damage, with elevated levels indicating more unfavourable prognosis (Hu et al. 2010, Jensen et al. 2011, Sun et al. 2013, Yokobori et al. 2013).

6.6 Neurological recovery

In both studies, the trend towards better neurologic status was observed in the RIPC group. However, in the neurological assessment, the 6-hour follow-up (II) might have been too short to evaluate thoroughly the neurologic recovery since the ketamine-based anaesthesia has individual and sometimes long-lasting effects. In Study I, with a 24-hour follow-up, the animals had been recovered from the general anaesthesia, making the neurological assessment more reliable, but the existence of bias due to anaesthesia still needs to be considered.

Moreover, the overall mortality was relatively high in Study I. The overnight mortality was higher in the RIPC group (3) compared with the control group (1). The small group size might partly explain this result. The main reason for death was respiratory failure due to excessive mucus secretion, and thus the experimental model was refined with glycopyrrolate in Study II.

6.7 Histopathology

Histopathologic findings were modest in all three studies, as seen in the sum scores. These results can partly be explained due to short follow-up periods and small group sizes. Furthermore, the histopathology changes require time to develop and thus survival models benefit more from these analyses.

Postoperatively, hyperthermia reactions were detected in the control groups of both spinal cord studies (I, II). The possible effects on the histopathologic findings cannot be excluded completely.
6.8 Immunohistochemical data

Expression of caspase 3 plays a role in the induction of DNA fragmentation and is associated in the activation of apoptosis (Sakurai et al. 2003). In Study II, in both groups strong nucleus staining reactions were detected, indicating varying grades of spinal cord injury. Study III demonstrated different grades of apoptotic events occurring in both study groups according to the caspase 3 findings, as well.

During ischemia, Nrf2 expression is associated with neuronal cell protection (Shih et al. 2005). Cytoplasmic Nrf2 staining reactions were clearly in favour of the remote ischemic preconditioning group in all sections of the spinal cord, suggesting better neuronal cell protection against oxidative stress (Dong et al. 2010). Moreover, cytoplasmic Nrf2 staining reaction was in favour of the RIPC group in the cerebellum, implying better regional neuronal cell protection against oxidative stress.

Nucleus staining reactions of Nrf2 were negative in all studied samples, which might be explained due to technical reasons, as well as time factors. The movement of Nrf2 from cytoplasm to nucleus causes the induction of cytoprotective antioxidant genes, whereas the degradation of Nrf2 occurs in the cytoplasm (Shibata et al. 2008, Sun et al. 2011, Ganán-Gómez et al. 2013, van der Wijst et al. 2014). The results of the studies indicate higher Nrf2 expression during the procedures, and consequent cytoprotective actions.

6.9 Summary

In conclusion, important information related to spinal cord protection by RIPC treatment was provided by these studies. RIPC in a porcine model induces electrophysiological changes and preserves spinal cord function, along with supporting the theory of the collateral network concept securing spinal cord blood supply. RIPC performed via blood pressure cuff or direct artery occlusion could provide a novel and beneficial adjunctive strategy to prevent spinal cord ischemic injury in thoracic aortic aneurysm repairs.

In addition to this, RIPC-induced effectors do not necessarily have to be proteins; circulating metabolites, non-peptide hormones, or combinations of several factors are other possibilities. Higher expression of Nrf2 in association with RIPC protocols suggests better protection against oxidative stress, revealing possible underlying mechanisms behind RIPC, though comprehensive protective actions of RIPC remain unclear. Additional and more analytically in-depth research
needs to be performed to identify global changes in peptides, non-peptide hormones, and other metabolites following the RIPC protocol.

It is noteworthy that these studies were experimental and thus satisfied expectations to provide clinically relevant models in search of adjunctive protection strategies against ischemic injury, along with the aim to assess their effectiveness and underlying mechanisms against ischemic injury.

Over the years, RIPC has shown its beneficial effects in organ protection, and has subsequently been applied to clinical settings. Controversial results in clinical studies have aroused suspicions regarding its effectiveness; however, careful consideration of and understanding about the underlying protective mechanisms together with cautious selection of suitable patients and clinical settings to be employed might show its full potential, and these features require further evaluation.
7 Conclusion

Conclusion I

RIPC preserves spinal cord function after occlusion of the left subclavian artery and segmental arteries, as indicated by the MEP amplitudes.

Conclusion II

RIPC in advance of spinal cord ischemia induces significant electrophysiological changes in the CNS that may confer spinal cord protection. The higher Nrf2 scores indicate better neuronal cell protection against oxidative stress in the RIPC group.

Conclusion III

The faster recovery of serum S100B levels and lower systemic lactate confer better cellular and mitochondrial function after global ischemia by RIPC. The higher regional Nrf2 scores suggest possible better neuronal cell protection against oxidative stress in the RIPC group. Postoperatively, higher cardiac index confirms the cardioprotective effects of RIPC.

Specific protein mediating the effect of RIPC was not identified in a porcine model of DHCA. RIPC-induced effectors do not necessarily have to be proteins, as circulating metabolites, non-peptides hormones, or combinations of several factors are other possibilities.
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Original publications


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Original publications are not included in the electronic version of the dissertation.
1405. Lepojärvi, Sannamari (2017) Normal variation of the tibiotalar joint in dynamic computed tomography
1408. Wang, Qin (2017) Epidemiological applications of quantitative serum NMR metabolomics: causal inference from observational studies
1410. Forstt, Anna-Kaisa (2017) Incidence, mortality, comorbidities, and treatment of bullous pemphigoid in Finland
1411. Asrnivala, Henri (2017) Deformational plagiocephaly: prevalence, quantification and prevention of acquired cranial asymmetry in infants
1412. Taurainen, Tuomas (2017) Complications associated with preoperative anemia, perioperative bleeding and blood transfusions after isolated coronary artery bypass grafting
1414. Pasanen, Ilkka (2017) Stromal cells of mesenchymal origin in breast cancer
1415. Kunnari, Marika (2017) Aikusväestön hyvinvointiin liittyvät huolet ja hyvinvoinnin heikentäjät
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