INTESTINAL TRANSPLANTATION

Experimental and clinical studies

Mihai Oltean

UNIVERSITY OF GOTHENBURG

Departments of Surgery and Transplantation
Institute of Clinical Sciences
Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden
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A Doctoral Thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted, or in manuscript)
“Make everything as simple as possible but not simpler”

Albert Einstein
Intestinal transplantation. Experimental and clinical studies
Mihai Oltean
Departments of Surgery and Transplantation, Institute of Clinical Sciences at Sahlgrenska Academy, University of Gothenburg
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ABSTRACT

Intestinal preservation-reperfusion injury may result in various degrees of mucosal injury. Interestingly, the preservation injury is similar when using the current preservation solutions, which are given as a intravascular flush. An extensive mucosal injury may ultimately preclude the use of organs that require longer preservation time. The intestine lacks a noninvasive rejection marker, as in the case of liver or kidney transplantation. Several bio-molecules have been suggested as biomarkers, yet their specificity is only partial.

Methods: Using a rat intestinal transplant model we studied the pharmacologic donor preconditioning and the intraluminal preservation with two different macromolecular solutions as means to decrease the intestinal preservation-reperfusion injury. We also investigated the impact of donor preconditioning on the ensuing systemic inflammatory response after transplantation. We analyzed resistin levels after clinical intestinal transplantation and seek to establish its significance and potential as rejection marker.

Results: Intraluminal introduction of low-sodium macromolecular solutions resulted in improved morphology after 8h and 14h of preservation compared with controls receiving only vascular flush with UW-solution. Moreover, intraluminal high-sodium solutions appear detrimental. These solutions also seem to influence differently the TJ conformation during preservation and de-localization of claudin-3 and ZO-1 was more prominent in intraluminal high-sodium solutions.

Following transplantation, pretreated grafts showed accelerated repair and improved morphology. Pretreated grafts revealed reduced NF-kappaB activation after reperfusion and subsequently blunted ICAM-1 expression and PMN sequestration. Pretreated graft recipients had milder liver injury and lower levels of the pro-inflammatory cytokines TNF-alpha, IL-1beta and IL-6 than recipients of untreated grafts.

Resistin levels were studied in seven patients receiving intestinal grafts. Resistin increased in all patients compared with controls and remained increased even during uneventful course. Resistin did not correlate with CRP, BMI, procalcitonin or WBC and it varied greatly between patients.

Conclusions: Preservation-reperfusion injury may be mitigated by the intraluminal introduction of macromolecular solutions or by donor pretreatment with Tacrolimus before graft harvesting. Tacrolimus-pretreated grafts trigger a lower remote organ injury and lower systemic inflammatory response. Plasma resistin levels greatly and were increased in all patients. However, the increase was unspecific and varied between individuals. Resistin appears unsuitable as rejection marker after intestinal transplantation.

Keywords: intestinal preservation, ischemia-reperfusion injury, tight junction, tacrolimus, resistin.

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LIST OF PUBLICATIONS

The thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV. Sustained elevations in plasma resistin, an inflammatory adipokine, after clinical intestinal transplantation. Oltean M, Herlenius G, Gäbel M, Karlsson-Parra, A, Olausson M. *Manuscript*
Tarmen är det organ som transplanteras mest sällan, delvis pga ett välfungerande alternativ i form av näringsdropp delvis pga allvariga komplikationer såsom avstötning eller infektioner som inte sällan leder till döden. Många av dessa komplikationer uppkommer som följd av en skadad tarmslemhinna. Slemhinnans integritet är viktigt för de två huvudfunktionerna. Dessa är upptagning av näringsämne från tarmen och barriärfunktionen, dvs att förhindra bakterierna som normalt finns i tarmen att ta sig till blodet och ge upphov till livshotande infektioner. En av de viktigaste moment när signifikant slemhinneskada förekommer är under och strax efter preservationstiden, dvs mellan uttaget från organdonatorn tills blodet släpps på genom transplantatet i mottagaren. Under preservationen förvaras organet i ett speciellt isbad efter har blivit genomspolat med speciella lösningar. Den låga temperaturen minskar ämnesomsättningen och fördröjer, men hindrar inte utvecklingen av skador under tiden organet måste förvaras fram till transplantationen.

I denna avhandlig har jag studerat två metoder att minska preservationskadan och dess effekter efter tarmtransplantation. Den ena metoden var att fylla tarmen med två olika lösningar som huvudsakligen skiljer sig endast genom Natrium innehållet. Lösningarna är kommersiellt tillgängliga och består egentligen av de tarmrengöringspreparat som används rutinmässig idag. Vi har upptäckt att införseln av lösningar med lågt natrium innehåll kan fördröja utvecklingen av slemhinneskada. Däremot kan högt Natrium innehåll kan innebära vätskeansamlingen i vävnaden och därmed vara skadlig.

Den andra metoden som jag har beskrivit var att framkalla skyddande proteiner i transplantatet genom en enkel behandling av tarmdonatorn. Läkemedlet som jag har studerat redan används inom transplantation, men ges vanligtvis till mottagaren.

Behandlingen visade en starkt skyddande effekt, förbättrad vävnadsarkitektur och även stimulerande effekter på vävnadsreparationen. Samtidigt upptäckte jag att behandlingen blockerar utsöndringen av vissa molekyler och faktorer som i ett senare skede även kan stimulera inflammation, som i sin tur kan framkalla organsvikt.

Efter tarmtransplantation är man idag tvungen att ta vävnadsprover för att diagnostisera förekomsten av avstötning, eftersom det inte finns blodprov som kan hjälpa ställa diagnosen, såsom vid njur- eller levertransplantation. Jag har studerat om ett visst protein (resistin), som visade sig öka i blodet på patienter med skov av inflammatorisk tarmsjukdom, kan användas som avstötningsskickl, och det har analyserat patientprover och har upptäckt att proteine tarmresistin ökar som förväntat, men detta sker även vid andra sammanhang såsom allvarliga infektioner. Detta begränsar det diagnostiska värden av resistin, men fyndet är mycket intressant och kan leda till en bättre förståelse av detta proteins egentliga roll i samband med andra sjukdomar.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>5</td>
</tr>
<tr>
<td>LIST OF PUBLICATIONS</td>
<td>6</td>
</tr>
<tr>
<td>POPULÄRVETENSKAPLIG SAMMANFATTNING</td>
<td>7</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>9</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>11</td>
</tr>
<tr>
<td>HISTORY OF INTESTINAL TRANSPLANTATION - A WORK IN PROGRESS</td>
<td>11</td>
</tr>
<tr>
<td>INDICATIONS, SURGICAL TECHNIQUES AND RESULTS</td>
<td>12</td>
</tr>
<tr>
<td>OBSTACLES, COMPLICATIONS AND SPECIAL ISSUES IN INTESTINAL TRANSPLANTATION</td>
<td>14</td>
</tr>
<tr>
<td>HIGH INTESTINAL SUSCEPTIBILITY TO ISCHEMIA-REPERFUSION INJURY</td>
<td>14</td>
</tr>
<tr>
<td>HIGH INTESTINAL IMMUNOGENICITY</td>
<td>14</td>
</tr>
<tr>
<td>IMMUNOSUPPRESSION AND ITS COMPLICATIONS</td>
<td>15</td>
</tr>
<tr>
<td>HIGH INCIDENCE OF ACUTE REJECTION</td>
<td>15</td>
</tr>
<tr>
<td>THE ABDOMINAL CAVITY</td>
<td>16</td>
</tr>
<tr>
<td>ISCHEMIA-REPERFUSION INJURY</td>
<td>16</td>
</tr>
<tr>
<td>BIOCHEMICAL AND MOLECULAR EVENTS DURING ISCHEMIA AND REPERFUSION</td>
<td>16</td>
</tr>
<tr>
<td>Transcription</td>
<td>17</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>18</td>
</tr>
<tr>
<td>LEUKOCYTE-ENDOTHELIAL INTERACTIONS</td>
<td>19</td>
</tr>
<tr>
<td>The endothelial interface</td>
<td>19</td>
</tr>
<tr>
<td>Tissue inflammation and phagocytosis</td>
<td>20</td>
</tr>
<tr>
<td>THE STRESS RESPONSE</td>
<td>20</td>
</tr>
<tr>
<td>The heat shock proteins- HSP</td>
<td>20</td>
</tr>
<tr>
<td>Danger activated molecular patterns – DAMP</td>
<td>21</td>
</tr>
<tr>
<td>THE SYSTEMIC CONSEQUENCES OF ISCHEMIA-REPERFUSION</td>
<td>22</td>
</tr>
<tr>
<td>The cytokine release</td>
<td>22</td>
</tr>
<tr>
<td>PATHOPHYSIOLOGY OF INTESTINAL ISCHEMIA-REPERFUSION INJURY</td>
<td>23</td>
</tr>
<tr>
<td>RELEVANT MICROSCOPIC ANATOMY</td>
<td>24</td>
</tr>
<tr>
<td>The intestinal lumen and the intestinal mucosa</td>
<td>24</td>
</tr>
<tr>
<td>The capillary ‘hairpin’ and the countercurrent exchange</td>
<td>24</td>
</tr>
<tr>
<td>The polarity of the intestinal epithelial cell</td>
<td>24</td>
</tr>
<tr>
<td>The junctional complex and the tight junctions</td>
<td>25</td>
</tr>
<tr>
<td>MAIN FEATURES DURING INTESTINAL ISCHEMIA AND REPERFUSION</td>
<td>26</td>
</tr>
<tr>
<td>REDUCING ISCHEMIA-REPERFUSION INJURY IN TRANSPLANTATION</td>
<td>27</td>
</tr>
<tr>
<td>MINIMIZING THE PRESERVATION INJURY</td>
<td>27</td>
</tr>
<tr>
<td>DONOR PRETREATMENT</td>
<td>28</td>
</tr>
<tr>
<td>Tacrolimus/FK506</td>
<td>29</td>
</tr>
<tr>
<td>Intestinal preservation</td>
<td>29</td>
</tr>
<tr>
<td>ACUTE REJECTION</td>
<td>30</td>
</tr>
<tr>
<td>MARKERS OF INTESTINAL ACUTE REJECTION</td>
<td>30</td>
</tr>
<tr>
<td>LYMPHOCYTES, CYTOKINES AND REJECTION</td>
<td>31</td>
</tr>
<tr>
<td>The CD8+ and CD4+ cells and the cellular rejection</td>
<td>31</td>
</tr>
<tr>
<td>The pro-inflammatory cytokines (Th1)</td>
<td>32</td>
</tr>
<tr>
<td>The anti-inflammatory cytokines (Th2)</td>
<td>33</td>
</tr>
<tr>
<td>RESISTIN</td>
<td>33</td>
</tr>
<tr>
<td>CONCLUDING INTRODUCTORY REMARKS</td>
<td>34</td>
</tr>
<tr>
<td>AIMS OF THE THESIS</td>
<td>35</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>37</td>
</tr>
<tr>
<td>PATIENTS</td>
<td>37</td>
</tr>
<tr>
<td>PATIENT MANAGEMENT AND SAMPLING</td>
<td>38</td>
</tr>
<tr>
<td>ANIMALS</td>
<td>EXPERIMENTAL SURGICAL PROCEDURES AND SAMPLING</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Intestinal transplantation in the rat (II, III)</td>
<td>Intestinal preservation (I)</td>
</tr>
<tr>
<td>Tissue and blood sampling (I, II, III)</td>
<td>Mean arterial pressure, heart rate and microcirculation (III)</td>
</tr>
</tbody>
</table>

| HISTOLOGY AND OTHER TISSUE ANALYSES AND TESTS | 40 |
| HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY |
| Neutrophils |
| Tight junctions (I) |
| Cell proliferation (II) |
| DISSACHARIDASE ASSAY (I, III) |
| WESTERN BLOT PROTEIN ANALYSIS (II, III) |
| ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) (II) |
| CASPASE ACTIVITY ASSAYS (II, III) |

| CYTOKINES, RESISTIN AND ENDOTOXIN IN PLASMA | 43 |
| ASSESSMENT OF PLASMA CYTOKINES USING ELISA (III) |
| ASSESSMENT OF PLASMA CYTOKINES USING MULTIPLEX ASSAY (IV) |
| ASSESSMENT OF PLASMA RESISTIN (IV) |
| ASSESSMENT OF PLASMA ENDOTOXIN (III) |

| STATISTICAL ANALYSES | 45 |
| RESULTS | 47 |
| PAPER I | 47 |
| PAPER II | 48 |
| PAPER III | 48 |
| PAPER IV | 50 |
| GENERAL DISCUSSIONS | 53 |
| CONCLUSIONS | 59 |

| REFLECTIVE STATEMENTS | 61 |
| ACKNOWLEDGEMENTS | 62 |
| REFERENCES | 64 |
| ABBREVIATIONS | 79 |
| APPENDIX – PAPER I-IV | 81 |
INTRODUCTION

The ischemic injury remains a major hurdle for a broader clinical application of the intestinal transplantation, since it may give rise to life-threatening infectious complications and triggers an intense systemic inflammatory response. The thesis explores two different experimental approaches intended to mitigate the preservation/reperfusion injury. Finally, the time-course and the significance of resistin after clinical intestinal transplantation is analysed.

HISTORY OF INTESTINAL TRANSPLANTATION – A WORK IN PROGRESS

Although not described in detail, the first transplantation of intestinal segments in dogs has been reported by Alexis Carrel in the early 1900s. Experimental work was re-initiated in the mid-1950s by Richard Lillehei from the University of Minnesota, particularly focusing on intestinal preservation and the surgical technique [1, 2]. The confidence regarding the technical feasibility, together with the wave of transplantation optimism in the early ‘60s, owing to several newly available immunosuppressants (steroids, azathioprine) led to the first clinical attempt in 1967 by Lillehei [3]. Alas, this was followed by fierce rejection, sepsis and patient death within 3 weeks. Other sporadic cases were followed by similar catastrophic results and intestinal transplantation virtually ceased over 1970s and ‘80s. Fortunately, the introduction of total parenteral nutrition (TPN) provided a salutary alternative for the patients with short bowel syndrome (SBS), representing the main patient population candidate for intestinal transplantation [4].

The advent of cyclosporine in the early 1980 renewed the interest for intestinal transplantation. The efficient immunosuppression and accumulating experience from transplanting other organs allowed the teams of Eberhard Deltz from Kiel, David Grant from London (Canada) and Olivier Goulet in Paris to achieve long term survival following combined or isolated intestinal transplantation (1989) [5-7]. These initial successes were later taken over, expanded and consolidated at the University of Pittsburgh during the 1990s [8, 9]. Refined surgical techniques and immunosuppressive regimens as well as protocols for patient selection and management were implemented, finally leading to continuously improving results and increasing frequency of the procedure [10-12]. As of 2009, intestinal transplantation has been performed in about eighty transplant units worldwide.
However, fewer than thirty centers currently perform intestinal transplantations routinely (David Grant, Intestinal Transplant Registry report, Bologna 2009).

The first two transplantations including a substantial part of the intestine in Sweden and in the Nordic countries were performed as cluster procedures in Gothenburg already in 1990. One patient survived for 11 months, while the other patient died at the time of surgery. A year later, the first transplantation of an isolated intestinal segment was attempted in a child by Staffan Meurling in Uppsala, but unfortunately, the recipient succumbed due to rejection and sepsis fifty days later. A second unsuccessful attempt was performed in Stockholm shortly thereafter. The first successful transplantation of an isolated intestine has been performed in Gothenburg by a team led by Gustaf Herlenius in 2007. The first combined liver-intestinal transplantation was performed in 2001 by Michael Olausson and Gustaf Herlenius while the first successful multivisceral transplantation has been performed already in the year 1998 by Michael Olausson, at the transplant unit in Gothenburg, Sweden [13].

INDICATIONS, SURGICAL TECHNIQUES AND RESULTS

Parenteral nutrition has achieved extended success for the majority of patients requiring prolonged treatment, however, complications leading to failure of TPN increase with the duration of therapy. These complications range from frequent infectious complications originating from the catheter or progressive thrombocytopenia to loss of venous access due to repeated thrombosis and cholestatic liver disease with or without portal hypertension [14, 15]. Intestinal transplantation is therefore considered in irreversible intestinal failure (IIF) patients with impending liver failure and frequent catheter-related septic complications, including vanishing central venous access [16-18].

Irreversible intestinal failure, the permanent reduction in the functional intestinal mass has different etiology in adult and pediatric population. Short bowel syndrome (SBS), whether congenital, functional or surgical is the main cause for IIF. In children, the main indication for intestinal transplantation is SBS after neonatal abdominal catastrophes, leading to massive intestinal loss (gastroschisis, necrotizing enterocolitis, volvulus)[19, 20]. Another group of indications are the intestinal functional diseases (microvillus inclusion disease, malabsorbtion, secretory diarrhea) or motility disorders (Hirschprung disease, pseudoobstruction).
In adults, massive intestinal loss is consecutive mainly to Crohn’s disease, ischemia and trauma. Certain gastrointestinal tumors requiring extensive intestinal resections as well as radiation enteritis are also among the causes behind IIF/SBS in adults [19, 21].

As briefly mentioned above, the intestine may be transplanted using three different approaches, namely as an isolated intestinal segment, together with the liver in the presence of liver failure (combined liver-intestinal transplantation) or as part of a composite graft that may contain other viscera (multivisceral transplantation) [22, 23]. When performing an isolated intestinal transplant the arterial inflow is achieved by anastomosing the graft’s superior mesenteric artery (either directly or through arterial grafts) to the recipient aorta. The venous drainage is performed either directly into the inferior vena cava (systemic drainage) or by anastomosing the graft’s portal vein to a branch of the recipient portal vein, usually the superior mesenteric vein (portal drainage), with similar metabolic results. During combined liver-intestine and multivisceral transplantations, the continuity of the portal axis is maintained and the hepato-intestinal graft is drained through the liver veins [22, 24, 25]. At the end of the transplantation, an ileostomy (terminal or lateral) is always performed to allow for the intestinal endoscopies, essential for rejection monitoring.

According to the latest Small Bowel Transplant Registry report (Bologna, September 2009), 2291 intestinal transplants have been performed in 2061 patients at 86 centers worldwide. Results are continuously improving due to increased expertise as well as refined patient selection and posttransplant management. Several analysis performed on this material found that results are dependent on pretransplant patient status (hospitalized vs. waiting at home), the inclusion of the liver into the graft and center volume (at least ten cases performed in total)[19].

Currently, one year survival rate is around 75% and five year survival rate is just above 50%, with the longest surviving patient now twenty years after transplantation. The main causes of patient loss are the infectious complications (≈50%), followed by rejection (≈ 10%) [26, 27].
OBSTACLES, COMPLICATIONS AND SPECIAL ISSUES IN INTESTINAL TRANSPLANTATION

The limited number of intestinal transplants performed to date compared with other transplantable organs explains the complexity of the procedure. The transplant candidates are often malnourished, with frequent hospitalizations, sometimes presenting with growth retardation. The very long and technically challenging surgical procedure frequently takes place in a scarred abdomen with extensive adhesions and fistulae following previous abdominal surgery. Several unique issues further complicate the management of intestinal graft recipients.

HIGH INTESTINAL SUSCEPTIBILITY TO ISCHEMIA-REPERFUSION INJURY

The intestine safely tolerates only up to 8-10 hours of cold ischemia, as compared with the 14-16h affordable in the case of the liver graft or the 24h or more in the case of the kidney [12, 28, 29]. The intestinal graft is unique among the transplantable organs due to its contaminated content. Bacteria and bacterial products may spread out in the recipient in case of mucosal barrier damage secondary to preservation-reperfusion injury, leading to early sepsis outbursts [30]. Therefore, the critical issue of alleviating ischemia-reperfusion injury, including by shortening the cold ischemia time puts considerable pressure on the procurement team, transplant coordinators and the transplantation team [12].

HIGH INTESTINAL IMMUNOGENICITY

Due to the expression of major histocompatibility complex (MHC) class 2 molecules on the enterocytes, the intestine has a very high immunogenicity [31-33]. The immunogenicity is further augmented by the great lymphocyte load, present within the mucosa, mesenteric lymph nodes and in the Peyer patches (gut-associated lymph tissue –GALT) [34, 35]. As a first consequence, acute rejection (AR) occurs and is directed against the epithelial layer of the intestinal mucosa and may lead very rapidly to mucosal barrier breakdown, mucosal ulcerations with subsequent bacterial translocation and ensuing sepsis [30]. The critical vascularization of the intestine, in particular at the villus level makes that any impending endothelial damage during AR may result in ischemia-like changes, further jeopardizing the mucosal barrier integrity [36]. In addition, a massive gut-associated lymphoid tissue (GALT) transfer may, at least theoretically, increase the risk of graft versus host disease (GVHD)[37, 38].
IMMUNOSUPPRESSION AND ITS COMPLICATIONS

The increased immunogenicity requires a more intensive immunosuppressive regimen compared with other organs [39, 40]. This most often includes antibody induction and relatively high Tacrolimus trough levels [41]. This intense immunosuppressive treatment renders the patient susceptible for bacterial, viral and fungal infections [42, 43]. Infections have a high incidence and their range is very broad [42]. The differential diagnosis with AR, based on the clinical findings and routine investigations is often impossible, particularly during enteritis [44]. Thus, it is often necessary to obtain endoscopic biopsies to exclude AR, since a wrong or delayed therapy may bear catastrophic consequences.

Besides increasing the risk of infections, the intense immunosuppressive treatment greatly increases the risk of malignancy, particularly post-transplant lymphoproliferative disease (PTLD) [45]. It also carries the cumulative risk, as well as nephrotoxicity that often leads to chronic kidney disease and even renal failure [46].

HIGH INCIDENCE OF ACUTE REJECTION

Despite intensive immunosuppression, AR is a greater hazard in intestinal than in any other organ transplantation, occurring in about 40-60 % of the cases [47, 48]. The early clinical signs of AR are unspecific (fever, nausea, increased stomal output) and there is no universally accepted serum marker of intestinal rejection, as in the case of kidney and liver. The diagnosis of AR is made on biopsies obtained through invasive and frequent endoscopies, requiring trained personnel, involving supplementary costs and time. Furthermore, it submits the patient to discomfort and potential complications [49]. Delay in the diagnosis and treatment by just hours to days may result in rapid progression from mild to severe exfoliative rejection, which is associated with a substantial risk of graft loss (up to 93%) and high mortality (50–70%) [50].

Several non-invasive markers have been suggested to be useful in diagnosing or predicting the development of AR [51-54]. Unfortunately, despite high sensitivity, many of these markers have low specificity or kinetics that limits their use during the early post-transplant period, when most of AR episodes are occurring [48, 55, 56].
THE ABDOMINAL CAVITY
Contracted abdominal cavity due to the content loss, previous stomas and fistula formation. This “loss of domain” is a major challenge when closing the operative wound over the transplanted intestine without the risk of, “abdominal compartment syndrome” [57, 58]. Several approaches to overcome this complication have been described to date, this including the use of prosthetic materials (Alloderm), vacuum-assisted abdominal closure (VAC), staged closure or transplantation of the abdominal wall [59, 60].

ISCHEMIA-REPERFUSION INJURY
Ischemia and reperfusion (IR) are unavoidable events in organ transplantation. This will result in complex metabolic and structural changes, whose extent mainly depends on the length of ischemia, surrounding temperature and type of tissue. Paradoxically, the reperfusion and subsequent reoxygenation initiate a cascade of biochemical and molecular changes that may lead to additional injury.

BIOCHEMICAL AND MOLECULAR EVENTS DURING ISCHEMIA AND REPERFUSION
Oxidative stress
At the core of the reperfusion injury lays an event occurring during ischemia, namely the adenosine triphosphate (ATP) degradation [61]. Upon reoxygenation, hypoxantine, an end-product of the anaerobic ATP degradation will be metabolized by xantine oxidase and generate oxygen free radicals (OFR) including the hydroxyl radical, superoxide and nitroperoxide ions [62-65]. The oxidative stress occurs early (i.e., minutes) after reoxygenation and will inflict further damage to numerous cellular elements such as mitochondria, proteins, nucleic acids and cellular membranes [65]. The stress may lead either to immediate cell death (necrosis), controlled cell death (apoptosis) or trigger changes of the cell phenotype in response to the injury (activation).
Besides local tissue injury, the oxidative stress may also act on the red blood cells increasing cell stiffness, reducing deformability and creating conditions for lysis. When lysis occurs, released free heme exacerbates the oxidative process, further generating OFR and a harmful iron chelate, which may promote deleterious cellular processes such as oxidative membrane damage.
These events occur in all cell types of the reperfused organ, yet the vascular endothelium is particularly important in this setting because of its role of interface between the intravascular and the extravascular compartments (Figure 1).

**Transcription**
The oxidative stress, either directly by OFR or through lipoperoxidation products, may initiate gene transcription as adaptation in response to the injury. Several transcription factors are activated by the oxidative stress, with nuclear factor-κB (NF-κB) as one of the most prominent [66, 67]. NF-κB has a central role in inflammation and innate immunity. NF-κB exists mainly as heterodimer, retained inactive in the cytoplasm by an inhibitory unit (IκB) and requires a signalling pathway for activation [68, 69]. Following IκB phosphorylation by IκB kinases (IKK), the IκB releases the heterodimer, which translocates into the nucleus, binds to specific DNA promoter sequences and initiates gene transcription. Among the numerous genes whose expression is regulated by NF-κB, there are adhesion molecules (vascular cell adhesion molecule (VCAM)-1, intercellular adhesion
molecule (ICAM-1), matrix metalloproteinases (MMP-2, MMP-9) and other genes that regulate endothelial cell survival, vasodilatation and angiogenesis. In addition to that, NF-κB promotes the expression of various genes essential in the inflammatory response such as TNF-α, IL-1, IL-6, IL-8, and GM-CSF [70] (Figure 2).

**Figure 2. The key molecular events leading to the activation of NF-κB**

*Apoptosis*

Apoptosis is an organized, energy-dependent cascade of events leading to controlled DNA fragmentation and cell death, followed by removal of the debris without inflammatory response. Apoptosis may be triggered by signals from within the cell (the intrinsic pathway) or by extracellular signals (the extrinsic pathway)[71, 72]. Although the initial stages of these two pathways are completely different, both pathways are mainly driven by a similar family of cysteine proteases (i.e., caspases) activated in cascade [71]. Moreover, the two apoptotic pathways converge at one point (caspase-3 activation) to follow a common route leading to the activation of endonucleases and DNA fragmentation [73]. Long time in the development of apoptosis the cellular membrane is maintained intact. In the final stages it fragments and surrounds the nuclear debris and the organelles to form the apoptotic bodies [74]. Recent evidence indicates that apoptotic cell death can signal neutrophil emigration in endotoxin shock and that blocking apoptosis with an
inhibitor of caspases can prevent neutrophil extravasation and hepatocyte necrosis [75, 76].

**LEUKOCYTE-ENDOTHELIAL INTERACTIONS**

The transcriptional changes triggered by IR injury generate complex changes towards an inflammatory phenotype in the surviving cells of the injured tissue, a process called activation [77, 78]. In addition, the various cell types in the injured tissue release numerous biomolecules (cytokines, chemokines) that may act as pro-inflammatory mediators [79, 80]. Secondary to their local or systemic release, these mediators both activate the endothelium and act upon circulating cells (mainly leukocytes) and promote leukocyte recruitment and enhanced leukocyte-endothelial interactions [81-84].

*The endothelial interface*

The leukocyte-endothelial interaction is initiated by rolling along the endothelial cell surface mediated by glycoproteins belonging to the selectin family, which are found on the surface of both leukocytes (L-selectin), platelets (P-selectin) and endothelial cells (P- and E-selectins) [85]. This weak leukocyte tethering to the vessel wall brings the leukocytes closer to chemoattractants that include “classical” chemoattractants (e.g., leukotriene B4, C5a, platelet activating factor (PAF) and the chemoattractant chemokines [86]. The tethering continues to firm adhesion, followed by transmigration into the tissue to remove the debris at the site of injury [87, 88]. The transmigration requires the expression of selectins and integrins (CD11a, CD11b, and CD11c/CD18 expressed on the leukocytes) and endothelial adhesion molecules (members of the immunoglobulin superfamily, e.g., ICAM-1, -2, -3, or VCAM-1) [89-92].

An essential factor for leukocyte extravasation is the chemotactic stimulus that attracts the leukocyte towards the site of injury. Chemokines are potent chemotactic factors and members of this class of mediators can have selective chemotactic properties for neutrophils, lymphocytes, monocytes, or eosinophils [93]. In addition, general cell injury may produce other chemotactic factors (i.e., leukotrienes) [94].
**Tissue inflammation and phagocytosis**

Leukocyte accumulation in the tissue, i.e., the inflammation, is the final phase of the response to injury [95, 96]. Temporally distinct patterns of expression of adhesion molecules and chemokines offer some specificity to the inflammatory infiltrate.

Although its primary role is the removal of tissue debris through phagocytosis, leukocyte infiltration paradoxically worsens the local injury [97]. It has long been thought that the oxidative stress upon reperfusion leads to cell death by lipid peroxidation. However, the early lipid peroxidation cannot entirely explain the prolonged and severe cell injury observed during reperfusion. The ability of antiproteases to reduce the late reperfusion injury revealed that polymorphonuclear neutrophils (PMN) themselves can cause tissue damage by releasing several proteolytic enzymes such as the elastase from cytoplasmic granules and by producing free radicals via the respiratory burst [98-101].

Another possible mechanism through which leukocytes can aggravate the reperfusion injury is by plugging the capillaries and thus impairing the microcirculation (the “no-reflow” phenomenon) [102, 103].

As a consequence and proof of these hypotheses, reduced leukocyte infiltration has been repeatedly associated with improved structure and function after IR injury [104-106].

**THE STRESS RESPONSE**

*The heat shock proteins- HSP*

Oxidative stress and temperature increase are major stimuli for heat shock protein (HSP) induction and graft reperfusion with warm, oxygenated blood leads to massive upregulation of various HSP [107-110].

HSPs are constitutive or inducible proteins mainly named according to their molecular weight. HPSs are primarily involved in protein homeostasis (chaperoning, folding and translocating proteins intracellularly). However, numerous studies signaled beneficial effects of preemptive HSPs overexpression in reducing IR injury as well as several interesting immunomodulatory roles [111, 112].

The molecular mechanisms behind the HSP-mediated cytoprotection certainly involve more than one pathway [113-116]. One mechanism suggested has been the HSP-mediated modulation of NF-κB activation [117-121]. HSP 60 may stimulate the innate immune response by acting as a ligand for several Toll-like receptors (TLR),
particularly TLR 4 [118, 122]. On the other hand, in macrophages submitted to heat stress, NF-κB DNA binding following lipopolysaccharide (LPS) exposure was effectively reduced, supposedly by preventing IκB degradation following its phosphorylation [123, 124]. The inhibition of NF-κB activation has been recorded when upregulating both members of the HSP70 and HSP32 (also known as HO-1) families. HO-1 has important roles in heme degradation, but previous studies also demonstrated that HO-1 may play many other vital functions in cellular homeostasis. It has also been suggested that the initial antioxidative protection conferred by the HO-1 and its byproducts will reset the molecular machinery of the injured cells [121, 125]. Hence, the response to stress leads to a protective phenotype. NF-κB pathway is redox sensitive and may be influenced by the antioxidant effects of HO-1 and its byproducts, while biliverdin may act as kinase by itself [121, 126]. Given its extreme pleiotropism, NF-κB inhibition by HSPs may explain many molecular events following heat-shock preconditioning [127-129]. Thus, HSP induction successfully reduced neutrophil sequestration both in models of warm ischemia reperfusion [129] and transplantation probably through a combination of cytoprotective effects, reducing the initial tissue damage as well as by interfering with various intracellular signaling pathways [121, 130, 131]. Several studies revealed that different HSP can activate dendritic cells and macrophages, making the HSP a large family of endogenous danger signals [132-135]. In nontransplant setting, this would have limited consequences; however in a transplantation setting these signals can prime and precipitate the alloimmune response [111, 136, 137].

_Danger activated molecular patterns – DAMP_

Recently, a new group of molecules has been described and grouped under the terms 'alarmins' or 'endokines'. These are either released by necrotic cells or actively secreted by activated cells after injury or stress response [118]. The molecules have been collectively suggested as danger-associated molecular patterns (DAMPs), in close similarity with PAMPs (pathogen-associated molecular patterns), a system within the innate immunity that alerts the organism to intruding pathogens and activates several signaling pathways [138]. Conversely, the extracellular presence of normal cell constituents released into the extracellular milieu during states of cellular stress or damage and may also signal cell injury and subsequently activate the immune system [139].

21
The ‘danger signal’ model proposed by Matzinger suggests that the immune system recognizes and reacts to damage signals in the body regardless of their origin [140]. The signaling cascade is initiated following the recognition of the danger molecule by the toll-like receptor (TLR) family, present on the membrane of numerous cell types [141]. After first being funneled by the myeloid differentiation primary response gene 88 (MyD88), the intracellular signal transduction pathways will ultimately lead to the activation of transcriptional factors such as NF-κB, or the expression of genes related to inflammatory and immune responses [142, 143]. A growing list of biomolecules has been proposed as DAMPs, including High mobility group box 1 (HMGB1), the S100 proteins, defensins, fibrinogen, soluble hyaluronan, hepatoma-derived growth factor (HGDF), uric acid, several HSPs, and IL-1α [144-149].

**THE SYSTEMIC CONSEQUENCES OF ISCHEMIA-REPERFUSION**

Apart of the organ-specific systemic effects observed after advanced ischemic injury (renal failure after severe renal IR, liver failure after liver IR), injury and dysfunction may occur in organs remote from the site of IR. Hepatic IR injury may lead to various degrees of acute cardiac, lung or kidney injury while lower body ischemia encountered during abdominal aorta aneurysm repair generates a strong inflammatory response and frequently, multiple organ dysfunction [150-155]. Moreover, intestinal IR is frequently associated with signs of liver, respiratory or kidney failure and significant associated mortality [156-158]. The exact mechanism behind the impairment of distant organs is unclear, though an excessive, sepsis-like inflammatory response is thought to underlie this phenomenon [159-161].

*The cytokine release*

Upon reperfusion, the oxidative stress stimulates several intracellular signaling pathways, ultimately leading to the transcriptional activation of numerous genes, including various pro-inflammatory cytokines [80, 162]. The transcription products depend on the degree of injury and cell type and their biologic effects are pleiotropic, often redundant, following the interaction with the equivalent structures (receptors). Most of the cytokines and chemokines have pro-inflammatory effects, but there are also molecules with anti-inflammatory role (i.e IL-4, IL-10, IL-13) [163, 164]. Pro-inflammatory cytokines act on mononuclear phagocytes or lymphocytes, modulating innate or acquired immunity [165]. Extensive evidence signals increases in tumor necrosis factor-alpha (TNF-α) in
various models of ischemia-reperfusion [166-168]. TNF-α is the prototype pro-
inflammatory cytokine and it is released by monocytes/macrophones, T lymphocytes, Natural killer cells (NK cells) and many other cell types found throughout the body. TNF-α activates the NF-κB transcription pathway, IL-6 and tissue factor secretion, has direct cytotoxicity, up-regulates adhesion molecules, stimulates nitric oxide (NO) synthesis and release, activates neutrophils and induces fever [169]. TNF-α may trigger apoptosis after binding to the death receptors and may also promote synthesis and release of other cytokines, most notably IL-1 and IL-6 [168, 170, 171].

Interleukin-1 (IL-1) is synthesized in precursor form and secreted under two forms (IL-1α and IL-1β) with similar actions. IL-1 mediates its biological effects after binding a membrane receptor that engages signal transduction pathways activating NF-kB and activator protein -1 (AP-1) transcription factors. At low concentrations, IL-1 is a local inflammation mediator (stimulates leukocyte adhesion on endothelium) but at high concentrations it acts as endogenous pyrogen and induces the synthesis of acute phase proteins in the liver [172].

Interleukin 6 (IL-6) is released by monocytes/macrophages, T lymphocytes, endothelial cells, fibroblasts, keratinocytes following TNF-α stimulation [173]. It is a major inductor of the acute phase response, stimulates B cell growth and increases NK activity as well as T cell differentiation [172, 174]. It increases in disease states such as malignancy, burn injury, pancreatitis or sepsis, and it is believed to have the potential of a prognostic indicator for mortality [175]. This is supported by several studies that revealed that deceased septic patients had higher levels of IL-6.

**PATHOPHYSIOLOGY OF INTESTINAL ISCHEMIA-REPERFUSION INJURY**

The pathophysiology of ischemia-reperfusion injury generally follows the steps mentioned earlier, irrespective of organ. However, due to structural and functional characteristics, each organ has several specific patterns of developing the injury. The following briefly outline the main features and consequences of the intestinal ischemia and reperfusion.
RELEVANT MICROSCOPIC ANATOMY

The intestinal lumen and the intestinal mucosa

An essential difference compared to other transplantable organs is that the intestine is a hollow organ, containing an epithelium with selective permeability. The different cell types making up the epithelium have a rapid turnover. From their origin in the crypts, where a self-renewing population of stem cells normally balances loss of effete enterocytes, the enterocytes continuously move upward towards the villus tip [176]. In the steady state in normal animals, around 10 new cells are produced in each crypt per hour. Thereafter, the enterocytes migrate from the crypt to the villus tip, a process that takes 2 to 3 days. At the tip, the enterocytes enter apoptosis and finally detach into the lumen [177].

The capillary ‘hairpin’ and the countercurrent exchange

Another morphologic feature relevant for the development of ischemic injury, particularly during hypotension and shock, is the hairpin shape of the capillaries inside the villus. Although capillaries run along the entire length of the villus, the proximity of the afferent and efferent capillary loop at the villus base may allow a short-circuit of the oxygen between these two. Consequently, a countercurrent exchange, oxygen shunting and a subsequent oxygen gradient between base and tip of the villus may occur, generating a relative hypoxia towards the villus tip.

The polarity of the intestinal epithelial cell

The enterocyte is a polarized cell, with the apical membrane biochemically and functionally different from the basolateral membrane [35]. Thus, the apical membrane is involved in digestion, absorption and secretion while basolateral membrane has basically only transport roles [178]. Cells are held together by junctional complexes, structures with selective permeability and rest on the basal membrane, an acellular structure covering the connective matrix that builds up the villus axis (lamina propria) [179]. Besides the matrix, lamina propria contains various cell types (e.g, lymphocytes), capillaries and lacteals [180, 181].
The junctional complex and the tight junctions

As mentioned above, all polarized epithelial cells are held together by an intricate structure called the junctional complex. Besides its mechanical role the junctional complex fulfills a dual function: a fence function, preventing the mixing of membrane proteins between the apical and basolateral membranes and a gate function which controls the intercellular passage of ions and solutes between the cells, preventing them to diffuse back into the lumen through the intercellular space. In certain physiological circumstances, but also in disease, the tight junctions’ (TJ) permeability may increase, allowing an increased flow of water and solutes, through the paracellular pathway [182-184].

Cell-to-cell adhesion is also provided by the desmosomes, button-like intercellular contacts that attach the cells together. Desmosomes are formed by a dense cytoplasmic plaque, connected by intracellular filaments to the cytoskeleton. The firm adhesion between plaques is provided by transmembrane proteins belonging to the Cadherin family [185, 186].

Figure 3. The intercellular space in a polarized epithelium (adapted after [186])

The TJ is the most apical component of the junctional complex and functions as the “fence” separating apical from basolateral domains. Several tight junction-associated proteins have been evidenced, such as occludin, zonula occludens-1 (ZO-1) and claudins. The protein content of this complex regulates the gate function.
of the paracellular space [187, 188]. Thus, the majority of channels established by claudin interactions regulate paracellular permeability and allow the passage of cations.

The junctional complex also contains the gap junctions. These mediate communication between cells by allowing small molecules to pass directly from cytoplasm to cytoplasm of adjacent cells [186].

**MAIN FEATURES DURING INTESTINAL ISCHEMIA AND REPERFUSION**

The intestinal ischemic injury presents as progressive subepithelial edema that will ultimately lead to the breakdown of the mucosal barrier. A subepithelial cleft (the Gruenhagen space) appears within one hour at the villus tip and extends towards the villus base, cleaving the epithelium from the lamina propria [189].

The continuity of the epithelium is ultimately lost and lamina propria will be exposed to the lumen, sustaining further structural damage. Extended ischemia leads to changes and structural alterations in the deeper intestinal layers. This sequence of events has been signaled both during normothermic and hypothermic ischemia and forms the basis of the Park score for grading of the intestinal ischemic injury [190].

At ultrastructural level, electron microscopy revealed structural mitochondrial alterations, an apparent redistribution of the intracellular organelles (lysosomes, endoplasmic reticulum), intracytoplasmic protein accumulations as well as widening of the intercellular space including proteinaceous deposits [191]. Upon reperfusion and the initial worsening, the repair process starts with flattening of the villi and increased lateral migration of the enterocytes to seal the denuded areas [192, 193]. Villus contraction has also been reported [194]. This is followed by intense proliferation in the crypts and, depending on the degree of injury, complete structural restoration within 24-48 hours. However, if advanced crypt damage (grade 6 or more on the Park scale) the complete restoration is unlikely and if the subject survives the reperfusion, chronic changes are likely to occur [195].

Microcirculation is also significantly impaired following intestinal IR and intestinal transplantation. This is due to leukocyte adhesion, direct impairment of the endothelium [103, 196, 197] or vasoactive mediators released at reperfusion [198].
REDUCING ISCHEMIA-REPERFUSION INJURY IN TRANSPLANTATION

Experimental and clinical studies indicated that IR injury can be successfully mitigated using a myriad of chemical or biological compounds as well as several maneuvers eliciting a biologic protective response (hyperthermia, ischemic preconditioning) [199-201]. However, most of these studies were performed by clamping and unclamping vascular pedicles (so-called warm ischemia/reperfusion) whereas the transplant setting implies several particularities such as the removal of the organ from the initial milieu, a period of hypothermia, additional warm ischemia as well as denervation. All these features generate several essential differences at cellular and metabolic level and thus only several of these observations are applicable in transplant setting [121, 202, 203]. Below the relevant approaches mitigating IR injury in transplantation will be briefly reviewed.

MINIMIZING THE PRESERVATION INJURY

Shortening cold ischemia time is by far the most significant measure to alleviate IR injury in transplantation [28, 204]. Slowing ATP depletion and maintaining viable ATP stores is very important during the energetic and metabolic recovery at reperfusion [205]. In addition, hypothermia reduces the anaerobic cell metabolism and slower catabolite formation in the ischemic organ. Perfusion with special solutions antagonizes the electrolyte shifts secondary to membrane pump dysfunction (mainly the Na/K ATPase). Moreover, the preservation solutions aim to prevent the osmotic water shifts and subsequent cell swelling, acidosis as well as attenuating the oxidative stress. All preservation solutions currently used generally follow these principles while sharing common characteristics and having particular traits [206].

Belzer-University of Wisconsin (UW) cold storage solution (Viaspan ®) revolutionized organ preservation in late 1980s and it is still considered the gold standard for abdominal organ preservation . It has an 'intracellular composition' containing a high potassium concentration (130 mmol/l) as well as high-molecular-weight impermeants (lactobionic acid and raffinose preventing intracellular edema secondary to ischemia), oncotic support (hydroxyethyl starch, HES), and redox agents (glutathione and allopurinol) mitigating the oxidative stress during reperfusion [207, 208].

An alternative preservation solution increasingly used is Histidine-triptophane-ketoglutarate (HTK, Custodiol ®). Similar to Belzer-UW solution, it has a powerful
buffer system (histidine) and impermeants (ketoglutarate) but its electrolyte contents resemble the extracellular milieu (high sodium and low potassium). Increasing evidence suggests that HTK solution yields comparable short term results with Belzer-UW solution for the preservation of livers and kidneys and also proved safe for intestinal preservation [209, 210]. Recent United Network for Organ Sharing (UNOS) registry analyses question its long term efficacy [211-213].

Celsior is another preservation solution with an ‘extracellular’ composition. It appears like a hybrid of the previous two solutions and combines reduced glutathione as well as high-molecular-weight impermeants (mannitol and lactobionic acid) like UW solution and high sodium concentration (100 mmol/l), low potassium and histidine as in HTK solution. Celsior solution proved to be suitable for preserving livers [214], kidneys and pancreas [215].

IGL-1 is a newly designed preservation solution intended for the preservation of abdominal organs developed by the French Institute George Lopez (IGL). It has similar key ingredients as Belzer-UW solution, but an ‘inverse’ composition (low potassium, high sodium), while using a new osmotic agent (polyethylene glycol, PEG 35) instead of HES. A recent clinical trial found IGL-1 at least comparable with Belzer-UW solution in kidney transplantation while an experimental study reveals similar results in liver transplantation [216, 217].

DONOR PRETREATMENT

The rationale for the interventions in the organ donor is the preemptive initiation of endogenous protective mechanisms within the graft that will be transferred with the organ and ultimately alleviate IR injury after reperfusion in the recipient. Two main types of interventions can be recognized, namely the ischemic and the pharmacologic preconditioning.

Ischemic preconditioning (IP) is defined as a brief period of ischemia followed by reperfusion prior to a sustained ischemic episode. IP has been reported to attenuate the tissue damage observed after IR in the heart, liver, kidney and intestine [218-220]. Moreover, the protective effect of IP has been demonstrated for normothermic ischemia and in transplantation setting [199, 221-223]. The mechanisms are only partly unraveled and appear exquisitely intricate and complex. The protective mechanisms behind IP include the involvement of HSP, nitric oxide, adenosine, inhibition of apoptosis, as well as the modulation of several kinases and signaling pathways [223-227].
Pharmacologic preconditioning (PP) aims at triggering one or several endogenous protective mechanisms following the administration of a drug or chemical compound. Several main mechanisms are operational, encountered either alone or in combination, so that virtually all the cellular and molecular events occurring during IR may be influenced by PP. Thus, the pharmacologic induction of a heat shock response, either through up-regulation of HSP32 (heme oxygenase-1, HO-1) or HSP70 has been shown to greatly reduce both the early damage due to IR and the chronic changes triggered by an advanced initial ischemic insult [228-230]. Brief treatments with immunosuppressive drugs or receptor blockade reduced the IR presumably through the modulation of tissue inflammation after reperfusion [231, 232].

**Tacrolimus/FK506**

Tacrolimus (TAC) is an immunosuppressive drug with a macrolide structure, currently used in liver, kidney, heart, pancreas and intestinal transplantation. Its immunosuppressive action is due to the inhibition of the phosphatase activity of calcineurin, that ultimately results in the inhibition of the nuclear factor of the activated T cells (NF-AT) and suppressed production of IL-2, essential for the clonal expansion of the T cells [233].

However, besides its immunosuppressive properties, a number of other biological actions were signaled [234-237]. Among these, numerous reports indicated a protective effect of TAC against ischemia reperfusion in various settings and suggested a multitude of putative mechanisms [238-242]. Common mechanisms identified by many studies were the modulation of the early inflammation and the decreased oxidative stress.

A single study explored the pretreatment the organ donor with TAC to reduce the ischemia/reperfusion-related changes after kidney transplantation [232]. Although the early reperfusion injury was only briefly analyzed, the study found improved morphology and long term function in animals receiving TAC pretreated renal grafts.

**Intestinal preservation**

Several standard preservation solutions have been tested for the intestinal preservation. Although superior to crystalloid solutions, such as Ringer or saline,
none provided consistently satisfactory results beyond 8-10 hours. The unsatisfactory results triggered intensive search for alternative solutions or approaches for the preservation of intestine. These included the experimental addition of chemicals (including gaseous compounds) mainly to the Belzer-UW solution that frequently resulted in some molecular and physiologic improvements. However, no notable prolongation of the cold storage time, while being able to achieve only mild ischemic injury, was reported [243, 244]. Another concept was represented by the intraluminal delivery of preservation solution or different tailored combinations [245-247]. This repeatedly resulted in superior morphology after extended preservation time and in some cases improved bioenergetics, suggesting that addressing the luminal compartment may prove beneficial in terms both of maintaining acceptable graft morphology and allowing the safe prolongation of the cold ischemia time [244, 248]. Since the cellular interactions maintaining the mucosal barrier, including the functioning of tight junction are energy-dependent processes, these approaches may definitely represent a particularly promising solution. A modification of the later alternative was represented by the intraluminal introduction of UW solution, followed by the immersion in continuously oxygenated perfluorodecaline (PFD), a variation of the “two layer method” (TLM) previously described in pancreas transplantation [249]. This strategy allowed a 75% recipient survival after 24 hours of cold storage, otherwise leading to 100% mortality. However, the approach is cumbersome and demands elaborated logistics.

**ACUTE REJECTION**

A detailed description of the rejection mechanisms is beyond the scope of this chapter. Below a short outline of several circulating markers and mediators during rejection, including cytokines is given.

**MARKERS OF INTESTINAL ACUTE REJECTION**

Unlike other transplantable organs, the intestine lacks a noninvasive rejection marker. Several tests or bio-molecules have been suggested throughout the years, but they have all ultimately been refuted [52, 250]. In the recent years, the nonessential aminoacid citrulline has been evaluated clinically [55, 251]. Citrulline level is a good indicator of the enterocyte mass and low levels of citrulline have
correlated with rejection [252]. Despite several drawbacks, such as the low levels during the first month after intestinal transplantation, a period when most rejection episodes occur, and dependence on the renal function, citrulline is considered a promising candidate for a noninvasive marker of intestinal acute rejection [56].

An equally interesting candidate-marker is fecal calprotectin. Calprotectin is present in several leukocyte subsets as well as constitutively in the cytoplasm of enterocytes. It is usually released as a result of cell disruption and death or during increased enterocyte shedding and consequently, calprotectin is considered an endogenous danger signal [253].

Increased fecal calprotectin has been reported during active inflammatory bowel disease [254]. A recent study identified increased calprotectin levels in the stomal effluent of patients with intestinal acute rejection, a finding that was later confirmed independently [53, 255]. However, the increase seems rather unspecific, hence independent investigators suggest its use as noninvasive screening marker to document the absence of rejection and thus reduce the need for blank endoscopies. Both these two markers are imperfect, and the search for a more accurate rejection marker continues.

LYMPHOCYTES, CYTOKINES AND REJECTION

The CD8+ and CD4+ cells and the cellular rejection

Cellular rejection starts with the recognition of the foreign antigens by the recipient immune cells during the process of allore cognition [256]. After antigen presentation to naïve T cells, some of these become activated, proliferate by clonal expansion, turn into antigen-specific cytotoxic T lymphocytes (CTLs, CD8+), attack the allograft and destroy it. This process usually takes days to weeks [257]. T cell proliferation requires several soluble molecules (cytokines) such as IL-2 and interferon-gamma (IFN-γ). These are provided either by the CD8+ cells themselves (autocrine) or by the CD4+ T-helper 1 cells.

The CD4+ T-helper 1 (Th1) cells constitute the other major effector limb of the T-cell response to an organ graft. After antigen stimulation, Th1 cells secrete cytokines, such as interferon-gamma (IFN-γ) and TNF-α. These cytokines have important pro-inflammatory actions and further stimulate the synthesis of various cytokines, stimulate B cell growth as well as antibody production. CD4+ T-cells may also
activate monocytes and macrophages, cell types that feature prominently in the cellular infiltrate during allograft rejection.

T-helper cells are also able to manifest anti-inflammatory effects; the subgroup is designated as Th2. The shift towards a pro-inflammatory or anti-inflammatory profile seems to depend on the cytokine environment and the original stimulus, but the exact circumstances that govern the shift are incompletely known [172, 257, 258]. The cytokine network is redundant and one cytokine may sometimes exert often opposite effects on the same cell type, depending on the presence of other factors in the microenvironment.

Two subsets of activated CD4+ T cells were primarily identified, i.e. the Th1 and Th2 cells. Th1 cells had IFN\(\gamma\) as the typical cytokine while Th2 cells produced IL-4 [259]. This straightforward, yet oversimplified classification has been repeatedly challenged and new functional subsets, such as the regulatory T cells (Tregs) or the Th-17 cells have been recognized [260]. However, for the sake of simplicity the literature still refers to a group of several pro-inflammatory cytokines as to Th1 cytokines and to some anti-inflammatory cytokines as Th2 cytokines [172].

**The pro-inflammatory cytokines (Th1)**

**IL-1\(\beta\)** and **TNF-\(\alpha\)** share a multitude of pro-inflammatory properties and appear to be critical to the amplification of mucosal inflammation in inflammatory bowel disease (IBD). Both cytokines are primarily secreted by monocytes and macrophages upon activation, and induce intestinal macrophages, neutrophils, fibroblasts, and smooth-muscle cells to elaborate prostaglandins, proteases, and other soluble mediators of inflammation and injury. Among the effects of TNF-\(\alpha\) in the intestine are the disruption of the epithelial barrier and induction of apoptosis of the villous epithelial cells. TNF-\(\alpha\) activates the endothelium and induces the expression of cytokines and chemokines. TNF-\(\alpha\) also activates neutrophils and macrophages and stimulates the production of IFN-\(\gamma\) by mucosal T cells.

**IL-2** is produced by naïve T cells after activation, simultaneously with the IL-2 receptor, in a classical autocrine and paracrine loop, promoting clonal expansion. IL-2 has important effects on other cells, including B cells, monocytes, and NK cells. **IL-8** is produced and released by endothelial cells and epithelial cells and is chiefly involved in neutrophil chemotaxis to the site of injury (chemokine).
IL-12 seems important for driving Th1 responses and IFN-γ production in the initial phases of an immune response, but conversely IL-12 may play a subsequent immunoregulatory role in late-stage inflammation.

IFN-γ has critical roles in both the innate and adaptive immunity. Among its many roles in the adaptive immunity are the upregulation of MHC class I and II expression, the endothelial activation and T cell adhesion and extravasation, as well as the differentiation of naïve CD4+ T cells towards a Th1 phenotype are directly instrumental in the development of rejection [172, 258].

The anti-inflammatory cytokines (Th2)
Th2 cytokines are thought to reduce the severity of allograft rejection by inhibiting the Th1-mediated cytotoxic lymphocyte (CTL) and delayed-type hypersensitivity (DTH) responses. These cytokines are IL-4, IL-5, IL-10, and IL-13.

IL-4 is the prototypic Th2 cytokine, polarizing activated CD4+ T cells to a Th2 phenotype. Systemic treatment with IL-4 of rat heart recipients significantly delayed rejection and inhibited Th1 responses within the graft, regional lymph nodes and spleen [261]. IL-4 also promotes the development of T-regs (CD4+CD25+Foxp3+) and thus the induction of transplantation tolerance [262].

IL-10 is produced by macrophages and inhibits macrophages and dendritic cells, thus creating a negative feedback loop. It inhibits the expression of MHC class II and diminishes Th1 cell activity by suppressing secretion of IL-2 and IFN-γ. IL-10 also inhibits the secretion of the pro-inflammatory cytokines IL-6, IL-8, IL-12, TNF-α, thereby attenuating mucosal inflammation [172]. The pivotal role played by IL-10 within the mucosal immune system has been extensively studied in mice lacking IL-10, that develop colitis due to an uncontrolled macrophage activation reacting to the intestinal bacteria [263]. Interestingly, IL-10 reduced the severity of both local and systemic inflammation in a murine model of intestinal ischemia-reperfusion when given either before or after reperfusion, allegedly by blocking the local cytokine production and remote organ inflammation [167]. Recent evidence indicates that IL-13 can prolong allograft survival and modify graft rejection by inhibition of dendritic cell and/or macrophage function [264, 265].

RESISTIN
Resistin is a recently described polypeptide, first identified in the adipose tissue (but not the leukocytes) of obese mice. Experimental evidence suggested that
resistin, together with adiponectin and leptin is involved in diet-induced insulin resistance, lipoprotein metabolism, obesity and atherosclerosis [266-268]. However, the clear cut conclusions from the animal studies seem only partly valid in humans. The protein sequences of murine and human resistin are only approximately 60% identical while the main resistin source in humans seems to be represented by the leukocytes. While a relationship between resistin and obesity and vascular disease is less clearly defined in humans [269-273], increasing evidence links resistin with inflammation and innate immunity. Thus, resistin increased after endotoxin challenge in healthy volunteers [274] and it was found increased in critically ill patients, proportional with the severity of the disease [275, 276]. Furthermore, resistin was normally found in the amniotic fluid and increased levels were reported during amniotic infection [277] and in the synovial fluid of patients with rheumatoid arthritis [278].

Two studies found increased plasma resistin in patients with inflammatory bowel disease [279, 280]. Resistin correlated with the activity of the disease as well as with C reactive protein (CRP) and white blood count (WBC). Subsequently, resistin has been suggested as a marker of an active intestinal inflammation.

The pathways through which resistin manifest its pro-inflammatory effects are quite unclear, yet it seems resistin can bind to TLR2 and TLR4 and the downstream signaling may involve p38, JNK MAP and NF-κB [281, 282].

There are only a few studies of the adipokines in transplanted patients. All reported elevated resistin levels after kidney transplantation [283, 284]. Although the information is scarce, it seems that that resistin levels were higher in kidney graft recipients than in normal individuals even in the absence of complications (rejection, infection).

**CONCLUDING INTRODUCTORY REMARKS**

Considering all the issues briefly summarized herein and the actual level of knowledge in intestinal transplantation, it is fair to conclude that we need to considerably increase the available relevant information on both preservation/reperfusion injury and rejection to improve both short and long term graft and patient survival.

This thesis explores two different experimental approaches intended to mitigate the preservation/reperfusion injury. Finally, the time-course and the significance of resistin after clinical intestinal transplantation are analyzed.
AIMS OF THE THESIS

The specific aims of this thesis were:

- to study if intraluminal introduction of different macromolecular solutions has beneficial effects on the preservation injury of the rat intestine
- to determine if donor pretreatment with a single dose of tacrolimus improves the preservation-reperfusion injury after rat intestinal transplantation
- to investigate if transplantation of intestines from donors pretreated with tacrolimus results in a different pattern of postoperative inflammatory response and remote organ injury after rat intestinal transplantation
- to analyze the adipokine resistin after clinical intestinal transplantation and assess its putative roles
MATERIALS AND METHODS

The main methods used in this thesis are shortly described below. More detailed descriptions are found in the ‘Materials and methods’ sections of papers I-IV.

PATIENTS

The patient material is represented by seven adult patients receiving intestinal allografts at the Transplant Institute/Sahlgrenska University Hospital in Gothenburg. Patient characteristics (age, diagnosis, type of graft, pretransplant body mass index) are summarized in Table 1. Informed consent was obtained at the time of the pretransplant evaluation.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>BMI</th>
<th>Diagnosis</th>
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<td>22,3</td>
<td>IPO</td>
<td>MV</td>
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<tr>
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<td>MV</td>
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<td>Crohn’s disease, SBS</td>
<td>MV+S</td>
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<td>20</td>
<td>20,8</td>
<td>IPO</td>
<td>MV</td>
</tr>
<tr>
<td>#5</td>
<td>F</td>
<td>22</td>
<td>17,8</td>
<td>SBS</td>
<td>IT</td>
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<tr>
<td>#6</td>
<td>M</td>
<td>48</td>
<td>25,6</td>
<td>Portomesenteric thrombosis</td>
<td>MV</td>
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<tr>
<td>#7</td>
<td>F</td>
<td>32</td>
<td>21</td>
<td>NEPT</td>
<td>MV</td>
</tr>
</tbody>
</table>

Table 1. Patient demographics, diagnosis, pretransplant body mass index and type of graft received: Abbreviations: M/F - male/female, BMI – body mass index, IPO – intestinal preusoobstruction, NEPT – neuroendocrine pancreas tumor with liver metastasis, SBS – short bowel syndrome, MV – multivisceral graft, MV+S-multivisceral and spleen, IT – isolate intestinal graft.

The intestine was transplanted either alone (one patient) or as part of a multivisceral graft (six patients) or using previously described surgical techniques [22]. The multivisceral grafts consisted in the stomach, duodenum, liver, pancreas and the small intestine.
The immunosuppression regimen consisted of antithymocyte globulin (Fresenius) induction with Tacrolimus (TAC) monotherapy in a steroid-free protocol described previously by the Pittsburgh group [39]. Target levels of TAC were 10 ng/mL during the first 6 months after transplantation and tapered thereafter to approximately 5 ng/mL by the end of the first year posttransplantation. Rejection was treated with a steroid bolus and thereafter the steroids were quickly tapered. Steroid resistant rejection episodes were treated with OKT-3 (Orthoclone).

**PATIENT MANAGEMENT AND SAMPLING**

Tacrolimus blood through levels, white cell blood count and CRP were obtained daily. Procalcitonin (PCT) was analyzed whenever clinically indicated. Rejection surveillance was achieved by the means of protocol ileoscopies performed twice a week during the first month starting at the end of the first posttransplant week, then weekly after the first month.

After informed consent, heparinized blood samples were obtained weekly for different time intervals after transplantation. Most of the samples (40/46) were taken during the first 8 weeks after transplantation. Samples were immediately centrifuged and plasma was collected, aliquoted and stored at -70 ºC until analyzed.

**ANIMALS**

Male SD rats (190-250 grams) were purchased from B&K Universal (Sollentuna, Sweden) and housed in the Experimental Biomedicine department of the University of Gothenburg, in 12 hours light-dark cycles. The studies followed the rules and regulations outlined by NIH and the European Union and had the approval of the local committee of the Swedish Animal Welfare Agency.

In papers I and II, TAC (Astellas, Osaka, Japan) at a dose of 0,3 mg/kg or equivalent volumes of saline were given intravenously to the donors six hours before graft harvesting (n = 30/group). Five untreated non-operated animals served as reference for histological and biochemical analyses
EXPERIMENTAL SURGICAL PROCEDURES AND SAMPLING

Intestinal transplantation in the rat (II, III)

Graft procurement and transplantation were performed as previously described by Kellersmann et al [285]. In brief, the first half of the intestine was transplanted heterotopically using microvascular anastomoses and microsurgical techniques. The arterial inflow was supplied from recipients’ aorta while the venous drainage was systemic, into the inferior vena cava. Graft perfusion and preservation were performed using iced saline (Papers II and III) or Belzer-UW solution (paper I).

Intestinal preservation (I)

Immediately after in-situ perfusion, the grafts were weighed and two different solutions having different compositions were introduced intraluminally. The compositions of the solutions are given in Table 2. In the control group, intraluminal solutions were omitted. Each graft end was ligated and the grafts were stored in 80 ml ice-chilled perfusion solution, without supernatant air. After eight, fourteen or twenty hours of cold storage the intraluminal solution was evacuated and recovered and the grafts were blotted dry and weighed, then tissue pieces were either placed in 4% buffered formalin or snap-frozen (n=10/time-point).

<table>
<thead>
<tr>
<th></th>
<th>UW</th>
<th>HSS</th>
<th>LSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium (mmol/L)</td>
<td>120</td>
<td>10</td>
<td>5,4</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>30</td>
<td>125</td>
<td>65</td>
</tr>
<tr>
<td>Magnesium (mmol/L)</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloride (mmol/L)</td>
<td>-</td>
<td>35</td>
<td>53</td>
</tr>
<tr>
<td>Sulphate (mmol/L)</td>
<td>5</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bicarbonate (mmol/L)</td>
<td>-</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>Glutathione (mmol/L)</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HES (mmol/L)</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose (mmol/L)</td>
<td>30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactobionate (mmol/L)</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PEG-3350 (g/L)</td>
<td>-</td>
<td>11,38</td>
<td>13,125</td>
</tr>
<tr>
<td>Allopurinol (mmol/L)</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenosine (mmol/L)</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>7,4</td>
<td>7,94</td>
<td>8,11</td>
</tr>
</tbody>
</table>

Table 2. Composition of the solutions used; UW-University of Wisconsin solution, HSS - high-sodium solution, LSS - low-sodium solution (Paper I)
Tissue and blood sampling (I, II, III)

Twenty minutes after reperfusion a three-cm-long graft segment was sampled from the distal graft end to study the early reperfusion injury and pieces were either placed in formalin or snap-frozen. In Paper II the recipients of either pretreated and control grafts assigned for the 12 or 24h endpoints underwent blood sampling from the tail after one or three hours of reperfusion.

After six, twelve or twenty-four hours, animals were reanesthetized and various measurements performed. Thereafter blood was collected through terminal heart puncture using endotoxin-free materials, spun and plasma was then stored at -76°C until analysed. Graft and liver tissue were obtained and stored in formalin or snap-frozen using the same technique.

Mean arterial pressure, heart rate and microcirculation (III)

Mean arterial pressure was recorded six, twelve and twenty-four hours after reperfusion, after cannulating the right femoral artery. After allowing animal to stabilize for 10 minutes, blood pressure and heart rate were recorded for further 10 minutes using a BioPac system (Harvard Apparatus, Cambridge, MA).

Superficial liver microcirculation was studied using laser Doppler Flowmetry (LDF). We used an adhesive probe connected to a Periflux 4001 base unit (Perimed, Järfälla, Sweden) gently applied on the liver surface. At least seven measurements were performed at different sites. The median of the measurements was calculated for each animal and the results were expressed as arbitrary Perfusion Units (PU).

HISTOLOGY AND OTHER TISSUE ANALYSES AND TESTS

Paraffin sections were cut and stained with hematoxylin-eosin. In Paper I and Paper III, intestinal grafts were examined blindly and the ischemia-reperfusion injury was graded according to the Park scoring system (Table 3)[190].

Morphometrical analyses were also performed (mucosal thickness, villus height). In Paper III paraffin liver sections were evaluated blindly regarding overall liver architecture, leukocyte adherence, inflammation and necrosis.
<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>normal mucosa</td>
</tr>
<tr>
<td>1</td>
<td>subepithelial space (bleb) at the villus tip</td>
</tr>
<tr>
<td>2</td>
<td>more extended subepithelial space (upper half of the villi)</td>
</tr>
<tr>
<td>3</td>
<td>epithelial lifting down the sides of the villi</td>
</tr>
<tr>
<td>4</td>
<td>epithelial breakdown</td>
</tr>
<tr>
<td>5</td>
<td>denuded villi</td>
</tr>
<tr>
<td>6</td>
<td>crypt layer infarction</td>
</tr>
<tr>
<td>7</td>
<td>transmucosal infarction</td>
</tr>
<tr>
<td>8</td>
<td>transmural infarction</td>
</tr>
</tbody>
</table>

Table 3 Park scoring system of the intestinal ischemic damage (Paper I, II).

HISTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Neutrophils

Intragraft or liver PMNs were stained using the Naphtol AS-D chloroacetate esterase kit (Sigma Chemicals, St.Louis, MO) on paraffinized sections. All samples were coded and positively stained PMNs were identified and counted in a blinded fashion at intermediate magnification (x200).

Tight junctions (I)

Immunofluorescence was used to study the co-localization of the two tight junction proteins zonula occludens-1 (ZO-1) and claudin-3 (double staining). Briefly, the slides were incubated overnight at 4°C with anti-ZO-1 (1:100, Invitrogen, Stockholm, Sweden) and anti-claudin-3 (1:100, Abcam, UK). Slides were then incubated with secondary antibody conjugated with Alexa 488 and Alexa 594 (1:100, Invitrogen), counterstained with DAPI and examined under the fluorescence microscope.
Cell proliferation (II)
Deparaffinized slides were incubated with Ki67 antibody (Novocastra, Newcastle/Tyne, UK) according to manufacturers’ instructions. Ki67 positive nuclei were blindly counted in fifteen randomly selected crypts sectioned transversally in three different sections under high magnification (x400).

Dissacharidase assay (I, III)
We measured the activity of sucrase and maltase, two dissacharidases found on the enterocyte brush border. Whole tissue homogenates were incubated with a substrate solution (2% maltose in 0.1 mol/L sodium maleate buffer, pH 6.0) for 1 h at 37°C according to the method of Dahlqvist [286]. The reaction was stopped by submerging the samples in boiling water for 5 min. One unit of maltase was defined as the quantity of enzyme hydrolyzing 1 μmol/L maltose to glucose in 1 minute. The resulting glucose was measured using the hexokinase assay and results were expressed as activity units/gram protein.

Western blot protein analysis (II, III)
Frozen intestinal or liver tissue was homogenised in ice-cold lysis buffer (0.2 M Tris-HCl, 0.1M NaCl, 0.1M EDTA, 0.5M DMSF, 1% Triton X-100, pH-7.4). Protein concentration in the supernatant was measured, and then proteins were separated by sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were transferred onto polyvinylidene fluoride (PVDF membranes) (Bio-Rad, Hercules, CA). The membranes were blocked in 5% skim milk and incubated for one hour with anti-HSP-72 antibody (1:3000, SPA-812, StressGen). Secondary antibody was added, and then blots were developed by the use of Advanced ECL kit (Amersham Biosciences Ltd, Buckinghamshire, UK).

For ICAM-1 determination, protein extraction, concentration measurements and blotting were performed as described above. Membranes were incubated with a mouse monoclonal antibody against ICAM-1 (1:3000, MCA773 Serotec, Oxford, UK) and an adequate secondary antibody. After development, the bands from immunoblots were quantified using computerized densitometry (Quantity One, BioRad). Results were normalized to β-tubullin and reported as optical density units (OD).

Electrophoretic mobility shift assay (EMSA) (II)
Frozen intestinal tissue was gently homogenized in 2 ml of hypotonic buffer at 4°C. Following centrifugation at 4°C 14000 g for 10 minutes, the supernatant was
carefully removed and the pellet was resuspended in ice-cold extraction buffer and extracted 2 hours at 4°C on a rotator. Cell particles were sedimented at 14000g at 4°C for 1 hour and the supernatant was collected, aliquoted and stored -80°C until the analysis of NF-κB was performed. EMSA was performed as described elsewhere [287]. Briefly, the double stranded consensus oligonucleotide (sc-2505, Santa Cruz Biotechnology, San Diego CA) was labeled with 32P (Amersham Pharmacia Biotech, Uppsala, Sweden) using T4-polynucleotide kinase (New England Biolabs, Ipswich, MA) and used in the binding reaction with the nuclear extracts. In some binding reactions 1 µl of an antibody against the p65 subunit of NF-κB (Santa Cruz Biotechnology, CA) was added and incubated for another 20 minutes (the supershift). The same mixture using unlabelled oligonucleotides were used as negative control. The protein-DNA complexes were resolved on a native 5% polyacrylamide gel, then the gel was vacuum-dried and exposed to x-ray film for 24-36 hours at -80°C. Band intensity was analyzed using computerized densitometry.

**CASPASE ACTIVITY ASSAYS (II, III)**
The activity of caspase-3 and caspase-9 was measured in whole tissue homogenates. The tissue homogenates were incubated at 37°C on a microtiter plate with caspase-specific substrates: Ac-DEVD-AMC for caspase-3 (Peptide Institute, Osaka, Japan) and Ac-LEHD-AFC (for caspase-9, Enzyme System Products, Livemore, CA). Caspase activity was measured using a Spectramax Gemini microplate fluorometer (excitation wavelength / emission wavelength 380/ 460 nm for caspase-3 and 400/505 nm for caspase-9) over 2 hours and expressed as pmol released AMC or AFC/µg protein/min. Rat testis homogenate was used as negative control.

**CYTOKINES, RESISTIN AND ENDOTOXIN IN PLASMA**
Several techniques were used to measure the circulating cytokines. These are described in detail in the respective papers. In short, the following methods were used.
ASSESSMENT OF PLASMA CYTOKINES USING ELISA (III)
Plasma levels of TNF-alpha, IL-1 beta and IL-6 at 1h, 3h, 6h, 12h and 24h postreperfusion were measured using commercially available, rat specific ELISA kits (R&D, Minneapolis, MN). The assay sensitivity for TNF-alfa, IL-1 beta and IL-6 were 5pg/mL, 5 pg/mL and 21pg/mL respectively.

ASSESSMENT OF PLASMA CYTOKINES USING MULTIPLEX ASSAY (IV)
Human plasma samples were analyzed for a panel of pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines. Plasma concentration of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p70, IL-13 and TNF-α was determined by the electro-chemiluminescence multiplex system Sector 2400 imager from Meso Scale Discovery (K15010A-4, Gaithersburg, MD, USA).

ASSESSMENT OF PLASMA RESISTIN (IV)
Plasma resistin concentrations were determined in 46 plasma samples (n=5-11 /patient using a commercial enzyme-linked immunosorbent assay (ELISA, DRSN00, RND systems, Minneapolis, MN) . This assay employs the quantitative sandwich enzyme immunoassay technique and a monoclonal antibody specific for human resistin. Measurements were performed using manufacturers’ instructions. Plasma samples from seven healthy individuals (live kidney donors) were used as controls. The results were correlated with CRP, WBC, immunosupression (TAC blood through levels), procalcitonin and BMI.

ASSESSMENT OF PLASMA ENDOTOXIN (III)
The Chromogenic Limulus Amoebocyte Lysate Assay test kit was used for duplicate determination of endotoxin plasma concentrations (Chromogenix, Mölndal, Sweden), according to manufacturers’ instruction. Escherichia coli O11B4 LPS was included in the test kit as standard LPS (100 pg of LPS corresponding to 1.2 endotoxin units). To remove inhibitors, the plasma samples were diluted 1/10 in pyrogen-free water and heat treated for 10 min at 75°C. The interassay coefficient of variation was 8%.
STATISTICAL ANALYSES

Nonparametric methods were used throughout the study, either due to small sample size or because Kolmogorov-Smirnov test showed non Normal data distribution. Differences between independent groups were calculated using the Kruskal-Wallis test followed by the Mann-Whitney U test (both non-parametric). In paper IV, correlations between different variables were assessed using the Spearman rank correlation test. P<0.05 was considered significant. Data were analyzed using GraphPrism 5 software.
RESULTS

PAPER I

The intestines which had low-sodium solution intraluminally had significantly improved preservation injury after both eight hours (median grade 2, range 1-3) and fourteen hours of preservation (median grade 3, range 2-5) compared both with grafts receiving intraluminal high-sodium solution (p<0.01) or undergoing vascular flush alone (p<0.05). After 20 h of cold storage, grafts in all groups had advanced ischemic injury consisting of complete loss of villi and injury to the deeper intestinal layers. After fourteen hours of cold preservation, grafts receiving intraluminal high-sodium solution had a tendency to generate a worse average preservation injury (4.5 range 3-6) compared with grafts receiving only vascular flush (4, range 3-5).

Tissue edema and water retention increased during preservation. However, there was always a tendency that tissue water content was highest in grafts receiving intraluminal high-sodium solutions. After 8 h of, the beneficial role of intraluminal preservation was most apparent in the group receiving intraluminal low-sodium macromolecular solutions, resulting in similar water retention compared with controls grafts. With more extended storage time, the water content gradually increased in all groups and all differences subsided.

The intraluminal solutions did not affect negatively the brush border enzymes. The sodium content into the intraluminal solution decreased in time, irrespective of the intraluminal solutions, indicating sodium absorption. The most significant absorption occurred during the first eight hours but continued thereafter, albeit at a slower rate.

In normal intestines, ZO-1 and claudin-3 co-localized in the crypts and in the villus epithelium mostly along the lateral surface of the enterocytes. The distribution of tight junction proteins changed in all three groups after cold preservation. After eight hours, control grafts showed signs of de-localization along the intercellular membrane owing to a decrease in claudin-3 expression. Co-localization was maintained in the region closer to the enterocyte base. Grafts receiving intraluminal high-sodium solution had marked de-localization on the enterocyte basolateral membrane and reduced claudin-3 expression. ZO-1 expression was maintained. Intestines receiving intraluminal low-sodium solution had maintained strong co-localization along the lateral membrane but reduced ZO-1 expression in the basal
region. After fourteen hours of preservation, the changes in tight junction structure progressed. The staining in the intercellular area appeared broadened and ZO-1 expression displayed a decreased, disrupted and reticular pattern. Claudin-3 was found greatly reduced and its pattern of expression changed from intercellular reticular to granular intracytoplasmic. These changes were recorded in all grafts, regardless of intraluminal treatment. Twenty hours of preservation generated advanced mucosal damage with broad areas of denuded submucosa. Despite frequent morphologically intact crypts, almost complete de-localization of ZO-1 and claudin-3 was also found in the crypts.

PAPER II

Grafts from donors pretreated with TAC had increased HSP72 expression at the time of harvesting. However, this did not influence preservation injury, which was similar between the pretreated and the control group. Pretreated grafts showed however a milder early reperfusion injury compared with controls. Thus, pretreated grafts maintained connective tissue (lamina propria) - (4,1 ± 0,1 vs. 5 ± 0,1 p<0,01). The significant improvement in graft morphology persisted after six and twelve hours post-reperfusion. Thus, pretreated grafts had improved morphological parameters (villus length, mucosal thickness) and milder neutrophil inflammation. Pretreated grafts also revealed an accelerated cell proliferation and enterocyte maturation, reflected by a higher proliferation index (Ki67 positive cells) in the crypts and by the superior dissacharidase levels.

At the same time, we found reduced NF-κB early after reperfusion and a uniform and persistent inhibition of the second wave of NF-κB activation. This inhibition was recorded at least during the first twenty-four hours following graft reperfusion. The effective and enduring abrogation of the NF-κB activation was also reflected by the lower ICAM-1 levels found in the pretreated grafts.

PAPER III

Biochemical evidence of liver dysfunction, revealed by increased levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), was present in all animals receiving intestinal grafts. The dysfunction seem to be directly related to the presence of the graft, since non-transplanted animals had virtually no
variations in plasma transaminase levels compared with normal animals. However, recipients of pretreated grafts had a milder dysfunction compared with recipients of control grafts. The hepatocellular injury seems to have occurred early but appeared to be short lived.

We found no morphological alterations that could have determined the transient liver injury, such as necrosis, or inflammation. However, we found evidence of ongoing apoptosis in the livers of graft recipients, revealing that the liver had been submitted to sublethal stress and pro-apoptotic stimuli, more intense in the recipients of control, untreated grafts.

Conversely, in the recipients of control grafts we identified a biphasic pattern of TNF-α release and an increasing trend of two pro-inflammatory cytokines (IL-1β and IL-6) throughout the first twelve hours after graft reperfusion. These interesting and characteristic patterns were absent or less obvious in recipients of pretreated grafts.

Looking at the local ischemia or hypoperfusion as a potential mechanism behind the transient liver dysfunction, microvascular blood-flow measurements at the surface of the liver indicated that microvascular perfusion was similar in all the studied groups at all times.
Circulating LPS had very low levels both after six and twelve hours after reperfusion and was found significantly increased only twenty-four hours after graft reperfusion, similar between the two groups.

PAPER IV
The six month and one year patient survival in the series presented herein was 100% and 85% respectively. Five out of seven patients (75%) have had early acute rejection episodes and the same proportion had severe bacterial infections or sepsis during the first two months after transplantation.

Resistin was detected in all healthy controls. Significantly increased resistin levels were found in all patients, already one week after transplantation. Resistin levels varied considerably between patients and remained increased throughout the first eight weeks after transplantation (Fig.5).

Figure5. Plotted resistin levels over the first eight weeks posttransplantation
Resistin continued to remain increased compared with controls after two and five years respectively, in the absence of infection or rejection.
Resistin levels were found increased in samples taken during acute rejection compared with samples taken during rejection-free course. Sepsis was also associated with increases in plasma resistin.

No significant positive correlation was found between plasma resistin and six different Th1 (pro-inflammatory) cytokines. In one patient we identified a significant negative correlation between IL-1β and IL-8 and resistin.

Several positive correlations were identified between resistin and some anti-inflammatory Th2 cytokines. Thus, IL-4 a regulatory cytokine promoting the Th2 phenotype positively correlated with resistin in two patients, of which one was rejection-free.

The analysis of WBC, BMI, TAC trough levels, CRP and PCT in relationship with plasma resistin revealed only one negative correlation with WBC in patient #7 ($r_s = -0.9$, $p<0.05$) and one positive correlation with TAC trough levels in patient #6 ($r_s = 0.68$, $p<0.05$). No other significant correlations whatsoever were identified.
GENERAL DISCUSSIONS

In contrast to previous clinical observations of harmful effects of the intraluminal bowel preparation solutions, the experimental results in this thesis suggest that a low sodium content solution may reduce the edema in the intestinal wall during preservation, which in its turn may have favorable effects after reperfusion. Furthermore, pretreatment of the donor with tacrolimus appears both to reduce the graft reperfusion injury and accelerate mucosal morphologic recovery after rat intestinal transplantation, as well as causing a milder systemic inflammatory response in the recipient.

The clinical study investigated the potential of resistin as an intestinal rejection marker. As the results in this small group indicate, despite uniformly increasing after intestinal transplantation, resistin is unsuitable for monitoring the acute rejection since the increase is present even in the absence of acute rejection.

Ischemia reperfusion is an extremely relevant issue in intestinal transplantation, because of the potentially contaminated intestinal content. Mucosal barrier breakdown may lead to bacterial translocation and endotoxemia, similarly with other conditions evolving with intestinal ischemia or hypoperfusion [288-290]. The ability of the ischemic intestine to promote sepsis and sepsis-like systemic inflammatory response has long been recognized [291, 292]. Thus, an intact mucosal barrier in these heavily immunosuppressed patients is paramount.

Throughout the 1990s procurement teams routinely performed donor bowel preparations using oral antibiotics and macromolecular solutions (i.e., Golytely) as well as luminal flush with Ringer solution (130 mmol/L NaCl) in an attempt to reduce the intestinal load [24, 293]. However, this approach is currently abandoned and most centers limit the donor preparation to intravenous antibiotics [12, 294].

The lumen has been recognized for a long time as a potential route to deliver nutrients to the mucosa during intestinal preservation. Oxygen, glucose, glutamine, perfluorodecaline and several customized solutions have been introduced intraluminally in an attempt to reduce the ischemic injury and most studies reported improvements [247, 248, 295, 296]. However, we believe that more ‘unspecific’ causes such as a better control over the composition and osmolarity of the intraluminal content may have contributed equally as the more specific research interventions [297, 298]. We argue that that the low metabolic activity at 4
degrees (either the glutamine metabolism or ATP synthesis) is too modest to decisively contribute to the improvements reported. Few studies were followed by transplantation, further raising doubt about the relevance of the methods.

As mentioned above, our current findings suggest that bowel preparation solutions may successfully be used, not only for cleansing the donor intestine but also as an intraluminal preservation solution, provided that a low-sodium solution is used. It is unknown if using HTK solution will yield similar results due to the different electrolyte composition UW solution. In addition, the optimal volume of solution to be introduced intraluminally should be further studied in large animal models or in the human intestine.

The morphological improvements described herein are not dramatic but rather modest. However, as Paper II and other previous reports show, reperfusion leads to an injury advancement corresponding to one grade on the Park scale, namely from grade 3 (i.e. subepithelial edema) to grade 4 (villus denudation, mucosal breakdown) [193]. In the transplantation setting Park et al showed that increasing reperfusion injury from grade 3 to grade 4 increases mortality from 0% to 70% while Haglind et al report similar results using a warm intestinal ischemia model [193, 299]. Thus, minor morphological improvements may translate into significant differences in distant organ injury, systemic inflammatory response and ultimately improved clinical course in the early post-transplant period.

Furthermore, besides the early benefits of an alleviated reperfusion injury may be reflected in less late changes, as chronic rejection is the new old foe the intestinal transplant community battles today [300]. Advanced preservation injury may increase graft immunogenicity and may precipitate rejection [79, 301]. Some reports demonstrate that alleviated reperfusion injury after donor preconditioning has also been followed by less chronic changes resembling rejection and improved long term graft function [232, 302-304].

The distant organ injury ensuing after intestinal ischemia has been the subject of extensive research [290]. Lung and liver injury have been reported after experimental and clinical intestinal ischemia including after surgery for abdominal aortic aneurysms [153, 305, 306]. The mediators of injury, initially described as "toxic factors from the intestine" seem to circulate both through the portal vein and the lymphatic drainage [160, 306-308] and interrupting the mesenteric lymph drainage resulted in a lower remote organ injury [309, 310]. Considering that the liver dysfunction seen in our model was mild and transient and the inflammatory response was quite modest, despite the rather advanced reperfusion injury, we hint
that the absence of the mesenteric lymph, or perhaps the systemic drainage, may have been beneficial. Furthermore, since the microvascular perfusion of the liver surface was similar in all groups, this virtually rules out local perfusion failure or ischemia as a cause of liver injury. Secondly, we do not believe that endotoxins from the bowel play a major role in our experiments, since circulating LPS had very low levels both after six and twelve hours after reperfusion and was found significantly increased only twenty-four hours after graft reperfusion, similar between the two groups. Thus, LPS did not seem responsible for the systemic inflammatory response, since maximal pro-inflammatory cytokine levels were attained in the presence of very low LPS levels.

The dysfunction seems driven by soluble mediators since we found no evidence of gross morphological abnormalities or neutrophil infiltration, despite earlier reports suggesting a causal role for the neutrophils [311]. Moreover, when transplanting intestines in neutrophil depleted rats we observed a pattern liver dysfunction which was similar with the control group of neutrophil-sufficient animals (own unpublished data). Extrapolating in a clinical perspective that may imply that induction with polyclonal antibodies may reduce the local reperfusion injury but it will not directly influence the remote organ injury.

Graft preconditioning through various pretreatment regimens applied to the organ donor has been increasingly explored and suggested to improve graft quality. Among the numerous approaches tested, pharmacological induction/upregulation of heme oxygenase-1 (HSP32) expression has probably been the most extensively investigated. The reason behind the choice of this inducible protein is the multitude of biological pathways and mechanisms that are presumably influenced by the enzyme and its byproducts [312, 313]. Some of the most interesting effects are the reduction of the oxidative stress and the inhibition of several transcription factors as well as the modulation of apoptosis. The biological potential of heme oxygenase-1 and its byproducts is however counterweighted by the reluctance towards the clinical use of carbon monoxide, a highly toxic gas and the large variability of the biological response in humans [312, 314].

Herein, we showed that donor preconditioning with an accepted drug (TAC) may significantly improve intestinal graft morphology, possibly through the upregulation of the cytoprotective HSP72. Whether the upregulation of heat shock proteins is a safe approach in an allogeneic setting, on both short and long run, remains to be further explored, since numerous studies confirmed the immunostimulatory properties of various heat shock proteins [315, 316]. Moreover, as mentioned
earlier, anti-heat shock protein reactivity has been repeatedly demonstrated in transplant and non-transplant setting (i.e., atherosclerosis) and suggested as a causal factor of undesirable events such as lymphocyte and dendritic cell activation [136, 317, 318]. We also recorded a significant and long lasting NF-κB inhibition in the pretreated grafts. Although NF-κB inhibition by TAC is not entirely new, this feature has not been reported earlier in this setting. Given the multifaceted effects of NF-κB inhibition, it is likely this contributed to the modulation of both the local and systemic inflammation, due to the assumed downregulation of adhesion molecules and reduced cytokine release by the graft.

The microenvironment is an essential factor in regulating cell proliferation [319, 320] In the present study the favorable microenvironmental conditions could have been represented by the lower inflammatory activation and the reduced tissue inflammation observed in the pretreated grafts. Conversely, cells remain quiescent in the presence of unfavorable circumstances (ie, cellular stress) and proliferation may be blocked or delayed [321]. On the other hand, TAC has been shown to stimulate cell proliferation regeneration after liver resection or neuroraphy [322, 323] and several mechanisms have been suggested, including growth factors and the FK-binding proteins. The latter proteins are responsible for the intracytoplasmic TAC transport and are also called immunophilins [324]. Whether the drug itself stimulated the crypt proliferation, seen in our experiments, or if it was the result of a chain of biological events resulting in a microenvironment more favorable for proliferation remains unclear.

As previously mentioned, TAC has been proved useful in reducing ischemia-reperfusion injury and its long term consequences in kidney transplantation in the rat The same study revealed very similar results when prednisolone, a potent synthetic steroid able to alter numerous signaling pathways, was given to the organ donors [232]. Donor pretreatment with steroids for the reduction of the alloindependent graft injury is probably the strategy with the highest chance to become a clinical routine in the future. Experimental studies revealed improved renal graft function after transplantation [325] and donor pretreatment with steroids blocked the organ activation and increased immunogenicity induced by the brain death [326, 327]. Moreover, a recent clinical trial demonstrated improved results using livers from donors systematically receiving methylprednisolone [328]. Lastly, the fact that
hormonal resuscitation is already currently used in thoracic organ donors with no adverse effects observed in other organs may advocate for the future routine use of steroids in pretreating the organ donors [329, 330].

At the moment resistin is considered a pro-inflammatory adipocytkine. While its association with inflammation is supported by extensive evidence [271, 276], a contributory role of resistin in inflammation is less clear. Resistin increases in a broad array of diseases evolving with tissue injury and inflammation, such as active inflammatory bowel disease, cancer, asthma, type 2 diabetes, chronic kidney disease and infections, but has little in common regarding the pathogenesis [277, 279, 331-333]. Hence, based on our observations showing an early rise in plasma resistin in the absence of rejection, in the presence of infection or even during uneventful course we are skeptical towards the hypothesis of a major pathogenetic role of resistin in inflammation as a ‘pro-inflammatory cytokine’. Instead for a role of ‘pro-inflammatory cytokine’ we suggest that resistin is a marker of inflammation or tissue injury. In the present study the increase was triggered by ischemia-reperfusion injury and/or the alloimmune injury.

In brief, arguments for a cytokine role of resistin are its production by leukocytes and its actions over several cell types, leading to changes in their phenotype. Arguments against a cytokine role for resistin are the very high levels, nanograms compared to picograms, compared with other acknowledged cytokines and that no specific stimulus or receptor for resistin has been identified. In addition, no counter-regulatory molecule or loop is yet recognized.

One of our aims was to investigate the potential of resistin as an intestinal rejection marker. This hypothesis was based on previous studies reporting specific increases during active inflammatory bowel disease [279]. We found a large inter-individual variation after transplantation as well as an unspecific increase during several complications, of which one was rejection. Moreover, we found resistin uniformly increased compared to healthy controls. We did not identify correlations with several key Th1 and Th2 cytokines, that could have allow us to speculate on the factors inducing the release of resistin. In contrast with other studies we could not identify any correlation between resistin or CRP or WBC either. This fact may be due to the immunosuppression, a feature absent in studies reporting such correlations [280, 334]. Thus, we believe that further studies on resistin in immunosuppressed subjects could shed more light on the biology of this intriguing molecule.
CONCLUSIONS

With support of the studies presented in this thesis I conclude that:

- the intraluminal introduction of macromolecular solutions having low sodium content may have beneficial effects on the preservation injury of the rat intestine

- donor pretreatment with a single dose of tacrolimus reduces graft reperfusion injury and accelerates mucosal morphologic recovery after rat intestinal transplantation

- transplantation of intestines from donors pretreated with tacrolimus is followed by a lesser liver dysfunction and a milder systemic inflammatory response compared with animals receiving untreated intestinal grafts

- resistin increases early after clinical intestinal transplantation irrespective of the presence of complications and has large individual variations, making it unsuitable as a diagnostic marker for acute rejection
REFLECTIVE STATEMENTS

• The lumen is an easy and obvious route to get direct access to the most sensitive part of the intestinal graft, i.e. the mucosa. Numerous interventions have been attempted over the time with various results but without a decisive breakthrough. Our results identified some morphological features underlying the permeability changes during ischemia. Moreover, the study suggests that intraluminal high-sodium solutions should be avoided when designing future intraluminal preservation solutions. Conversely, previous observations might have missed the importance of the right sodium content in the right compartment and a clinical reassessment in the setting of a multicenter randomized controlled trial may be beneficial.

• This study showed once again that targeted interventions in the organ donor may successfully reduce the initial ischemia/reperfusion injury. Whether the present methodology and the conclusions can be directly translated clinically is unknown, since TAC metabolism differs between rats and humans. However, considering the fact that:
  i TAC is a major immunosuppresant already in clinical use
  ii the graft will be submitted to the same drug shortly after reperfusion
  iii similar beneficial results following TAC pretreatment have been described in other transplantable organs and
  iv the intervention is feasible and straightforward

may open the perspective for a future clinical trial.

• Although resistin does not seem a reliable acute rejection marker in clinical intestinal transplantation, the need for such a tool is still a high priority. This first report on resistin in a field quite remote from obesity, atherosclerosis, chronic inflammation suggest that resistin is a versatile molecule that is probably involved in many biological processes.
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REFERENCES


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
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<td>AR</td>
<td>Acute Rejection</td>
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<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocytes - CD8+ cells</td>
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<tr>
<td>DAMP</td>
<td>Danger-Associated Molecular Pattern</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DTH</td>
<td>Delayed-type Hypersensitivity</td>
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<td>EMSA</td>
<td>Electrophoretic Mobility Shift Assay</td>
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<td>GALT</td>
<td>Gut Associated Lymph Tissue</td>
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<td>GVHD</td>
<td>Graft Versus Host Disease</td>
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<td>Hepatoma-Derived Growth Factor</td>
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<td>high sodium solution</td>
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<td>IR</td>
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<td>Inhibitory-unit κB</td>
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<td>Platelet activating factor</td>
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