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Chapter 1

Introduction
Introduction

Liver Transplantation

Orthotopic Liver transplantation (OLT) has evolved into a routine treatment with excellent short and long-term patient survival for various end-stage liver diseases. Several improvements, including those in surgical technique and immunosuppression, have contributed to improved survival. Possibly, a reduction in infectious complications after OLT may have contributed to this improved patient survival, but this is not well known. Improved patient survival may also be attributed to better donor management, organ preservation, as well as intensive care treatment protocols. Recently, it has been shown that, next to the antibiotic regimen, donor and recipient genetic polymorphisms in innate immunity contribute to the risk of infection after OLT. In addition, graft loss due to rejection has virtually disappeared since the introduction of tacrolimus.

The success of treating patients with end-stage liver disease with liver transplantation has led to an increased demand for donor livers while the number of suitable grafts has remained static over the last decades and this resulted in longer waiting lists. Many centers have expanded their criteria, in consensus, for potential donor livers. These include extended criteria donor livers and livers from donation after cardiac death (DCD). According to the Maastricht criteria a no touch period is warranted in DCD donors to ensure that cardiac arrest had become irreversible as opposed to donors from donation after brain death (DBD), where the circulation in the donor is still intact. DCD donors therefore have an additional and inevitable donor warm ischemia time (DWIT), which is defined as the time between circulatory arrest and cold flush in the donor. In addition, a short episode of hypotension between ventilation switch-off and cardiac arrest precedes DWIT. This additional period in DCD of less and no perfusion respectively, and warm ischemia precedes the cold ischemia time (CIT) which starts with cold flush in the donor and persists during transportation. CIT ends at the time of removal of the donor liver from ice at which the recipient warm ischemia time (RWIT)
starts. RWIT ends at reperfusion of the donor liver in the recipient. Unlike DWIT in DCD, the CIT and RWIT are also present in donation after brain death (DBD). Despite donor selection and reduction of ischemia times, livers from DCD donors are more prone to develop severe complications such as primary nonfunction (PNF), delayed graft function and, especially, biliary nonanastomotic strictures (NAS).\textsuperscript{25-31}

In 2001, a national protocol in the Netherlands has been introduced for multi-organ donation from DCD donors to fulfill the increasing demand and compensate for the decreasing DBD donation. As to date, approximately 20\% to 30\% of all OLTs in our country are performed using livers from DCD donors.\textsuperscript{32} NAS can occur in up to 35\% of grafts using DCD donors and are considered a significant source of morbidity.\textsuperscript{33} The exact pathogenesis of the development of NAS remains unclear but several theories exist.\textsuperscript{34}

\textbf{Biliary complications}

Biliary complications can occur at any time after OLT. Biliary complications include anastomosis leakage leading to bilomas and stricture formation.\textsuperscript{35} Biliary strictures can be divided into two different entities: anastomotic strictures (AS) occur at the surgical notch of the bile duct anastomosis.\textsuperscript{36,37} Local inflammation may lead to scarring which in turn leads to narrowing of the anastomosis. NAS are considered as strictures or irregularities occurring at least 1 cm above the anastomosis.\textsuperscript{35,38,39} NAS form a heterogeneous group with considerable variation and NAS incidence rates may vary widely, perhaps due to lack of a clear definition. In addition, time of presentation may also vary greatly.\textsuperscript{40} NAS may occur early after OLT, i.e. within the first twelve months, or late, even after several years. One study showed that NAS occurring early after OLT (< 1 year) was associated with preservation related factors, such as cold ischemia time (CIT) and WIT as opposed to late presenting NAS, which was more related to the indication for which OLT was performed, especially primary sclerosing cholangitis (PSC).\textsuperscript{40} In OLT for PSC, one of the difficulties is that to date there are no diagnostic modalities to distinguish recurrent PSC from NAS related to OLT.

Grafts from DCD are known to develop more NAS than grafts from DBD.\textsuperscript{41} NAS – also known as ischemic-type biliary lesions (ITBL) – are associated
with an increased risk of bacterial cholangitis, frequent admissions to the hospital, endoscopic treatment and retransplantation.\textsuperscript{42} NAS are most likely the result of a complex mechanism involving ischemic, immunologic and toxic processes which all affect the biliary tree or its vascular system.\textsuperscript{43,45} The microvascular supply of the biliary tree, the peri-biliary plexus, stems from the hepatic artery branches and flows into the hepatic sinusoids. A decreased blood flow in the peri-biliary plexus after orthotopic liver transplantation may be involved in the development of NAS.\textsuperscript{46,47} This might be the result of microthrombi that develop during the DWIT, CIT and RWIT.\textsuperscript{48,49} The most frequently used preservation fluid, University of Wisconsin (UW) solution and Histidine-tryptophan-ketoglutarate (HTK) solution, which was until recently also used in the Netherlands for liver preservation, may be far from perfect.\textsuperscript{50} Several attempts are being made to improve preservation and to reduce ischemia-reperfusion injury (IRI) of liver grafts, including machine liver perfusion but also “flush” protocols in which fibrinolytic agents are being used to dissolve microthrombi in the microvascular system of the biliary tree during procurement of the donor liver.\textsuperscript{49,51}

Apart from IRI and possible microthrombi, there are several other theories on the development of biliary strictures. One theory encompasses an altered composition of bile after IRI, which occurs after OLT.\textsuperscript{44} In a porcine model of DCD liver transplantation, a warm ischemia period of 30 minutes and longer produced bile with a significantly higher bile salt-to-phospholipids ratio after transplantation than livers from donors with 0 or 15 minutes warm ischemia in the donor.\textsuperscript{45} Secretion of bile salts might be impaired due to an imbalance between hepatobiliary transporter proteins which secrete bile salts and multidrug-resistance protein 3 (MDR3) which secretes less phospholipids resulting in an increase of the bile salt/phospholipids ratio. Toxic bile salts might lead to injury of the biliary epithelium, especially in case of a reduced ‘bicarbonate umbrella’.\textsuperscript{52,53} In the past, ABO-incompatibility in liver transplantation was also associated with the development of NAS, indicating that immunological processes are involved in the development of NAS.\textsuperscript{54} In ABO-incompatibility this could partially be explained by the fact that the ABH-antigen is consistently present on biliary epithelial cells—in contrast to hepatocytes—which in turn can initiate an immune reaction that causes local
damage to the biliary tissue.\textsuperscript{55}

One theory that supports the involvement of the immune system is the loss-of-function mutation of chemokine receptor 5 delta 32 (CCR5Δ32) which has been associated with the development of NAS in recent literature.\textsuperscript{43} CCR5Δ32 is a protein, which is located on the surface of macrophages, CD4+, CD8+ but also natural killer cells, and its main function involves attracting immune cells to damaged tissue sites. Impaired functioning of the CCR5Δ32 might lead to less attraction of immune cells to the biliary tract and subsequent healing of injured tissue. However, the contribution of CCR5Δ32 in the development of NAS has been questioned and needs replication in larger cohorts to determine its exact role in the development of NAS.\textsuperscript{56}

Activated immune cells also release specific members of the tissue remodeling matrix metalloproteinases (MMP). MMPs have been associated with numerous conditions such as IBD and cancer and there is strong evidence suggesting involvement in IRI as well.\textsuperscript{57-59}

**Matrix metalloproteinases**

Jerome Gross and Charles Lapierre made the first discovery of MMPs in 1962 and the first MMPs discovered in human neutrophilic granulocytes, was in 1968.\textsuperscript{60,61} MMPs comprise a large family of proteolytic enzymes that are important in physiological and disease-related extracellular matrix (ECM) remodelling processes.\textsuperscript{62} MMPs consist of a signalpeptide or prepeptide, a pro-peptide region, a catalytic domain with a zinc binding region and a hemopexine domain which is connected with a so called hinge region with varying length to the catalytic domain.\textsuperscript{63}

The signalpeptide or pre-peptide consists of a sequence of 17-20 hydrophobic amino acids. The hydrophobic portion of the signal peptide is responsible for the secretion of MMPs in the endoplasmic reticulum, from which they can be released into the extracellular space.\textsuperscript{64} The propeptide, contains 80 amino acids with an N-terminal hydrophobic rest. Near the C-terminal end of the propeptide is a highly conserved sequence region around cysteine: PRCGVPD.\textsuperscript{65,66} The catalytic domain consists of 160 - 170 amino acid
residues and contains binding sites for calcium and zinc ions. The catalytic domain is connected to the hemopexin domain by a so-called hinge region. This connection region is important for the substrate specificity of MMPs which can bind to the substrate itself or establish the binding orientation of the catalytic and hemopexin-domain. The hemopexin-like domain comprises about 200 amino acids that contain four so-called repeats, each with about 48 amino acids. The hemopexin domain seems to be important for substrate specificity of the MMPs and contributes to binding of the substrates which makes it a key player in activating and inhibiting MMPs. MMPs hydrolyze most components of the ECM and play a central role in many biological processes such as normal tissue remodeling, embryogenesis, wound healing and angiogenesis. Currently about 26 MMPs have been identified, and most are multidomain zinc endopeptidases. According to their substrate the members of the family are divided in collagenases, stromelysines, gelatinases, membrane-type-(MT)MMPs and others. Stromelysins (MMP-3 and MMP-11 or stromelysin-1 and -2) and matrilysin exhibit the ability to degrade a broad range of substrates, including proteoglycans, fibronectin, laminin, gelatin, collagens-III, -IV, and -V, and elastin. The membrane-type MMPs (MT-MMPs) differ from other MMPs in having a C-terminal transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP) or are anchored to glycosyl phosphatidyl inositol (MT4-MMP, MT6-MMP), which localizes these enzymes to the surface of cells. MT-MMPs have a broad substrate specificity and can degrade interstitial collagens III and I, as well as fibronectin, vitronectin, and cartilage proteoglycans. In healthy tissue a strict regulation of MMPs is critical in order to maintain proper ECM homeostasis. Among other levels of regulation, MMPs are precisely regulated by their main endogenous protein inhibitors (TIMPs). Disruption of this balance results in serious diseases such as fibrosis, arthritis, and tumour growth. Certain MMPs such as gelatinases (MMP-2, MMP-9) have specific characteristics such as digesting components of connective tissue matrix and type IV collagen. MMPs can no longer be solely thought of as ECM destructionists, but as part of a delicate equilibrium system through which epithelial and immune cells interact with the stroma.
Table 1. List of MMPs and their substrate specificity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Pro-MMP</th>
<th>Substrate</th>
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</thead>
<tbody>
<tr>
<td><strong>Collagenase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagenase 1</td>
<td>MMP-1</td>
<td>Type I, II and III collagen</td>
</tr>
<tr>
<td>Collagenase 2</td>
<td>MMP-8</td>
<td>Type I, II and III collagen</td>
</tr>
<tr>
<td>Collagenase 3</td>
<td>MMP-13</td>
<td>Type I collagen</td>
</tr>
<tr>
<td>Collagenase 4</td>
<td>MMP-18</td>
<td>Not found in humans</td>
</tr>
<tr>
<td><strong>Gelatinases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatinase A</td>
<td>MMP-2</td>
<td>Type IV, V, VII, IX and X collagen, gelatins</td>
</tr>
<tr>
<td>Gelatinase B</td>
<td>MMP-9</td>
<td>Type IV, V, XI, and XVI collagen, laminin, elastin, decorin</td>
</tr>
<tr>
<td><strong>Stromelysin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromelysin 1</td>
<td>MMP-3</td>
<td>Basement membrane glycoproteins, fibronectin, E-cadherine, activates plasminogen and MMP-2</td>
</tr>
<tr>
<td>Stromelysin 2</td>
<td>MMP-10</td>
<td>Basement membrane glycoproteins</td>
</tr>
<tr>
<td>Stromelysin 3</td>
<td>MMP-11</td>
<td>Basement membrane glycoproteins</td>
</tr>
<tr>
<td><strong>Matrilysin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrilysin 1</td>
<td>MMP-7</td>
<td>Fibronectin, elastin</td>
</tr>
<tr>
<td>Matrilysin 2</td>
<td>MMP-26</td>
<td>Type IV collagen, fibronectin, fibrinogen</td>
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<tr>
<td><strong>Membrane-type MMPs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(MT-MMP)(A)</td>
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<td></td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>MMP-14</td>
<td>Type I, II and III collagen, activates MMP-2 and MMP-13</td>
</tr>
<tr>
<td>MT2-MMP</td>
<td>MMP-15</td>
<td>Fibronectin, laminin, activates MMP-2</td>
</tr>
<tr>
<td>MT3-MMP</td>
<td>MMP-16</td>
<td>Type III collagen, fibronectin</td>
</tr>
<tr>
<td>MT5-MMP</td>
<td>MMP-24</td>
<td>Proteoglycans</td>
</tr>
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<td>MT4-MMP</td>
<td>MMP-17</td>
<td>Fibrinogen, fibrin</td>
</tr>
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<td>MT6-MMP</td>
<td>MMP-25</td>
<td>Type IV collagen, fibronectin, fibrin, casein</td>
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<tr>
<td><strong>Others</strong></td>
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<td></td>
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<tr>
<td>Macrophage elastase</td>
<td>MMP-12</td>
<td>Type IV collagen, elastin, gelatins, fibronectin</td>
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<td>MMP-19</td>
<td>Type IV collagen, fibronectin</td>
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<tr>
<td>Enamelysin</td>
<td>MMP-20</td>
<td>Amelogenin, aggrecan</td>
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<tr>
<td>-</td>
<td>MMP-21</td>
<td>Type IV, V, VII, IX and X collagen</td>
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<td>MMP-23</td>
<td>Unknown</td>
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<tr>
<td>-</td>
<td>MMP-27</td>
<td>Unknown</td>
</tr>
<tr>
<td>Epilysin</td>
<td>MMP-28</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

MMP-2 and MMP-9

The gelatinases A (MMP-2 or 72-kDa type IV collagenase), and B (MMP-9, 92-kDa type IV collagenase) can degrade denatured interstitial collagens (gelatins), type V collagen, and intact type IV collagen, which is an important component of basement membranes. The baseline structure of MMP-2 is homologous to MMP-9 and is constitutively expressed in almost all human tissues but mainly by hepatic stellate cells (HSC), endothelial and epithelial cells. MMP-2 is secreted in its zymogen form (pro-MMP-2) and is tightly regulated by complex signaling through TIMP-2, TIMP-3 and TIMP-4 which all display relevant affinity for the MMP-2 and their adequate secretion is...
required for a balanced MMP-2/TIMP ratio and MT1-MMP. The membrane bound activation of pro-MMP-2 ensures that proteolytic activity is localized to specific regions of the cell-surface. MMP-2 cleaves a vast repertoire of substrates, including cytokines, growth factors, and receptors or binding factors but is primarily known for its cleaving properties of gelatin, and types IV, V, VII, IX and X collagen which makes MMP-2 a key player in degrading ECM. Within the catalytic domain of MMP-2 and MMP-9 a threefold sequence consisting of 58 amino acids exists of fibronectin type II that can bind to gelatin and collagen, which makes these MMPs capable of breaking down ECM substrates.

MMP-9 is secreted in monomeric form as zymogen (pro-MMP-9) predominantly from neutrophils and macrophages but the main source in the liver is thought to be the Kupffer cells and activation of pro-MMP-9 is amongst others mediated by the plasminogen activator/plasmin (PA/plasmin) system. Its expression level and activity is regulated through TIMP-1 and TIMP-3 but in vivo experiments have shown that MMP-9 activity can also be mediated by trypsin, chemotrypsin, cathepsin B and a variety of cytokines and growth factors including interleukins (IL-1), interferons, epidermal growth factor (EGF), nerve growth factor (NGF), basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet derived growth (PDGF), tumor necrosis factor-alpha (TNF-α), transforming growth factor (TGF-β) and the extracellular matrix metalloproteinase inducer (EMMPRIN). The active form of MMP-9 is able to digest decorin, elastin, fibrillin, laminin, gelatin (denatured collagen), and types IV, V, XI and XVI collagen and also activates growth factors like pro-TGF-β and pro-TNF-β.

Genetic variants and its functional implications

MMP-2 and MMP-9 have been associated with numerous diseases such as cancer, autoimmune disorders, coronary diseases and diseases, which involve degradation such as Alzheimer’s disease. The majority of MMP-2 studies have focused on demonstrating an essential role in promoting cell invasiveness during tumor angiogenesis, arthritis, and atherogenesis, as well as tumor metastasis where levels of MMP-2 expression can be correlated
with tumor grade. Studies have shown that natural occurring variants of MMP-2 affect expression and thus might impact progression of pathophysiological processes. Single nucleotide polymorphisms (SNP) in the promoter region of the MMP-2 gene have shown to affect gene regulation. The C -> T transition at −1306 of the MMP-2 promoter gene interrupts a Sp1 binding site. Sp1 is a multifunctional protein that can directly interact with the basal transcription complex or alternatively function as a more general transcription factor and play an important role in directing tissue-specific expression. A variant that abolishes Sp1 binding, such as the MMP-2 −1306 C/T polymorphism, has the potential to affect the level and specificity of gene transcription. One study showed that the reporter gene expression of the T allele in at -1306 was 0.71 lower than the C allele indicating less transcriptional activity. Several other SNPs in the promoter region have been discovered such as the −1575 G/A, −790 C/T and −735 C/T, but these are in complete linkage equilibrium with the −1306 polymorphism.

MMP-9 expression is very low in most tissues but increases in response to local secretion of inflammatory cytokines and growth factors, most notably IL-1 and TNF-α, two highly potent inducers of MMP-9 gene activation. MMP-9 has two promoter gene polymorphisms that have proven to be functionally relevant, namely a (CA)n microsatellite polymorphism at position -90 and a SNP polymorphism at position −1562. Other nonsynonomous SNPs have been described but these show no functionally relevant changes in levels or activity. With the −1562C/T polymorphism in the MMP-9 promoter there is a cytosine to thymidine transition at the polymorphic position −1562. Several studies have found that this MMP-9 C->T transition polymorphism exerts an allelic effect with higher transcriptional activity and higher functional MMP-9 serum activity and it has been associated with a higher risk of cardiovascular complications in HIV patients and severe coronary atherosclerosis.

**Matrix metalloproteinases in liver diseases**

MMPs have also been implicated in numerous conditions involving the liver. For example, plasma MMP-2 levels were significantly higher in patients with hepatocellular carcinoma (HCC) and chronic liver diseases as compared to healthy controls and they were more elevated if the Child-Pugh class was
higher. In contrast, MMP-2 is very low in normal liver tissue. These findings imply that MMP-2 may be actively involved in both development and progression of various liver diseases or that breakdown and excretion of MMP-2 is impaired in this situation. MMPs are also involved in OLT and studies have shown changed hepatic expression of various members of the MMP/TIMP family during cold preservation injury in both humans and rats. Hepatic ischemic injury leads to swelling of endothelial cells and Kupffer cell activation which in turns leads to secretion of particularly MMP-9 and to a lesser extent MMP-2 via HSC. During OLT MMP-2 plasma levels gradually decrease but MMP-9 plasma levels increase further from the anhepatic phase on until 30 minutes after reperfusion, and are related to the degree of tissue injury and thus seem to be involved in early IRI. Furthermore, after OLT, neutrophil infiltration and matrix degradation was observed, which is accompanied by an increase of MMP-9 in patients with rejection in the first week after OLT, while neither MMP2 level nor MMP9 level are related to peak ALT in the first week after OLT. Inhibitors of MMP have been studied in rat models mimicking IRI showing significant improvement in liver function and liver injury. In addition, several other studies have studied the relationship between MMP-2 and MMP-9 and the development of acute cellular rejection after OLT suggesting that MMPs might also be involved in immunological processes after OLT.

Outline of the studies described in this thesis

Liver transplantation for end-stage liver disease has become a standard treatment for end-stage liver diseases with excellent long-term results. However, due to scarcity of DBD donor livers many centers have expanded their donor pool by using donor livers with “extended criteria” and DCD livers. Chapter 2 elaborates on 20 years of OLT for chronic liver disease at the Leiden University Medical Center and describes long-term outcome after OLT using livers from both DBD and DCD. The analysis focuses on differences in patient survival and graft survival in the first and second decade, and after DBD- versus DCD-OLT. Causes of recipient mortality and changes in these parameters were studied. The evaluation not only includes indications for OLT but also parameters of the surgical technique such as intraoperative blood loss.
Influence of other aspects such as ischemia-reperfusion variables for both DBD and DCD donors is described. In addition indications for re-OLT were assessed for both decades. Biliary complications, especially NAS, frequently occur after OLT and development is often insidious. NAS development after OLT seems predominantly the result of IRI and probably subsequent collagen deposition surrounding the bile ducts. One of the commonly accepted markers of IRI is an increased serum level (peak) of alanine aminotransferase (ALT), which occurs within the first week post OLT. This elevation is more marked for DCD-OLT than for DBD-OLT and the evaluation of the relationship between peak ALT and the development of NAS after OLT is described in chapter 3. When there is a clinical suspicion of NAS after OLT, i.e., due to jaundice, fever or itching, invasive procedures such as endoscopic retrograde cholangiography (ERC) or percutaneous transhepatic cholangiography (PTC) or magnetic resonance cholangiography (MRC) are the choices of modality for confirmation. However, these are invasive and expensive and may have side effects. Most centers routinely assess patients in the outpatient clinic with serum liver enzymes and abdominal ultrasound (US). The study on the predictive value of US and serum liver enzymes assessments for detecting the development of NAS post OLT is described in chapter 4.

MMP-2 and MMP-9 are the most potent degrading enzymes of type IV collagen; the main component of the extracellular matrix (ECM). Functional single nucleotide polymorphisms (SNPs) of MMP-2 and MMP-9, which affect their activity, may therefore play a role in development of biliary strictures and thereby potentially influence the incidence of NAS. Chapter 5 reports on the relationship between gene promoter polymorphisms of MMP-2 and MMP-9 in both recipient and donor and the incidence of NAS after OLT. In primary sclerosing cholangitis (PSC) the hallmark of the disease, like in NAS, is stricture formation of the bile ducts. OLT is often indicated in PSC, which is considered a chronic inflammatory disease of the bile ducts. The results of a study on the relationship between gene promoter polymorphisms of MMP-2 and MMP-9 and disease severity in patents with PSC, as defined by patient mortality or OLT, are described in chapter 6. Gene polymorphisms might have the potential to be used as a marker to
evaluate chimerism after OLT. Chimerism in transplantation refers to the coexistence of two different populations of (genetically) distinct cells that originate from both donor and recipient. **Chapter 7** describes the use of MMP-2 and MMP-9 donor/recipient gene promoter profiles in liver biopsies and in blood after OLT as a marker for chimerism. These findings were related to clinical outcomes such as acute cellular rejection. In **chapter 8** the results of the different studies described in this thesis are summarized and discussed.
Introduction
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