General introduction and aim of the thesis
Before Tumor Necrosis Factor

The first report of the beneficial effect of intravenously administered nitrogen-mustard on tumor growth appeared just after the second world war.\(^1\) Soon afterwards reports were published on the advantageous effect of intra-arterially administered nitrogen-mustard on malignant tumors.\(^2\)\(^-\)\(^4\) Using technology to support extracorporeal circulation developed for cardiac surgery in the 1950s, the surgical oncologists Creech, Krementz, Ryan and Winblad of the Tulane University in New Orleans developed the technique of isolated limb perfusion (ILP).\(^5\) In this procedure the blood circulation of a tumor bearing limb is isolated from the circulation of the rest of the body by clamping the major artery and vein and tightening a tourniquet around the root of the limb. The major artery and vein are subsequently connected to a heart-lung machine and the cytotoxic drug is administered through this isolated circuit. Key point in ILP is that the dose of chemotherapeutics used, can be 15-20 fold the maximum systemic tolerated dose, since vital organs are isolated from the perfusion circuit.\(^6\)\(^-\)\(^8\)

The original patient population treated with ILP was a subgroup of melanoma patients who had extensive local recurrence in the arm or leg. The initial drug used for ILP to treat extremity melanoma was melphalan (L-phenylalanine mustard). Melphalan is an alkylating agent of the bischloroethylamine type comprising nitrogen mustard and phenylalanine. Phenylanaline is a metabolite of melanin and therefore melphalan specifically targets melanocytes and melanoma cells. Its cytotoxicity appears to be related to the extent of its interstrand cross-linking with DNA. Like other bifunctional alkylating agents, it is effective against both resting and rapidly dividing tumor cells. In 1959 Creech, Krementz and Ryan described their initial results of patients treated with regional perfusion. The first patient was a 76 year old male with multiple melanoma satellites on his upper leg. After regional perfusion with melphalan the satellites disappeared completely and the patient died at the age of 92 with no local recurrence. The case history of this patient was frequently illustrated at lectures and a poster with pictures of this patient decorated the entrance of the surgical ward of the Tulane University for many years. Cavaliere and co-workers investigated the addition of hyperthermia in the treatment of cancer and, as this appeared to augment the anti-tumor effects of melphalan, in doing so they laid the basis for hyperthermic isolated limb perfusion (HILP).\(^9\) At temperatures of 41.5 degrees C and higher a direct anti-tumor effect was observed however, this was accompanied with unacceptable local toxicity.\(^10\) To avert this increased local toxicity it was established that mild hyperthermia with temperatures of 39 to 40 degrees C was best used. Wieberdink introduced the optimal dose calculations of melphalan based on limb volume instead of patient weight, since the latter may lead to under- or overtreatment of an individual dependent on body habitus.\(^11\) An essential component of HILP is
monitoring the perfusion leakage to the systemic circulation and being able to make adjustments during treatment to reduce this leakage. Different methods to measure leakage are used. Stehlin and associates were the first to describe a method of continuous external leakage monitoring with radioactive Iodine-131 labeled human serum albumin (RISA). This is still the method most frequently used nowadays. It places a gamma counter over the precordium with RISA in the perfusion circuit, which allows continuous readings and estimations of the leak of the perfusion solution into the systemic circulation.

From 1969 until recently, ILP with hyperthermia and melphalan was the gold standard for regional treatment of in-transit melanoma. The response rates to this therapeutic HILP are considerably higher to any other systemic therapy for this type of tumor. Objective response rates have been reported as high as 70% to 100%, with complete response rates between 54% and 65%. The median duration of responses is approximately 9 months, and some patients experience a long-term disease control with this regional therapy.

Many publications on HILP for melanoma combine adjuvant perfusions with therapeutic perfusions, often with different treatment schedules, making the interpretation of available data very difficult. A publication on the 35-year experience with HILP of the Tulane Hospital serves as a good example for this problem. Over 1100 cases were reported with a median follow-up longer than 10 years. However, an evidence based conclusion about the benefit of the procedure could not be made. A prospective randomized German study published in the 1980s reported a significant improvement in survival after adjuvant HILP. However, the numbers of patients treated were small, and the outcome in the control group was much worse than expected compared to historical controls, which meant that this trial could not be used in arguing for adjuvant HILP. The value of HILP as an adjuvant treatment modality in patients with high risk stage I disease (more than 1.5 mm Breslow thickness), was recently evaluated in a prospective randomized trial by the European Organization for Research on Treatment of Cancer (EORTC). This study showed no overall survival benefit for patients treated with HILP with melphalan followed by local excision compared to patients that had undergone local excision only. However, a slight benefit in disease free survival was seen in the perfusion group. With the publication of this study as a negative trial, no adjuvant HILP should be performed after resection of primary melanoma. Another patient population that may benefit from a adjuvant HILP are those who have developed in-transit metastases that have been excisionally biopsied. These patients are at a much greater risk for additional recurrences in the limb than patients with high-risk primary cutaneous melanoma who have not had a regional recurrence. A small prospective study from
Sweden found a significant improvement in tumor free survival in the perfusion group, however no overall survival benefit was demonstrated.\textsuperscript{20} In conclusion, adjuvant HILP with melphalan should not be used for high-risk primary melanoma and should only be used as an adjuvant in the setting of a clinical trial with patients with in-transit metastases.

Other chemotherapeutic agents used in HILP for melanoma have shown much lower subjective response rates often with a higher toxicity. Cisplatin as one of the most successful alternatives with a 50% to 60% response rate showed a high frequency of peripheral neuropathy.\textsuperscript{21-23} The most successful systemic treatment agent for melanoma is DTIC but used in regional perfusion this agent leads to a complete response rate of 11% and a partial response of only 26%.\textsuperscript{24}

Although HILP was most frequently used in the treatment of extremity melanoma, the procedure was also applied to soft tissue sarcomas (STS) of the extremity. Krementz described their initial results in 113 patients. Fifty-four patients treated with HILP without surgical excision of the tumor showed an early response rate of 83%, however only four patients had a complete regression of the tumor.\textsuperscript{25} Several studies were published on the treatment of STS with HILP and melphalan, these studies also have the problem of being heterogeneous as to the type of STS, disease stage and therapy performed, making comparison difficult. The local recurrence rates range from 0% to 25% with a 5-year survival rate of 56% to 69%.\textsuperscript{26-31} Other perfusion agents have been investigated in the treatment of STS with HILP. Klaase et al. described the use of doxorubicin as the sole perfusion agent but this was ineffective. The complete remissions observed in four patients occurred after perfusion with doxorubicin combined with melphalan. Local toxicity was high, and tissue necrosis necessitated amputation in three cases.\textsuperscript{32} However in a study of Rossi et al, tumor necrosis was more than 50% in 17 patients (74%) and limb-sparing surgery was feasible in 20 patients (91%). They concluded that HILP with doxorubicin is an active and well-tolerated procedure within a multidisciplinary approach of the treatment of limb sarcomas.\textsuperscript{33} Pommier and Di Filippo investigated cisplatin as a perfusion agent in the treatment of STS.\textsuperscript{34,35} Seventeen patients whose sarcomas were measured prior to HILP, none of the patients showed a complete response, three had a partial response (18%), five had a minimal response (29%), seven had no change (41%), and two had progression (12%).\textsuperscript{34} In conclusion, results with HILP for STS were not impressive and alternative strategies for limb preservation by intravenous and intra-arterial adriamycin with preoperative or postoperative radiation therapy followed by compartmental excisions, were able to provide adequate local control for most extremity STS.\textsuperscript{36-39}
Introducing Tumor Necrosis Factor

William Coley, a surgeon who lived and worked in New York City during the second half of the 19th century, was the first to investigate the phenomenon of tumor necrosis, occurring in patients suffering from severe infections. By administering preparations of gram-positive and gram-negative bacteria or their products to patients with inoperable neoplastic diseases, Coley hoped to bring about an involution of the tumor. The side effects of Coley’s regimen were unacceptable, however, and his treatment ultimately fell into disrepute.\(^{40,41}\) Shear and co-workers, seeking to isolate an active therapeutic fraction from Coley’s toxins, purified what they called the “bacterial polysaccharide” from \textit{Serratia marcescens} organisms.\(^{42-44}\) This molecule, now known as lipopolysaccharide (LPS), was shown to induce hemorrhagic necrosis of transplantable tumors in mice.\(^{45}\) A major conceptual advance occurred with the work of O’Malley, et al., who reported that an endogenous factor appeared in the serum of animals treated with LPS, which could induce hemorrhagic necrosis of tumors grown in animals that had not been exposed to LPS. This information, though published in a prominent journal, was largely overlooked for over 20 years.\(^{46}\) The transferability of tumor-necrotizing activity from one animal to another was then identified by Old and co-workers, who showed that a factor produced in mice pretreated with Bacillus Calmette-Guérin (BCG) and subsequently challenged with LPS was capable of causing hemorrhagic necrosis of the meth A sarcoma, grown in the skin of a recipient animal.\(^{47}\) The factor was dubbed “tumor necrosis factor” (TNF). A large number of studies reveal that TNF is produced principally by macrophages.\(^{48-51}\) A long period of time elapsed between the identification of TNF and its isolation in pure form. TNF from a human source was first isolated by Aggarwal and colleagues at Genentec.\(^{52}\) The molecular cloning of the TNF DNA was accomplished almost simultaneously by a number of workers at separate biotechnology firms and the cloning of the human TNF locus followed soon afterwards.\(^{53-56}\)

A lot of articles published both in scientific literature and in popular press claimed, that this molecule would prove to be a revolutionary tool in the battle against cancer. However, phase I and II clinical trials of systemic TNF were very disappointing. An overall response rate of 1-2% was seen in almost 1000 patients treated with systemic TNF.\(^{57-60}\) The dose-limiting toxicity of TNF was typical hypotension, clearly delineating the central role of this cytokine as a mediator of the pathophysiology of septic shock.\(^{61-64}\) This dose-limiting toxicity in patients kept the peak intravascular level achievable in humans 100-fold lower than the level needed for an anti-tumor effect in a mouse model.\(^{65,66}\)

Because it seemed impossible to achieve effective systemic concentrations of TNF in patients, and because it appeared to act very rapidly with a short, single treatment
in animal models, TNF was ideally suited for use in HILP. Ferdy Lejeune and Danielle Lienard, surgical oncologists working in Brussels at the time, were the first to link high-dose TNF and HILP to treat 19 patients with cutaneous melanoma and 4 patients with STS in the early 1990s. In this setting, the equivalent intravascular levels that led to responses in mice (1-3 µg/ml) could be achieved in the perfusion circuit. In a pilot study of 3 patients with TNF as the sole perfusion agent, one complete response of 7 months, one partial response of 21 days, and one minor response lasting for 1 month were observed. Posner described these 3 patients and another 3, treated with HILP and TNF as the sole perfusion agent. One patient had a complete response, 2 patients had a partial response of less than 1 month’s duration and no response was seen in 3 patients. HILP with TNF as the sole perfusion agent showed inadequate activity. Three of these 6 patients had been reperfused with TNF and melphalan resulting in 2 complete responses and 1 partial response. In vitro and vivo studies had already shown an enhanced cytotoxic activity of TNF when chemotherapeutic drugs, especially alkylating agents were added. The treatment regimen conceived by Lejeune was a combination of preoperative subcutaneous interferon-gamma (IFN) and perfusion with low-dose IFN, high-dose TNF and melphalan for a 90-minute treatment period. The IFN was added to the regimen because it synergized with TNF in pre-clinical studies. In all 23 cases, an early and spectacular softening of the tumors was seen within the first 3 days after treatment, consistent with the TNF effect seen in the murine models. Sixteen of 19 patients with melanoma (84%) and 3 out of 4 patients with a STS (75%) showed a complete response. Three melanoma (16%) and 1 STS (25%) showed a partial response. Based on the initial study, two prospective randomized trials were initiated. In Europe, Lejeune and colleagues started a prospective randomized phase II study of patients with advanced melanoma of the limbs with in-transit metastasis. They compared 32 patients who received melphalan plus TNF and IFN to 32 patients who received melphalan plus TNF only. The overall response rate and the complete response rate were higher for the patients treated with IFN compared to the ones treated with melphalan TNF only, 100% vs. 91% and 78% vs. 69% respectively, but the differences were not significant. In the United States a trial comparing melphalan alone to the identical dose of melphalan combined with TNF and IFN was initiated by Fraker in patients with in-transit melanoma of the extremity with no known disease outside the extremity. At an interim analysis of this study the complete response rate for melphalan, TNF and IFN perfusion arm was 80% and 61% for the melphalan alone perfusion arm. In a subgroup of patients with a high tumor burden of the extremity, the melphalan, TNF and IFN perfusion arm had a much more dramatic effect (67% complete responses) than what could be achieved by melphalan alone (17% complete
responses). Patients with low tumor burden or small tumors showed equivalent results with both of these two perfusion regimens, 87% complete responses with TNF versus 81% with melphalan only. The complete response rate seen with melphalan alone in this study is somewhat better than that reported by other investigators and in order to draw conclusions about the value of TNF as an adjunct to HILP in melanoma patients, more patients need to be included.

When the benefit of TNF with melphalan in HILP for bulky melanoma was observed, the same regimen was applied to STS. The results were much more positive in this combination compared to melphalan alone, and several series have been published demonstrating limb preservation in patients deemed to have unresectable tumors with amputation as the only surgical option. The overall approach with large extremity sarcomas that have no local resection options because of their relationship to neurovascular and bony structures, is to conduct HILP with TNF and melphalan. This treatment results in significant tumor shrinkage in 6 to 12 weeks. A second procedure is performed after this period to resect the remaining tumor that is often reduced in size. Patients with multifocal sarcoma do not undergo the secondary resection, similar to those patients suffering from in-transit melanoma. The European trial of 186 patients showed complete responses in 18% and partial responses in 57% of the cases measuring tumor size. HILP with TNF and melphalan was also feasible in patients with locally advanced extremity STS with disseminated disease as local control improved the quality of life. These studies on bulky extremity sarcomas demonstrated that TNF acts by attacking the tumor vasculature with rapid elimination of tumor blood flow within days after treatment. Other more unusual tumors of the extremity such as Merkel cell carcinoma, which often spreads by in-transit metastases within the limb, as well as eccrine adenocarcinoma and basal and squamous cell skin carcinoma have been reported to respond to HILP with melphalan plus TNF. Again, because this treatment acts via an apparent antiangiogenic mechanism, it may be applicable against all solid malignancies, with the tumor endothelium as the target tissue, which is similar across several histologies.

**Toxicity of HILP**

Toxicity of HILP can be categorized as a side effect from systemic exposure to the drugs and as a side effect due to the regional effects of high-dose exposure. The systemic exposure depends not only on the adequacy of the isolation during HILP, but is also caused by systemic exposure to the perfused drug during reperfusion. Although the limb is flushed after perfusion, residual active agents still remain in the limb either within the intravascular space or in the interstitial fluid, which results in a systemic peak of drug concentration following the re-establishment of normal
vascular flow to the extremity. Systemic leakage of melphalan has been described and consisted of nausea and vomiting (22%), bone marrow depression in 4% and miscellaneous systemic side-effects, including fever and minimal scalp hair loss, occurring in 19 patients (5%). With the introduction of high-dose TNF at levels 10 times the maximum tolerated systemic intravenous bolus, isolation was all the more important, but it introduced also another path to systemic toxicity namely the induction of secondary host mediators during HILP that are subsequently released into the systemic circulation after the perfusion. For standard chemotherapeutics, there is little or no induction of host mediators. The systemic effects of TNF HILP reflect the reported toxicity present in phase 1 systemic TNF trials. The most serious complication is hypotension. In the first report by Lienard, 23% (7/31) of the patients treated experienced hypotension, and 10% (3/31) showed severe hypotension. All patients in this initial trial received dopamine (3 mg/kg/min) at the time of TNF injection into the perfusate as a prophylaxis against hypotension. The most significant toxicity of TNF limb perfusions can be summarized as a so called Systemic Inflammatory Response Syndrome (SIRS). This was observed in all patients and was accompanied by fever, rise in cardiac output, fall in systemic vascular resistance and the need for fluid resuscitation and inotropes. Perfusion with melphalan as the sole perfusion agent did not trigger these effects. Detailed analysis showed positive correlations between maximum TNF concentrations and systemic vascular resistance and cardiac index. The National Cancer Institute perfusion group demonstrated the relation between the vascular response and the need for vasopressor support and systemic TNF levels in patients with TNF leakage as well. Lejeune also demonstrated severe toxicity in patients with leaks of >5%. Vrouwenraets et al. reported an absence of severe systemic toxicity of TNF in patients without systemic leakage. Stam et al. observed only a mild postoperative toxicity in the event of significant leakage during perfusion. This was easily managed on the ICU with fluid substitution and, in some cases, with vasopressors. All these systemical side effects of TNF HILP were minimal, transient, and could easily be managed with appropriate resuscitative techniques.

The normal tissues in the limb that are perfused such as skin, muscle, peripheral nerves, blood vessels, bone, cartilage, and synovium comprising the skeletal system, are also exposed to the same concentrations of anti-neoplastic agents active against the tumor. Wieberdink developed a grading system to score these regional toxicities. The toxicities seen with melphalan are skin erythema, some with areas of blistering and subcutaneous edema, in virtually all patients. The skin changes as well as this edema universally returns to normal after several months. The most important toxicities are the effects on muscle and peripheral nerves. Myopathy can occur with
mild muscle discomfort and in the worst case may cause a compartment syndrome with potential muscle necrosis and subsequent limb loss. This is the main reason why a prophylactic fasciotomy is performed after HILP at the University Hospital in Groningen. Long term analysis of limb function after fasciotomy showed no impaired function of the perfused limb compared to the contralateral none perfused limb. This was in contrast with other reports claiming approximately 5% to 10% of the patients have significant long-term discomfort in their extremity after HILP, a difference that can be possibly explained by the prophylactic fasciotomy. Initial reports from Lienard et al. indicate that TNF and IFN add little to the regional toxicity of limb perfusions compared to melphalan alone. Skin erythema and desquamation, edema, joint stiffness, and peripheral neuropathy appear to occur in the same number of patients as after melphalan alone perfusions.

**Positron Emission Tomography**

Positron Emission Tomography (PET) is a non invasive, diagnostic imaging technique for measuring the metabolic activity of cells in the human body with the aid of short-lived positron emitting radiopharmaceuticals. Traditional diagnostic techniques, such as x-rays, CT scans or MRI, produce images of the body’s anatomy or structure.

The first step in a PET-study is to label a selected compound with a positron emitting radionuclide. Starting from non-radioactive atoms, a cyclotron is used to produce radionuclides. In a cyclotron, particles such as protons or deuterons (hydrogen and deuterium atoms without their orbital electrons) are brought to high energies by traversing several hundred orbits within the cyclotron. When the protons or deuterons orbits near the maximum radius of the cyclotron, they are removed through electrostatic or magnetic deflection and are impinged upon small volume hollow metallic cylinders filled with a nonradioactive gas or liquid. Nuclear reactions take place within the cylinder (target) between the high energy particle (proton or deuteron) and the contents of the target. With different target materials, different radioactive products can be obtained. These are then separated from the target material and can be used in the synthesis of more complex radiopharmaceuticals. The most frequently applied radionuclides in PET are carbon-11 ($^{11}$C, half-life 20 minutes), nitrogen-13 ($^{13}$N half-life 10 minutes), oxygen-15 ($^{15}$O half-life 2 minutes) and fluorine-18 ($^{18}$F half-life 110 minutes).

The production of the radiopharmaceutical is performed with the use of automated synthesis systems. These are located within lead-walled (5-6 cm thick) cabinets so called “hot cells”. The precise composition of the radiopharmaceutical is assured by testing the products with e.g. high pressure liquid chromatography before administrating them to the patient. Sterility and pyrogen testing are performed on
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every dose afterwards.
The radionuclides now incorporated within the radiopharmaceutical, have a surplus of positive nuclear particles. Because this is an unstable situation, these radionuclides either capture an electron or emit a positron (which is a particle with the same weight as an electron, but with a positive charge) to achieve stability, depending on the energy of the nucleus. After a positron is emitted, it is rapidly slowed down by interactions within the surrounding tissue until all its kinetic energy (velocity) is lost. At this point, the positron combines momentarily with an electron. The combination of particles (positron and electron) then totally annihilates or disintegrates and results in two diametrically (180° apart) photons of exactly 511 keV energy. The pairs of photons are emitted equally from the body in all directions. In general, several million events (photon pairs) are accumulated for each PET image.
The next step in PET is to detect the emitted photons with the PET camera. The PET camera used for this study at the University of Groningen contains 8192 crystals oriented into 16 rings arranged in two rings of 64 detector blocks each 512 detectors per ring. The 16 rings are used to collect 16 planes (slices) of data and an additional 15 cross-planes (slices) are obtained by collecting photon interactions between adjacent direct planes for a total of 31 planes. The scanner has a 10.4 cm axial field of view. Patients are positioned comfortably on a table which moves through the opening of the scanner. Some patients require only one field of view (10 cm) to visualize a particular area of the body while others are moved through the scanner using 9-10 bed positions (90-100 cm) to complete whole body imaging. PET cameras make use of the fact that the two annihilation quanta have opposite directions. Emitted photons can be absorbed by the detectors in the camera. Each detector has connections with many opposite detectors. A signal is said to be caused by annihilation if the capture of a photon by two opposite detectors coincides within 20 nsec. Simultaneous detection of two of these photons by detectors on opposite sides of an object places the site of the annihilation or on about a line connecting the centers of the two detectors. At this point mapping the distribution of annihilations in the field of view by a computer is possible and an image can be reconstructed. If the annihilation originates outside the volume between the two detectors, only one of the photons can be detected, and since the detection of a single photon does not satisfy the coincidence condition, the event is rejected. The image achieved is generally presented as a gray scale image of a cross-section of the patient, with the intensity of each picture element proportional to the isotope concentration at that point in the patient.
Fluorine-18 labeled 2-fluoro-2-deoxy-D-glucose (FDG) is one of the most widely used radiopharmaceuticals used in PET and has proven to be of value in the visualization of various types of tumors.95,96 The use of FDG is based on Warburg’s
observation of increased glycolysis in cancer cells. The citric acid cycle, which is more efficient in adenosine triphosphate generation, is suppressed.\textsuperscript{97} As a result, cancer cells accumulate the glucose analog FDG which is trapped intracellularly as FDG phosphate. The FDG consumption, and since FDG acts in the same way as glucose, the glucose consumption can be determined with the use of a three-compartment model: plasma-FDG, tissue-FDG and tissue-FDG-6-phosphate, as described by Sokoloff.\textsuperscript{98} The tissue components can be measured by the PET camera and the plasma components can be measured by counting the activity in blood samples. With the compartment model, the glucose consumption can be calculated in $\mu$mol per 100 grams of tissue per minute.

The majority of the PET studies with amino acid tracers have been performed with L-[methyl-1\textsuperscript{11}C]-methionine (MET).\textsuperscript{99-101} MET reflects amino acid uptake rather than protein synthesis and because it is involved in other metabolic pathways such as transmethylation and polyamine synthesis, this may lead to accumulation of a variety of nonprotein metabolites in tumor tissue.\textsuperscript{102-104} This complicated metabolism of methionine has made it impossible to create a precise metabolic model. Carboxyl-labeled amino acids, such as L-[1-\textsuperscript{11}C]-tyrosine (TYR), L-[1-\textsuperscript{11}C]-methionine and L-[1-\textsuperscript{11}C]-leucine, appear to be more appropriate compounds to determine protein synthesis in tumors.\textsuperscript{103,105} The main metabolite of these amino acids is $^{11}$CO$_2$, which is rapidly cleared from tissue and exhaled and does not contribute to the PET-measured $^{11}$C radioactivity in tumor tissue. Using a method developed at the PET Center Groningen, the protein synthesis rate can be determined using $^{11}$C labeled L-amino acids with a four-compartment model: plasma-amino-acid, tissue-nonprotein-amino-acid, metabolites and protein-incorporated-amino-acid.\textsuperscript{106}

**The aim of this thesis**

Hyperthermic isolated limb perfusion is a major surgical procedure and over the years new developments have been initiated and examined. Traditionally the University Hospital Groningen plays an important role in the history of regional perfusion and therefore this thesis describes different aspects of regional perfusion during the last decade.

1. What are the short term effects of HILP with cisplatin in the local treatment of spontaneous osteosarcoma in dogs?
2. Is HILP with TNF and cisplatin feasible in the canine model?
3. What are the results of HILP with cisplatin in patients with sarcomas of soft tissue and bone?
4. What is the relation between the tumor vascularization and the vascular changes after irradiation therapy?

5. How does HILP influence the glucose metabolism and protein metabolism as studied by PET, and is it possible to predict the outcome of therapy?

6. Is it worthwhile to monitor continuous leakage with RISA during HILP with TNF and melphalan?
Introduction

References

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Introduction


Chapter 1


