imILT®

Scientific Background

Preclinical and clinical studies

TRANBERG®Thermal Therapy System

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1 List of abbreviations

AE	Adverse Event
APC	Antigen Presenting Cell
CIS	Cancer *In Situ*
CT	Computed Tomography
CTLA-4	Cytotoxic T-Lymphocyte-Associated protein 4
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cell
ECOG	Eastern Cooperative Oncology Group
EIT	Electrical Impedance Tomography
FLA	Focal Laser Ablation
HIFU	High Focused Ultrasound
HSP	Heat Shock Protein
ICD	Immunogenic Cell Death
IFN-γ	Interferon-gamma
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-18	Interleukin-18
ILT	Interstitial Laser Thermotherapy
imILT	Immune stimulating Interstitial Laser Thermotherapy
Laser	Light Amplification by Stimulated Emission of Radiation
LITT	Laser-Induced ThermoTherapy
MW	Microwave
NIR	Near Infrared
ND-YAG	NeoDynium-Yttrium Aluminium Garnet
PD-1	Programmed cell Death protein 1
SAE	Serious Adverse Event
SADE	Serious Adverse Device Event
SD	Standard Deviation
TGF-β	Transforming Growth Factor beta

2 Executive summary

Immune stimulating Interstitial Laser Thermotherapy (ImILT) using the Tranberg® Thermal Therapy System (TTT System), consist of a precision temperature-controlled destruction of a tumor using a laser. The protocol ensures hyperthermia at the tumor boarder below coagulating temperature, resulting in immunogenic cell death and with the potential to affect metastasis elsewhere.

There are a large number of well designed, preclinical studies in clinically relevant models available. Using the ImILT protocol has been shown to evoke a local control of the treated tumor, a powerful immune reaction and a complete eradication of secondary tumors (abscopal effect) in preclinical settings where the ingoing parameters can be controlled. In a few patients in the clinical setting, a possible abscopal effect on secondary tumors has been reported. There are limited clinical data available, and additional clinical trials are planned to identify the tumor properties and patient groups that will have the optimal response to the treatment.

3 Introduction

In the late 19th century the German physician Busch reported that a facial sarcoma disappeared after a prolonged episode of fever due to erysipelas (1). Later an American surgeon used heat (fever) induced by bacteria or toxins injected locally into the tumor to treat cancer (2) (3). Using bacteria or toxins to induce fever in order to provide hyperthermic treatment and activation of the immune system is impractical and risky and therefore focus has been on externally induced hyperthermia which was suggested in 1898 by a Swedish gynecologist who used local hyperthermia on advanced cervical cancer (4). During the late 20th century several methods for treatment with heat were introduced; Whole Body Hyperthermia, Regional Hyperthermia, and Local Hyperthermia using several different methods to induce heat either externally or internally. External methods include microwave or radiofrequency therapy or focused ultrasound. The problems with external methods are that a very precise focusing is needed to avoid damage to non-tumorous tissue and in some cases high water content or e.g. bone may hinder the heating at the preferred location (tumor). Internal methods depend on probes/needles inserted into the target tumor/tissue and include radiofrequency, microwave, and laser ablation.

Whole body hyperthermia differs from local hyperthermia in that the whole body is heated. The body temperature is kept between 41.5°C and 42°C for one hour or more and has been suggested for patients with advanced cancer. The method may not be used in patients with decreased left ventricular ejection fraction, decreased pulmonary vital capacity, brain metastases, etc. (5). The method has been used in combination with chemotherapy, but results are few; 27 studies are listed at clinicaltrials.gov but no results have been posted.

Local hyperthermia has primarily been developed as tools to ablate/eradicate local tumors when surgical excision is deemed impossible. For this purpose, focal treatments other than hyperthermia have been developed such as cryoablation and radiotherapy. During the last decades data has emerged indicating that in-situ destruction of tumors may induce tumor antigen release which may stimulate antigen specific cellular immunity. This hypothesis is further supported by the finding that shrinkage of untreated tumors occurs concurrently with shrinkage of the focally treated tumor(s), the abscopal effect (‘ab’ - away from, ‘scopus’ – target). These findings have boosted the interest in focal ablation of tumors.

Immune stimulating Interstitial Laser Thermotheraphy (ImILT) using the Tranberg® Thermal Therapy System (TTT System), consist of a precision temperature-controlled destruction of a tumor using a laser. The protocol ensures hyperthermia at the tumor boarder below coagulating temperature, resulting in immunogenic cell death and with the potential to affect metastasis elsewhere. This whitepaper on ImILT will present the technical development, the preclinical and clinical data of the TTT System.

4 Hyperthermia

4.1 Hyper-thermic tissue effects

Supraphysiological temperatures (>40°C) causes thermal damage to the tissue. In principle, four different stages of thermal damage can be distinguished: 1) hyperthermia, 2) coagulation, 3) boiling of water and 4) carbonization, ablation, and vaporization. The different stages are dependent on the temperature and exposure time in combination with the specific tissue properties and will cause changes at the molecular, cellular, and structural level.

At temperatures between 41 and 43°C the damage to the tissue/cells may not be visible on microscopy. However, the nuclear matrix may undergo significant changes already at 40°C which may affect transcription and replication. In addition, these temperatures may affect plasma membrane permeability and alter the mitochondrial membrane potential (6). These changes may result in cell death during replication but may also affect e.g. cell respiration.

Between 43 and 45°C the macroscopic appearance is erythema and edema in the tissue and is visible in the microscope as shrinkage of the cytoplasm and condensation of the chromatin. The extent of the damage will be dependent on the temperature and the exposure time, i.e. at lower temperatures and exposure times the damage is reversible while longer exposure time at a low temperature will result in irreversible damages. One of the major changes is unfolding and aggregation of proteins with a significant impact in the nucleus due to the large number of proteins. These changes will result in inability to DNA repair and thus increase the unrepaired DNA damage. However, in principle these changes are reversible by the presence of molecular chaperons such as Heat Shock Protein (HSP) 70 (6). The cell death is mainly promoted through apoptosis and the extent of damage may not be detected until 48-72 hours after temperature increase and subsequent cell death because of difficulties to distinguish cells that can recover from those that will not (7).

At temperatures above 45°C the unfolding of proteins becomes more evident. If direct DNA damage is caused by higher temperatures or not is not clear, it may be that the damage is caused by the inability to DNA repair due to unfolded and/or denatured proteins (6).

At temperatures lower than 60°C (and above 42°C), irreversible cell death, without instantaneous coagulation, occurs mainly due to inactivation of vital enzymes, and the time needed for cell death is longer the lower the treatment temperature (8) (9) (10). It has been shown that a temperature of 45°C leads to inactivation of vital enzymes and intracellular processes, which will lead to irreversible cell damage within 30 min (8). The tight dependence on the temperature level was shown in another tumor system; heating for 30 min at 43, 43.5 and 44°C produced apoptosis, heating at 45°C produced a mixture of apoptosis and necrosis whereas heating at 46-47°C produced only necrosis (10). Immunostimulating interstitial laser thermotherapy (the imILT® protocol) at 46°C required treatment for 30 minutes to ensure radicality in tumor-bearing rats (see chapter 9).

Temperatures above 60°C produce protein denaturation and coagulation, which immediately leads to coagulative necrosis and destroys tumor antigens. The coagulative necrosis is an irreversible injury and detectable on microscopy immediately after the damage. These temperatures also destroy vessels, which prevents transfer of antigens into the lymph vessels and systemic circulation. At 100°C the water in the tissue vaporizes and the cells will rupture due to steam bubbles. Above approximately 200° C the tissue will carbonize.

4.2 Other effects of hyperthermia

When normal tissue is heated, the blood perfusion increases due to dilatation of vessels and increased permeability of the vascular wall. Between 44 and 46°C blood perfusion begins to decline, due to damage to the endothelium, hemorrhage, or thrombosis. In tumors the blood perfusion is greater near the tumor margin than in the neighboring normal tissue. The blood flow in tumor tissue does not increase to the same extent as in normal tissue on heating since the blood flow is already at or near the full capacity. Therefore, the blood flow decreases at lower temperature levels (40.5-43.5°C) and after shorter exposure times in tumor tissue compared to normal tissue (11). This difference in tissue perfusion leads to a greater rise of tissue temperature in tumors during therapy, resulting in a greater proportion of cell death, compared to normal tissue.

Due to the decreased blood flow the metabolism in the heated tissue will decrease and the acidity will increase due to anaerobic metabolism (11). Since cells in acidic environment are more sensitive to heat this will also affect the heat induced cell death.

5 Immunology and focal tumor ablation

Since only a few years back immunotherapy is an established tool in the cancer clinic. Research in the tumor immunology field has moved fast during the last decade and a series of therapeutic monoclonal antibodies have shown extraordinary anti-cancer effects in several tumor types (12). The new therapeutic antibodies for systemic immunotherapy of cancer are primarily directed to block immune checkpoints like CTLA-4 and PD-1 and thereby release suppressed anti-tumor T lymphocyte activity. Therapeutic blockade of immune checkpoints has revolutionized treatment of metastatic melanoma and other types of cancer, but only less than half of treated patients experience durable responses (13). Therefore, combinations with checkpoint blockade and other immune therapies or conventional therapies are necessary to further broaden and enhance their effects.

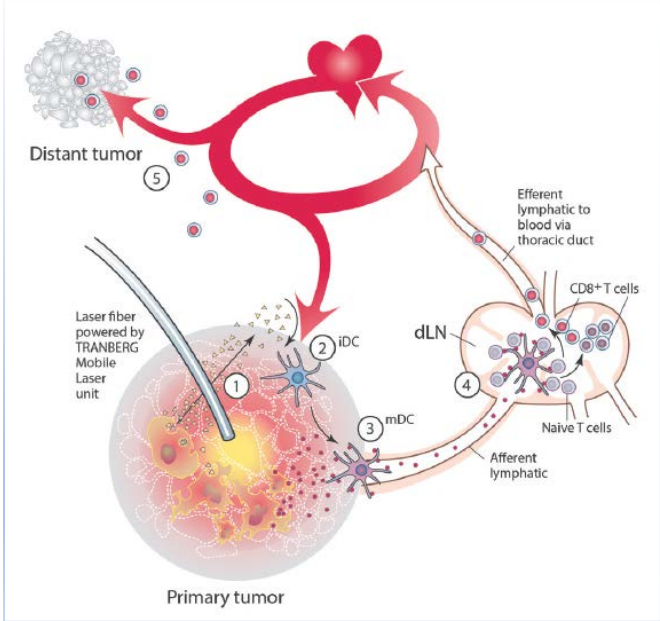

Figure 1.Overview for the proposed mechanism of action for imILT. 1. Laser induced hyperthermia induces cell rupture and cytokine release (yellow triangles). 2. This recruits antigen presenting cells (APCs) to the tumour. 3. APCs take up tumour cells/antigens (red circles) and are activated. 4. Activated APCs travel to draining lymph nodes (dLN) and cross-present tumour antigens to T lymphocytes, in turn activating and turning them into cytotoxic T lymphocytes (CD8+ T cells). 5. CD8+ T cells traffic through the circulation and attack untreated tumour cells.

To date the immune response activated by local ablative treatment is recognized but not fully understood. It is assumed that the ablative treatment releases tumor antigens that eventually will result in a systemic anti-tumor response (Figure 1). Methods such as high-temperature laser ablation have been developed for focal malignancies and in recent years a large volume of data has emerged on the effect of *in situ* tumor destruction on inflammatory and immune components resulting in systemic anti-tumor reactions (14) (15). An important feature to stimulate anti-tumor response is to induce immunogenic cell death (ICD) of the tumor cells (16) (17). It is evident that *in situ* tumor destruction involves ICD with tumor antigen release, cross presentation and the release of Damage Associated Molecular Pattern (DAMP) molecules to make the tumor its own cellular vaccine (14). *In situ* tumor tissue destruction may stimulate antigen-specific cellular immunity by activating inflammatory cells and converting the tumor environment from immune suppression towards immune activity. Dendritic Cells (DCs) are attracted to this microenvironment, undergo maturation after internalizing cellular debris containing tumor antigens and are exposed to DAMP molecules. Mature DCs activate antigen-specific cellular immunity via presentation of processed antigens to T cells. Different T cell types develop into tumor selective effector cells capable to kill tumor cells. The immunomodulation exhibited by *in situ* destruction by carefully using energy sources like laser light mediating distinct temperatures in the tumor tissue induces ICD and immune responses directed to the tumor and its tumor antigens. The spreading of this immunity to also eliminate other non-treated tumors resulting in tumor shrinkage is often called the abscopal effect and is now regarded an established immune mediated phenomenon (15) (18).

Immune stimulating Interstitial Laser Thermotherapy (imILT®) is a treatment protocol that, when applied, may result in immunological changes with the potential to activate a systemic immune response against remaining tumors. This is achieved by using laser light energy to increase temperature in the tumor tissue and controlling the temperature in the tumor periphery at a lower level during a prolonged treatment time. This induces ICD of the tumor cells (Figure 1) and necrotic cells that, as opposed to apoptotic cells, can produce mature DCs that are capable to induce antigen-specific T cells (19) (20). The imILT® protocol is a precision laser ablation protocol and applies selected treatment temperatures and durations for induction of ICD. The preclinical proof-of-principle and proof-of-concept studies indicates that the imILT® protocol evokes an immune-medicated abscopal anti-tumor effect.

A majority of cancer patients with advanced disease still lack effective treatment also when treated with checkpoint blockade. Next generation of immunotherapies for malignant diseases will therefore include combinations of checkpoint blockade and e.g. immune stimulation and/or vaccination (13). One such tool to stimulate immunity may constitute of the use of the imILT® protocol.

6 Laser Physics

Laser is an acronym for **l**ight **a**mplification by **s**timulated **e**mission of **r**adiation. Gamma rays, x-rays, ultraviolet, visible, and infrared light, microwaves, and radio waves are all forms of electromagnetic radiation and make up the electromagnetic spectrum. Wavelength, frequency, or energy of a photon are used to describe electromagnetic radiation. The energy of electromagnetic radiation is emitted in the form of photons.

A laser emits light coherently, spatially, and temporally, through a process of optical amplification based on the stimulated emission of electromagnetic radiation. The laser energy is the product of the delivered power and exposure time. During laser induced thermotherapy light causes damage in tissue due to direct absorption of light and through heat conduction into the tissue of the absorbed energy. Therefore, laser thermotherapy produces an ablation size that is larger than the volume where light is absorbed due to this heat conduction.

Dependent on the wavelength, the emitted light can be absorbed or scattered by the tissue in different ratios. This determines how much light is transmitted through the tissue. Absorption is determined by the tissue properties and composition (type and quantity of chromophores like water and hemoglobin, which is the main absorber in the near infrared (NIR) wavelength). Scattering occurs because of inhomogeneities of the tissue structure (e.g. membranes, nuclei, etc.). Scattering also increases when the tissue is coagulated, and the proteins are unfolded. The penetration depth of laser light in tissue is basically determined by scattering and absorption and is defined as the distance at which the light is attenuated to 1/e (37% of original intensity). The penetration depth can be used to describe the volume in which the main part of the laser energy is absorbed in tissue.

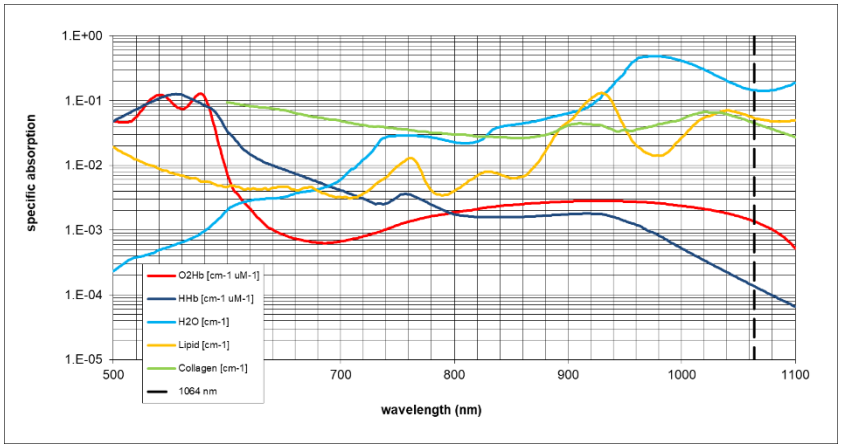

Figure 2. Absorption spectra of tissue components in the window 500-1100 nm. Dotted line at 1064 nm.

Penetration depth can also be used to determine how the risk for carbonization (charring by temperatures above 150°C) during ablation is affected by the choice of the wavelength. Low penetration depth indicates a higher power density in tissue and thereby a higher risk of carbonization.

The absorption of a specific tissue depends on the tissue composition. Each component has a specific absorption spectrum. In Figure 2, the absorption spectra of the major absorbers in the near-infrared window in biological tissue are shown. Light at 1064nm was chosen for the TRANBERG system because of its relatively high penetration depth in tissue and its suitability for the treatment of a variety of tissues.

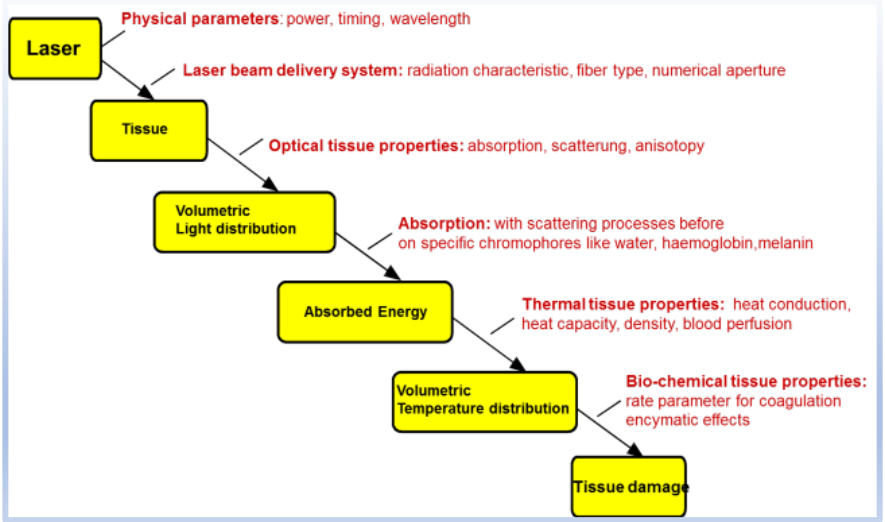

Figure 3. Factors influencing the capacity of laser induced ablation

Thus, laser induced ablation is not only dependent on the laser device properties but also on tissue properties such as absorption and scattering of the laser light, heat conduction in the tissue, biochemical tissue properties, etc. (Figure 3).

7 Local destruction therapies

Ablation is synonymous to surgical removal of a body part or tissue but is used to describe a method to destroy/evaporate tissue, rather than surgically removing it. Ablative therapy is used to treat not only tumors but also for treatment of atrial fibrillation, other cardiac disorders, epilepsy, Parkinson's disease, etc. For tumor treatment, most methods used aim at treating tumors that are not resectable due to anatomical difficulties or metastatic tumors that cause local problems.

Methods for local tumor destruction that are in clinical use include laser-induced thermotherapy (LITT or focal laser ablation (FLA)), radiofrequency ablation (RFA), high-focused ultrasound (HIFU), cryotherapy, microwave coagulation (MW), ethanol or acetic acid injection, photodynamic therapy (PDT), and radiation. In recent years, clinical use has focused on MW, RFA and LITT since they appear superior to the other methods with respect to efficacy, performance, and safety. These methods can be classified as hyperthermic treatments. The TRANBERG®Thermal Therapy System and the imILT®protocol treatment is a method that combines local tumor destruction by FLA, with an activation of the body's immune system to attack other tumors and metastases, as shown in preclinical studies.

8 imILT®

Immune stimulating Interstitial Laser Thermotherapy induces a precision temperature-controlled destruction of a tumor. It is based on using LITT (laser-induced thermotherapy) and applies a special treatment protocol including temperature regulation of the laser energy delivered to achieve necrosis at non-coagulative temperatures in the tumor periphery, resulting in immunogenic cell death (ICD).

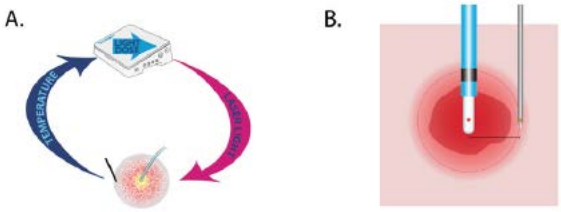

Figure 4. Overview of the setup for imILT® protocol. A. Graphical representation of the feedback system of the TRANBERG Thermal Therapy System. B. Placement of optical fibre applicator delivering laser light and temperature probe measuring the temperature within the targeted tumour tissue.

Thereby, the neoplastic cells in the periphery of the tumor will be exposed to heating at 46 – 57°C inducing necrosis and ICD in this part of the treated tumor. This part of the tissue will then release intact (not coagulated) antigenic material that may create an immune response towards tumor cells (Figure 1). The antigenic material will induce an immunological reaction that has the potential to affect metastasis elsewhere (abscopal effect) and to lower the rate of recurrence of the tumor.

Treatment of tumors with the TRANBERG®|Thermal Therapy System, using the imILT® protocol is indicated for palliative treatment in adult patients with primary pancreatic cancer, primary or secondary cancer in the liver and breast cancer who are not eligible for other treatment options. The use of the imILT® protocol is restricted to clinical studies. The system consists of a laser generator providing laser light to the tissue through a silica optical fibre, which is inserted into the tumor, causing heat as the light is absorbed by the tumor tissue. A temperature probe connected to the laser unit is placed in the periphery or just outside of the tumor and regulates the laser output, keeping the temperature at the rim of the tumor constant (Figure 4). The energy source is a built-in laser diode that emits light at a wavelength of 1064 nm up to 25 W continuous wave. The wavelength used by this system is in the invisible near infrared region and is commonly used for laser procedures because of its deeper penetration capabilities.

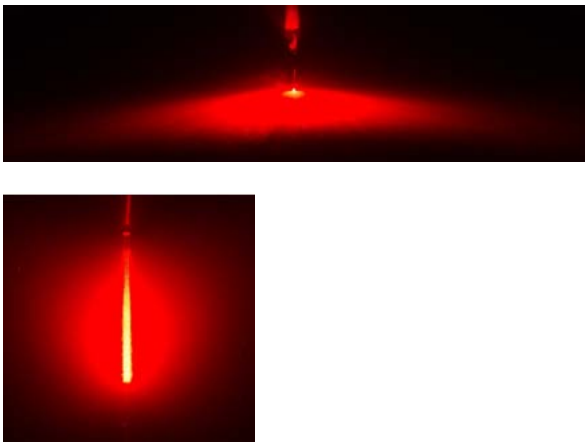

Figure 5. Spread of laser light at 1064 nm as illustrated by red light with lesser wavelength. Upper panel: Radial applicator creates spherical volumes; Lower panel: Diffusor applicator

Radial emitting laser applicator creates spherical ablation volumes and diffuser tipped laser applicator results in ellipsoidal ablation volumes (Figure 5). The distal end of both laser applicators consists of a silica cap with a diameter of 1.7 mm/15 G. There are diffusor tips with an active length of 15 and 25 mm. Both types of laser applicator are used together with an introducer with a stylet to insert the laser applicator into the tissue.

9 Preclinical studies on efficacy of imILT

All preclinical studies were performed using a prototype of the laser system with temperature feedback modality for enabling equivalent treatment as the imILT®protocol for small animals. In general, the laser fibre was placed centrally in the tumor and the feedback thermistor was placed 3 mm outside of the tumor margin. Below follows a summary of experiments with imILT.

The tumors used in the majority of the preclinical studies are dimethylhydrazine-induced, weakly immunogenic adenocarcinomas of the rat colon. This type is known to have a poor immunogenicity, which is the ability to induce an adaptive immune response. Weak antigenicity is the root cause of why the immune system ultimately fails to control tumor growth; weak tumor antigens stimulate a weak, and thus slow, immune response that provides the opportunity and time for tumor cells to develop immune evasion mechanisms and to ultimately gain the upper hand. Human tumors show great variation in immunogenicity, why the weakly immunogenic adenocarcinoma is considered as a relevant worst case compared to the clinical situation. Induced colon cancer was selected because it is common for colon cancer to metastasize and grow well in the liver, hence, the tumor will be exposed to a relevant microenvironment. The implanted (or injected) tumors metastasize from the implantation site, which also correlates well with the clinical situation. In conclusion, the selection of type of tumor and the implantation site are clinically relevant factors for assessing the potential immunological effect following treatment with Laser Interstitial Thermal Therapy using imILT® protocol.

9.1 Interstitial laser thermotherapy of adenocarcinoma transplanted into rat liver (21)

The aim of the study was to define the effect of different temperatures and exposure times in imILT.

Two sets of experiments were conducted: 1/ the effect of imILT treatment at different target temperatures on local tumor control, 2/ the effect of different treatment times with imILT. In the first series of experiment 24 male Wistar Furth rats were randomly assigned to imILT treatment with a target temperature of 43, 46, or 50°C for 30 minutes. In the second series of experiment 24 rats were randomly assigned to imILT treatment at a target temperature of 46°C for 10, 20 or 30 minutes. A dimethyl-hydrazine induced adenocarcinoma was implanted in the left lateral lobe of the liver. For the first set of experiments the treatment was conducted 6 days and for the second 8 days after inoculation of the tumor.

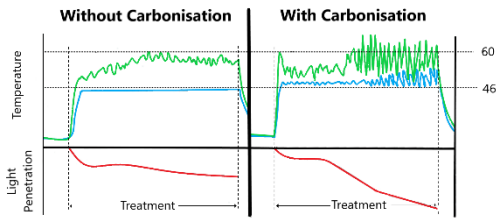

Figure 6. Schematic drawing of temperature and light penetration. The left panel shows a treatment session without carbonisation and the right during which carbonisation occurred. One thermistor (green) is located at the tumour margin, and one thermistor (blue), the feed-back thermistor is placed 3 mm outside of the tumour margin. The temperature is controlled at 46° C and light penetration is measured 10 mm from the laser fibre.

Carbonization occurred in 3/8 at a target temperature of 43°C, 4/8 at 46° C, and 8/8 at 50° C. In the second series carbonization occurred in 50% of the animals,

regardless of treatment time. Carbonization resulted in a substantial decrease in light penetration (Figure 6).

imILT produced necrosis of the entire tumor in all rats treated for 30 minutes, regardless of treatment temperature. However, if treatment time was less than 30 minutes (10 and 20 minutes)), 2 rats in each group did not display complete tumor necrosis.

In conclusion, in this model aiming at low temperatures at the tumor margin, treatment time seems more essential than the target temperature to achieve complete tumor eradication.

9.2 Comparison between interstitial laser thermotherapy and excision of an adenocarcinoma transplanted into rat liver (22)

The aim of the study was to define tumor burden after treatment comparing interstitial laser thermotherapy with excision of liver tumor.

Five experimental groups were investigated; 1/ imILT of tumor, 2/ Sham imILT of tumor, 3/ Left lateral liver lobe resection (tumor bearing lobe), 4/ Median lobe liver resection (no tumor), 5/ Sham liver resection. One hundred and twenty male Wistar Furth rats were randomly allocated to either of the groups. A dimethyl-hydrazine induced adenocarcinoma was implanted in the left lateral lobe of the liver. Treatments were performed 8 days later with a target temperature of 46°C for 30 minutes. The rats were sacrificed 6, 12 or 24 days later and examined for tumor burden.

In a supplementary study the effect of varying treatment times with imILT was investigated. Target temperature was 46°C and treatment times were 10, 20 and 30 minutes. In addition, one group had an intended incomplete treatment with a target temperate of 44°C at the tumor border for 30 minutes.

Six days after imILT there was complete tumor necrosis in all imILT treated animals, after liver resection there was tumor growth at the resection margin in 2/8 rats and intraperitoneal tumor implant in 1/8.

At 12 and 24 days there was tumor growth in the liver of imILT treated rats in 7/16 rats and intraperitoneal spread in 6/16, after liver resection there was tumor growth in the resection margin in 6/16 rats and intraperitoneal spread in 11/16.

Six days after imILT for 10 minutes small areas of viable tumor cells were found, and 2/8 rats displayed intraperitoneal tumor growth. At 20 minutes treatment time small areas of viable tumor cells were found in the treatment area in 2/8 rats and 2/8 rats displayed intraperitoneal tumor growth. Finally, at 30 minutes treatment and 6 days later no remaining tumor was found in the liver while 1/8 rats displayed intraperitoneal tumor growth. The group of rats that were treated suboptimal, i.e. at 44°C at the tumor margin for 30 minutes all displayed tumors at the treatment site and 1/8 showed intraperitoneal tumor growth.

In conclusion, imILT treatment reduces the spread of an experimental liver tumor compared to liver resection. The local treatment effect is temperature and time sensitive, but suboptimal local treatment, either due to too low target temperature or too short treatment time, still reduces intraperitoneal spread. This suggests that immunologic mechanisms are involved.

The findings in refs 21 and 23 led to the conclusion that imILT with radical intent should be performed for 30 min, with the laser fibre placed centrally in the tumor, and with a target temperature of 46 °C at the feedback thermistor that is placed 3 mm outside of the tumor margin. This set-up was used in all subsequent treatments with locally radical intent.

9.3 Interstitial laser thermotherapy of a rat liver adenocarcinoma (23)

The aim of the study was to define tumor growth after treatment of liver adenocarcinoma comparing imILT with liver resection.

In the first series of experiments, 72 inbred Wistar Furth rats were randomly assigned to three study groups: 1/ imILT, 2/ sham imILT, 3/ resection of tumor bearing liver lobe. A dimethyl-hydrazine induced adenocarcinoma of the rat colon was implanted in the left lateral liver lobe. Treatments with either imILT (target temperature 46° C), or sham imILT, for 30 minutes or resection of the tumor bearing left lateral liver lobe were performed 8 days after implantation. The rats were sacrificed 6, 12 or 24 days later and investigated for tumor burden, macroscopically and microscopically.

Six days after imILT there was complete tumor necrosis in all rats with no signs of tumor cells. At 12- and 24-days tumor growth was found in 7/16 rats, associated with intraperitoneal spread in 6 rats. Six days after liver resection there was tumor growth in the resection margin in 2/8 rats, associated with intraperitoneal spread in 1. At 12- and 24-days tumor growth was found in 6/16 rats and intraperitoneal spread in 11/16 animals.

In a second series of experiments thirty-three rats were inoculated with tumor cell suspensions prepared from BN7005, an adenocarcinoma induced by dimethylhydrazine in a Brown Norwegian (BN) rat. Seventeen BN rats had tumor cell inoculations (as opposed to tumor implantation) in both the left lateral and the median liver lobes. 15-16 days later the left lateral tumor was treated with imILT or sham ILT with the tumor in the median lobe serving as a read-out tumor for a possible systemic effect. In addition, sixteen rats had inoculation into the median lobe alone and 15-16 days later these rats underwent laparotomy alone. All rats were sacrificed 2 weeks after the treatment.

In rats with twin tumors, both take and growth of the untreated tumor in the median lobe was smaller after imILT than after sham imILT and smaller than the single tumor seen after sham laparotomy.

It is concluded in the study that imILT 1) reduces spread of liver tumors as compared to resection and 2) decreases the growth of concomitant, untreated tumors, i.e., that it produces an abscopal effect in the preclinical setting. It is proposed that this is due to an immunologic effect evoked by the imILT treatment.

9.4 Heat shock protein 70 (HSP70) after laser thermotherapy of an adenocarcinoma transplanted into rat liver (24)

The aim of the study was to define the effect of imILT on HSP70 in tumor and immune cells.

Three groups were investigated: 1/ imILT, 2/ sham imILT, 3/ control (no treatment). Experiments were performed in Wistar Furth rats using a dimethyl-hydrazine induced adenocarcinoma in the left lateral lobe of the liver. Sixty-six rats were randomly assigned to the study groups and imILT treatment were performed 6 days after tumor implantation. Rats (6 per group and post treatment time) were sacrificed 15 minutes, 5, 10, 15 hours or 12 days after treatment. At sacrifice the liver tissue with the tumor area was collected and later analyzed for ED1 macrophages, HSP70, and $CD8^+$-cells.

There was an increase of HSP70 immunoreactivity in tumors treated with imILT as compared to controls and HSP70 displayed a shift from the cytoplasm to the nucleus in the imILT treated animals. Also, tumor infiltrating ED1 macrophages showed an increase in the imILT treated rats and these cells showed an increased presence of HSP70 in the membrane and cytoplasm as compared to control

rats. Only few CD8⁺-cells were found. Finally, serum HSP70 increased during the first 15 hours after imILT.

In conclusion, imILT caused an increase in HSP70 within tumors and a shift of HSP70 from cytoplasm to nucleus. Furthermore, there was an increased number of HSP-positive tumor-infiltrating macrophages after treatment.

9.5 Resistance to tumour challenge after tumour laser thermotherapy is associated with a cellular immune response (25)

The aim of the study was to define the time-response relationship of the imILT induced immunization and the cellular response of macrophages and lymphocytes.

Four groups were investigated: 1/ imILT, 2/ resection of tumor bearing liver lobe, 3/ resection of normal liver lobe in rats without tumor, 4/ imILT of normal liver parenchyma in rats without tumor. Two hundred and eighty-eight Wistar Furth rats were randomly assigned to the study groups and a dimethyl-hydrazine induced adenocarcinoma were implanted in the liver. The first implantation (the tumor to be treated) was done in the left lateral lobe (study group 1 and 2) and the second (challenging) tumor was implanted in the median lobe (all study groups). imILT treatment was performed 6-8 days after implantation of the first tumor and the challenging tumor was implanted 2, 5, or 10 weeks later. The rats were sacrificed 6, 12, or 48 days after challenging, unless they showed signs of inactivity or distress at an earlier time. Tumor burden, ED1 and ED2 macrophages, and CD4⁺ and CD8⁺ lymphocytes were evaluated.

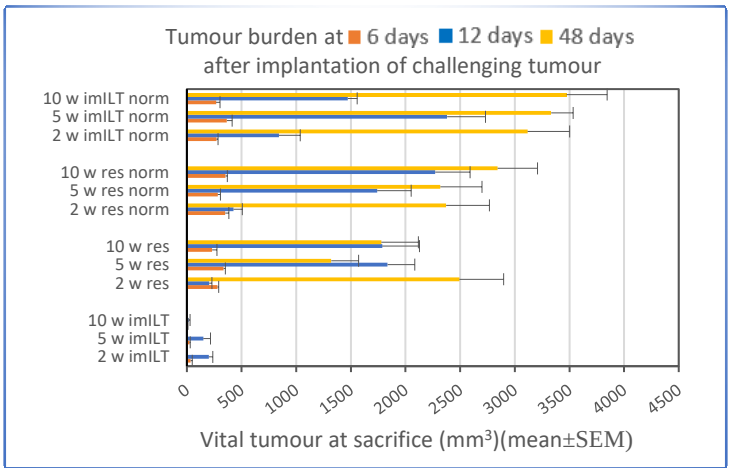

Figure 7. Only rats having been treated with imILT of primary tumour survived for 48 days after implantation of challenging tumour. All other rats in the 48 days study group had to be euthanised within 10-30 days after the tumour challenge due to extensive tumour burden. imILTnorm: imILT treatment of normal liver tissue; res norm: resection of normal liver lobe; res: resection of lateral liver lobe with tumour; imILT: imILT treatment of tumour in lateral liver lobe. These treatments were performed 2, 5, or 10 weeks before implantation of challenging tumour.

In the rats that 2, 5, or 10 weeks previously had undergone imILT for a tumor the reimplantation of a second tumor (challenge) was followed by eradication of the tumor at day 48 (7). For the rats that had undergone liver resection of the primary tumor the reimplantation of a second tumor was followed by a substantial tumor growth to the extent that the rats did not survive for 48 days. Likewise, the rats that were treated with liver resection or imILT *without* having a primary tumor displayed a substantial growth of the challenging tumor and did not survive for 48 days (). At day 12 after challenge ED1 macrophages in viable tumor and tumor capsule was significantly larger in the imILT group (group 1) than in the liver resection group (group 2). Similarly, there was a significant but more obvious difference in number of CD8⁺ cells in these two groups at both 6 and 12 days after challenge. ED2 and CD4 cells were always few and no differences between the groups could be demonstrated.

The study showed that the used temperature and time was adequate for destruction of the tumor and that the treatment resulted in immunological changes that activated a systemic immune

response that completely eradicated a secondary tumor of the same sort as the first tumor in all 72 rats.

In addition, the study clearly demonstrates that treatment of normal liver tissue (treatment group III & IV) does not induce any immunologic effect, further strengthening the statement that the immunologic response is specific and induced by an earlier tumor cell necrosis. Thus, treating a viable tumor with imILT evoked a strong immune response which prevents future identical tumors from being able to grow and spread. Most likely this response is a cellular immunologic response as demonstrated by the increased number of CD8+ T cells in the challenging tumors.

9.6 Additional studies

Additional studies have been performed during the development of the ImILT protocol. One study on Wistar Furth rats evaluated the effect of hepatic inflow occlusion on tumor growth in combination with ImILT treatment (26). The relative tumor growth was similar in controls and hepatic inflow occlusion while both imILT treated groups displayed lower ratios. The combination of imILT and hepatic inflow occlusion was most efficient. Hepatic inflow occlusion has also been evaluated in pigs (27). In conclusion the hepatic blood flow had a strong influence on ablation size in imILT. Furthermore, increase in power does not seem to be sufficient to counteract the cooling effect of the blood flow.

A combination of Linomide and ImILT has been evaluated in Wistar Furth rats (28). Linomide has been reported to have immunomodulatory and/or anti-angiogenetic effects in various tumor models. In the study, Linomide further reduced the liver tumor growth and intraperitoneal spread in rats treated with imILT, which was associated with a decrease of tumor associated macrophages and newly formed blood vessels. In Linomide treatment alone, a reduced tumor growth was also seen but no effect was demonstrated on intraperitoneal spread or on macrophages or newly formed vessels. Thus, imILT induced necrosis and reduced the number of tumor-associated macrophages seemingly adding to the Linomide capability to induce a reduction in newly formed vessels.

The immunologic pathway was evaluated in another study (29). A dimethylhydrazine-induced adenocarcinoma (HID2) was implanted into the liver of syngeneic rats. Intraperitoneal injection of irradiated, IL-18 transfected HID2 tumor cells was performed 7 days later. Rats were allocated to treatment with imILT or sham imILT + resection of the tumor-bearing lobe, 9 days after tumor implantation. Spleen and lymph node anti-tumor lymphocyte proliferation and cytokine production in response to wild-type and IL-18 transfected tumor stimulator cells were studied 11, 30 and 50 days after treatment. The study showed that imILT induced an anti-tumor lymphocyte proliferative response that started in tumor-draining lymph nodes and subsequently developed in other lymph nodes and in the spleen. The main effects on lymphocyte cytokine production were an increased release of IFN-γ and IL-10 from tumor-draining lymph nodes and a decreased splenic release of IL-10.

One study was performed with the aim to define the effect of imILT on local blood flow when treating normal liver parenchyma (30). The study was performed in normal rats without liver tumor(s) assigned to three different groups; 1/ imILT, target temperature 41°C, 2/ imILT, target temperature 44°C, and 3/ sham treatment. Treatment time was 30 minutes.

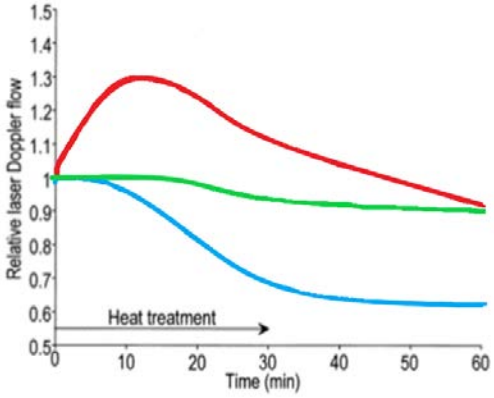

Figure 8. Schematic drawing of perfusion given in relative perfusion units. Red: 41°C feed-back thermistor location; Green: Control; Blue: 44°C feed-back thermistor location.

In conclusion, the target temperature in imILT is of importance regarding the blood flow in the periphery of treated lesion and there seems to be a zone between 41°C and 44°C where the blood flow changes from increasing to decreasing which may have an impact on a potential immunologic reaction (Figure 9).

9.7 Summary, preclinical studies

The main preclinical studies evaluating the effect of the imILT protocol has been summarized in the table below.

Table 1. Summary of the main preclinical studies

Study	Groups	Tumor	Treatment temp/time	Results (for feedback protocol)
Ivarsson 2005 (25)	ILT Resection of tumor bearing lobe Resection of normal liver lobe ILT of normal liver	Dimethylhydrazine-induced adenocarcinoma	46°C, 30 min	Challenging tumor completely eradicated No spread of tumor Memory effect
Tranberg 2002 (23) Series 1	ILT Sham ILT Resection of tumor bearing lobe	Dimethylhydrazine-induced adenocarcinoma	46°C, 30 min	Equivalent results as resection in local control Reduced spread of tumor Systemic effect (lowered metastatic spread)
Tranberg 2002 (23) Series 2	ILT (treatment of 1/2 tumors) Sham ILT (treatment of 1/2 tumors) Laparotomy (single tumor)	Tumor cells from dimethylhydrazine-induced adenocarcinoma	46°C, 30 min	Less growth of untreated tumor compared to Sham ILT and laparotomy. Abscopal effect

Möller 1997 (21)	ILT Sham ILT	Tumor cells from dimethylhydrazine-induced adenocarcinoma Dimethylhydrazine-induced adenocarcinoma	43, 46, 50°C, 30 min 46°C, 10, 20, 30 min	The protocol was superior in complete necrosis (100 %) The treatment is time sensitive to achieve necrosis
Möller 1998 (22)	ILT Sham ILT Resection of tumor bearing lobe Resection of normal liver lobe Sham liver resection	Dimethylhydrazine-induced adenocarcinoma	46°C, 30 min 46°C, 10, 20, 30 min 44°C, 30 min	Equivalent results as resection in local control Reduction of tumor spread compared to resection. Systemic effect (lowered metastatic spread). Local treatment effect is temp and time sensitive, but suboptimal treatment still reduces spread.

10 Clinical studies on imILT, pre-CE mark and CLS initiated

10.1 Interstitial laser treatment of malignant tumours: initial experience (31)

The aim of the study was to show the feasibility of interstitial laser treatment using two different treatment modes.

Twelve patients (median age 61 years), 7 with recurrent colorectal carcinoma (liver metastases or local recurrences), 3 patients with pancreatic cancer, and 2 patients with primary liver cancer were treated. Two different treatment protocols were used: interstitial laser photocoagulation (FLA) (7 patients) and interstitial laser thermotherapy (imILT) (5 patients). imILT was used for treatment of pancreatic cancer and local recurrences.

Using the imILT mode the temperature control was good, and no carbonization occurred. Target temperature was 45°C - 48°C and actual temperatures were close to the intended steady-state temperatures. In one patient with a pancreatic head cancer pain relief was exceptional and remained so for the rest of his life (8 months). Another patient with a pancreatic body cancer was completely relived of pain for 3 of his 4.5 remaining months. imILT also alleviated the symptoms for 40 months in a patient with a thoracic wall tumor and for 3 months in a patient with an abdominal wall tumor and finally a patient with a perineal local recurrence symptom were relieved for at least 5 months.

The study indicated that it is possible to achieve long-term control and symptom relive of malignant tumors with the imILT treatment.

10.2 Induction of a distant anti-tumour effect by interstitial laser thermotherapy (imILT) in a patient with malignant melanoma (32)

A 60-year old male with a thoracic malignant melanoma developed axillary disease two years after surgery. He was treated for the next three years with surgical excisions, external irradiation, cytostatics and immuno¬modulating substances such as interferon-γ, granulocyte-macrophage-colony-stimulating-factor, interleukin-2 and 13-cisretinoic acid. The disease continued to progress, and numerous biopsies failed to show any evidence of immunologic activity. The patient was ultimately treated with interstitial laser thermotherapy (ILT) to palliate a large ulcerating tumor that covered most of the left thoracic cage and the left axilla. ILT sessions, using a multi-fibre system, consisted of treatment at a temperature of 48°C for 30 min at one or two sites.

Locally, ILT had a good effect with improvement of symptoms and obvious tumor shrinkage of the thoracic-axillary tumor. Due to abdominal pain, a large, untreated intraabdominal metastasis was removed after 4 ILT sessions. Histology showed that this ILT untreated tumor was largely destroyed, as opposed to previous vital tumor, and was heavily infiltrated with macrophages and CD4 T-helper lymphocytes, CD3 pan lymphocytes and CD8 cytotoxic lymphocytes, cells that had been completely absent from all previous biopsies. Thus, in this patient imILT induced an abscopal effect as demonstrated by necrosis and infiltration of immunocompetent cells in a non-treated tumor.

10.3 Interstitial laser thermotherapy (imILT) of breast cancer (33)

The aim of the study was to show feasibility and anti-tumor effect by imILT for radical treatment of breast cancer.

Twenty-four patients with invasive breast cancer were treated with imILT at a target temperature of 48°C for 30 minutes. Treatment was performed under local anesthesia on an outpatient basis. The laser fibre and the temperature probe were placed under ultrasound guidance in all but 5 patients. Standard surgical excision was performed 12 (4-23) days after imILT treatment.

The patients mean age was 61 (39-84), the tumor was an invasive ductal carcinoma in 15 patients, a lobular carcinoma in 8 patients and a lobular-ductal cancer in 1 patient. Average tumor diameter was 14 mm on ultrasound (5-35). In 8 patients there was CIS (cancer *in situ*) outside the invasive cancer. The treatment was well tolerated but many patients had a sensation of warmth locally at the beginning of treatment. Pain was reported by 15 patients during treatment and tenderness in the treatment area was reported by 5 patients after treatment. Two patients experienced minor skin necrosis, the treated tumors being 3.4 and 15 mm respectively from the skin surface.

During the following surgical excision, the surgeons experienced that the tumors was larger than expected and that it was more difficult than usual to palpate the tumor border probably due to the inflammation induced by imILT. At pathological examination, the tumors were mean 23 mm (7-55) large.

The extent of laser induced damage could not be judged with ultrasound. At microscopy the cancer necrosis varied between 0 – 100%; in the first 4 patients the laser fibre was placed 3 mm outside the tumor border resulting in necrosis between 0 and 33%, in 8 patients 4 laser fibres were placed at the tumor border with the feedback thermistor 5 mm outside the tumor border resulting in a necrosis of 0 – 60%, in 2 patients 4 laser fibres were used and placed at the tumor border with the thermistor in the tumor center resulting in 83% and 100% necrosis; finally 10 patients had one laser fibre placed in the center of the tumor with the thermistor at or outside the tumor border resulting in necrosis of 5 – 100%. In addition, microscopy showed that the tumors usually had very irregular borders and carbonization was found in few tumors only. Two patients died due to metastatic disease one year after treatment, they both had lymphatic glandular metastases at initial surgery.

The difficulties in achieving local radicality agrees with those observed in other ablative therapies and may be due to problems to estimate the size on imaging. Therefore, the need for improved pre- and intra-operative imaging is crucial for ablative therapies to be locally radical. The treatment was well tolerated with few and mild adverse events.

10.4 Changes in immunocompetent cells after interstitial laser thermotherapy of breast cancer (34)

The aim of this study was to show the effect of imILT of breast carcinoma on immune cells in the tumor and regional lymph nodes.

Seventeen women (17/24 patients in the study above (sect. 10.3) who had radical surgical excision after imILT and another 6 patients operated for breast cancer without prior imILT served as controls. imILT was performed at a target temperature of 48°C for 30 minutes. Surgical excision was performed 12 (6-23) days after the imILT procedure. Immunohistology was performed on core needle biopsies obtained prior to imILT and surgery and compared to excised specimens.

The imILT treated patients had a 29% (1-98) tumor necrosis after the imILT treatment. Changes in immune cell density showed the same pattern in both tumor border and within the tumor (Figure 10). $CD8^+$ cells were significantly increased within the tumor and a tendency to increase was also seen at the tumor border.

For the dendritic cells there were no significant difference in $CD1a^+$ immature cells while the mature dendritic cells ($CD83^+$) were increased at the tumor border indicating an ongoing immune response.

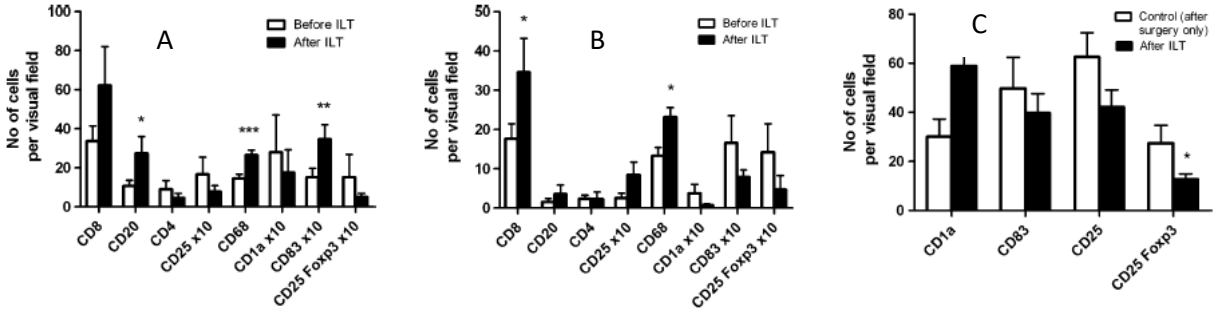

Figure 9. Graphs showing effect of imILT on immune cells. A/ Tumour border before and after imILT; B/ Within tumour before and after imILT. C/ Regional lymph nodes after imILT and in control patients not imILT treated.

In the lymph nodes there was no statistical difference compared to non-imILT treated patients but a trend towards increase in $CD1a^+$ cells could be noted.

The number of T_{reg} ($CD25^+Foxp3^+$) cells within the tumor or at the tumor border did not change significantly, although there was a trend towards a decrease in $CD25^+$ and $CD25^+Foxp3^+$ cells. However, in the lymph nodes without metastases the imILT treated patients had a significantly lower number of $CD25^+Foxp3^+$ cells than the control patients (Figure 10C).

imILT induced changes in immunocompetent cells in treated as compared to non-treated patients. Thus, imILT induced a significant increase in $CD8^+$ cytotoxic lymphocytes and mature $CD83^+$ dendritic cells at the tumor and a decrease in $CD25^+Foxp3^+$ T_{reg} lymphocytes in regional lymph nodes. It is concluded that the stimulation of the immune system is an added feature of imILT treatment.

10.5 Long-term follow-up after interstitial laser thermotherapy of breast cancer (35)

The aim of the study was to show the effect of immunological changes induced by imILT on long-term outcome of patients with breast cancer.

This was a long-term follow-up including all (24) patients treated with imILT (see section 10.3) The patients were followed-up according to the standard protocol established for southern Sweden and included postoperative clinical check-up 2 weeks after surgery and then clinical examination and mammography, and in some cases ultrasound, every year for 3 years. No patient was lost to follow-up.

Follow-up was median 116 (91-136) months. Patients operated with partial mastectomy had radiotherapy after surgery and those with estrogen-positive tumors received adjuvant anti-estrogen therapy. No patient experienced local recurrence. Sixteen patients were N0 and none developed recurrent disease. Eight patients were N1-3, whereof 5 developed metastases. For those 5 patients the median tumor necrosis after imILT was 19 (0-60) %. Comparing the occurrence of immunocompetent cells (see chapter 10.4) with the outcome showed lower number of $CD8^+$ cells, both before and after treatment, in patients without recurrence than in those with recurrence. Most other immunocompetent cells did not differ between patients with or without recurrence.

The increase in immunocompetent cells in patients treated with imILT could not be linked to favorable outcome in terms of recurrence. However, the number of patients is too low to adjust for receptor status (e.g. estrogen receptors, progesterone-receptors, Herceptin receptors), tumor stage, the total necrosis volume induced by imILT or the following surgical intervention. Thus, although imILT does induce an immunologic reaction apart from inducing necrosis of the tumor, larger patient groups are needed to understand the importance of the immunologic reaction for the final outcome.

10.6 Interstitial laser thermotherapy (imILT) as a treatment option in breast cancer patients not suitable for surgical excision (CSR-2015-009, data on file)

The aim of the study was to provide evidence of patient safety and additional evidence of apparatus usability and to analyze the local effect of imILT.

Patients were eligible if they had histologically confirmed breast cancer, T1-3 and N0-2 disease, and had assessable tumors by MRI or ultrasound. This was a descriptive and exploratory study and no statistical consideration regarding sample size or power calculation was made for this investigation. Treatment was carried out with the TRANBERG®Thermal Therapy System. The laser fibre was placed in the tumor using image guidance such as ultrasound. To facilitate placement an introducer set was used to create a channel for the laser fibre within the target tissue. A master temperature probe sensor was placed about 0-3 mm outside the tumor border. The tumor was heated by the laser beam using temperature feedback control so that treatment gave a steady-state target temperature of 46° C for 30 min at the feed-back temperature probe. Treatment was performed with one laser fibre. The evaluation of tumor response used the sum of the two largest perpendicular diameters of target lesions (SPD) and followed the immune-related response criteria (irRC).

Three patients were recruited, one patient withdrew consent before treatment. Thus, only 2 patients were treated.

No patient had any other tumors than the one treated. One patient had a tumor size of 17 mm at screening, and after treatment the tumor was either not visible or not measurable. The second patient had a tumor size of 23 mm at screening. No new MRI was done until 7 months later, when the tumor measured 26 mm. At ultrasound measurements post-operatively and at 28 days post treatment the tumor appeared somewhat reduced in size. No adverse events were reported. Pain score before treatment was 0 for both patients and increased to 4 during treatment.

Based on only two treated patients it is impossible to draw any firm conclusions regarding efficacy. However, the use of the TRANBERG®Thermal Therapy System seems safe in treatment of breast cancer, no adverse events was reported

10.7 To Evaluate the Safety & Palliative Treatment Effect on Patients with Solid Cancers by Immunostimulating Interstitial Laser Thermotherapy (imILT) (CSR-2014-003, data on file), Frankfurt

The aim of the study was to evaluate safety and adverse events and to describe and evaluate the imILT-induced changes with imaging methods (MRI and CT) in patients with solid tumors, including metastases, that are unresectable or inoperable.

The study patients were adults with solid organ malignancies with or without regional and distant metastases and were inoperable or had unresectable disease.

The laser fibre was placed in the tumor using image guidance such as ultrasound or CT to facilitate placement, an introducer set was used to create a channel within the target tissue for the laser fibre. Depending on tumor size, a master temperature probe was placed about 3 mm outside the tumor border or within the tumor for regulation of laser output. The tumor was heated by the laser beam using temperature feedback control so that treatment gave a steady-state target temperature of 46° C for 30 min at the feed-back temperature probe. Treatment was typically performed with one laser fibre which typically can create an ablation size of about 30 mm in diameter. If re-treatment were considered beneficial, it could be repeated once or twice with a 4-week interval between treatments.

Re-treatment should not be done if there was evidence of progressive disease after the preceding treatment. Response was evaluated according to RECIST and irRC criteria. Immuno-monitoring blood work, core needle biopsy of target lesion, and core needle biopsy of non-target lesion were also to be obtained.

Only three patients were included and treated once in the study. All patients were followed up according to the protocol at day 56 – 70. No patients were treated a second time. One patient did not fulfil the criteria for retreatment, one patient was withdrawn by the investigator due to AE, and one patient did not want repeated treatment. One patient had lymph node metastases from a breast cancer, two patients had liver metastases from a colon cancer.

The laser treatment can create an ablation size of approximately 30-35 mm. Two of the patients had tumors larger than this size while only one patient had a tumor with an adequate size. Hence, in 2 patients the treatment probably was suboptimal and did not have any obvious impact on the tumors that continued to grow, although the treated tumors were considered stable at end-of-study according to evaluation criteria. But in both patients', other metastases showed progressive disease. However, in patient no 3, the tumor had an adequate size at treatment and did not show any signs of continuous growth after treatment, this tumor was stable at the 2-month follow-up. In addition, non-treated metastases also displayed a stable disease at follow-up.

No serious adverse events were reported during or after the treatment. Several mild adverse events were recorded during the 2-month follow up period, but none was considered related to the treatment.

In conclusion the imILT treatment was safe. There were too few patients to evaluate efficacy but it was noticed that the patient who had a complete treatment of the target tumor displayed not only stable disease regarding the treated tumor but also regarding other simultaneous tumors as an indication of a possible abscopal effect.

10.8 Pancreatic cancer metastasis, Department of interventional radiology, Portuguese Oncology Institute of Porto (CTP-2015-006 Data on file)

This was an open-label, double-arm study to assess the effect of treatment with a combination of imILT protocol and standard chemotherapy treatment compared to chemotherapy standard treatment alone. The study assessed the clinical and radiologic response together with pain registration (VAS). Adverse events were also registered together with performance and user-friendliness.

The patients in the experimental arm was treated with imILT and standard chemotherapy and the patients in the control group was treated with the standard chemotherapy. imILT was given, at least two weeks before chemotherapy, to eliminate the possibility of the immune activation effect reduced by a prior cytotoxic chemotherapy influence.

For both locally advanced disease and for stage IV disease, three alternative chemotherapy schedules for non-resectable patients were employed as standard treatment. These were the FOLFIRINOX (folic acid, 5-fluorouracil, irinotectan, oxaliplatin), gemcitabine alone or gemcitabine plus nab-paclitaxel.

The imILT treatment in this open-labelled investigation was not blinded. The control group consisted of 10 patients fulfilling the inclusion/exclusion criteria and treated with chemotherapy standard treatment. The control group was matched as to age, gender and grading of pancreas cancer.

- Seven patients were included in the study, five to active treatment and two as controls. One patient was allocated to active treatment, but surgery could not be performed. The patient was lost to follow-up and treated at another hospital. Thus, five patients were treated, two patients served as controls, and one patient was lost-to-follow-up
- Three patients were male, three were female and for one patient gender was missing. Age was between 42-69 years, with a mean age of 58.
- Actively treated patients:
    - One patient had a target tumor in the liver, being 15 mm at screening. The tumor was completely disappeared at 28 days but multiple liver metastasis, not reported at screening, were detected. This was assessed as a complete local response for the target lesion and progressive disease for non-target lesions. At 3 months, the tumor was back and had a size of 29 mm. The spread of multiple liver metastasis was assessed as worse.
    - One patient had treatment of a liver lesion. The lesion was slightly smaller at day 28 and at 3 months. The pancreatic tumor was 39*34 mm at screening and was measured to 39*35 mm at 28 days and 3 months. A segment VI/VII liver lesion was 23*12 mm at screening and was measured to 5 mm at 28 days and 3 months. No assessment according to RECIST criteria was done.
    - One patient had a liver lesion treated. The tumor size was 6 mm at screening and had increased in size to 36*16 mm post-operatively and then shrinking to 24*11 mm at 28 days. At 3 months the lesion was not measured, and it was evaluated as complete response. The pancreatic tumor remained the same size at all measurements and was evaluated as stable disease at 3 months.
    - One patient had a primary pancreatic tumor treated during open surgery. The tumor remained the same size as at screening at all follow-up measurements. No assessment according to RECIST criteria done.
    - One patient treated per operatively in November 2018 due to a locally advanced pancreatic cancer. No available data at this point.
- Control Patients:
    - One patient had a pancreatic tumor of less than 1 cm at screening. No follow-up measurements are available. The tumor was assessed as stable at 28 days, 3 months and 6 months. However, at 3 months peritoneal carcinomatosis was found, which indicated progressive disease. Progressive disease was then confirmed at 6 months.
    - One patient had a pancreatic lesion of 40 mm at screening. The tumor was growing and reached 60 mm at 6 months. No assessment according to RECIST criteria.

10.9 Prospective, open, non-comparative trial to evaluate safety and feasibility of immunostimulating Interstitial Laser Thermotherapy (imILT®) in Stage III pancreatic carcinoma, Marseille (CSR-2015-001 Data on file)

The primary objective was to evaluate the safety, the feasibility, and the adverse events of imILT® protocol in patients with locally advanced stage-III ductal adenocarcinoma of the pancreas, not further responsive to chemo- or chemoradiotherapy. The study included patients between 18-80 years who were diagnosed with Stage III pancreatic ductal pancreatic adenocarcinoma and did not

need palliative surgery at time of diagnosis. The patients selected for inclusion were patients that had already received chemotherapy or (chemo)radiotherapy and in whom further such treatments had been discontinued because they were not able to induce a downstaging of the disease and/or because of side effects. Only limited and incomplete data is available and collected in an interim report. Five patients were treated.

At the latest follow up, all but one patient was alive. The deceased patient died of due to a moderate primary infection of central catheter sepsis 2 years after the treatment. One patient had survived 24 months, one 18 months and one 16 months. The two last patients have survived 3 months or less but are both well. The two patients that have survived 18 and 16 months respectively have recurrence of the disease, while the first patient who has survived for 24 months have a suspicion of recurrence. An overview of the efficacy on the tumors as well as survival is presented in the table below.

Table 2. Efficacy and survival

Patient #	**Target tumor size at screening (mm^2)**	**Efficacy target tumor**	**Efficacy non-target tumor**	**Patient status at latest follow up**
1	1394	Tumor size reduced in size until month 9, then growth was detected.	Size reduced until 18 months after surgery	Local and systemic progression
2	272	Tumor size reduced at 3 months follow up, at 6 months and onwards, size had increased.	Only target tumor	Local and systemic progression
3	899	Tumor size reduced at 3 months follow up without local progress. Multiple liver nodes.	Only target tumor	Local and systemic progression
4	945	Reduced tumor size post op, at month 3 growth was detected (still below original size), but at 9 months size was substantially larger than before treatment. Multiple hepatic lesions.	Only target tumor	Local and systemic progression
5	414	Reduced size until month 6, following substantially increase at month 9.	Only target tumor	Local and systemic progression

If the patient has locally advanced disease, the median survival is six to eleven months (36). At the last reported patient follow up in this study, three of the five patients had survived for over 15 months. Another interesting finding is a possible abscopal effect seen in one patient. However, since this is the only patient with a measurable secondary tumor that was not treated, no conclusions can be drawn from this. Based on QoL measurements it seems like all patients are feeling worse initially after the laser-treatment. However, for patients where additional follow-up data is available, this seems to be related to the surgery, and patients was recovering well.

No major safety concerns were noted regarding the imILT® treatment as none of the AEs or SAEs were related to the treatment, but most likely rather related to the status of the patient population or other treatments. This indicates that the treatment with imILT® is safe and well tolerated.

10.10 Exploratory study on Immunostimulating Interstitial Laser Thermotherapy in Malignant Melanoma (CTP-2015-004 Data on file)

The objective of the study was to analyze the local effect of imILT protocol, determine if there is a significant distant and thus probably immune-mediated effect on non-laser-treated tumor mass, and to provide additional evidence of patient safety and apparatus usability in patients with advanced melanoma disease.

Six patients were screened for the study. Two patients failed screening and 4 patients signed Informed Consent. Two patients did not receive treatment, one because of lack of availability of surgical ward and one because of the site of the tumor. Only two patients were therefore included and treated in the study. Both patients were included in Q4 2015 and both patients completed all study visits.

In general, the device was perceived as very easy or easy to handle, except for handling and placing the introducer, which was perceived as difficult for both patients. One patient was treated in a tumor in the right shoulder. The tumor was stable at assessment one month after treatment. At three months follow up, the tumor had doubled its size and was assessed as progressive disease. One patient was treated in a tumor in the right axilla. The tumor was significantly enlarged at 1 month follow up and was assessed as progressive disease. At three months the tumor had continued to grow even further. No effect on the treated tumors or other metastasis could be demonstrated. One patient had pain and swelling at the treated location one month after treatment, and this was judged as related to laser treatment by the treating Physician. No additional information about this adverse event is available. There were no other safety related findings due to the laser treatment in these two patients.

11 Technical development of the imILT device

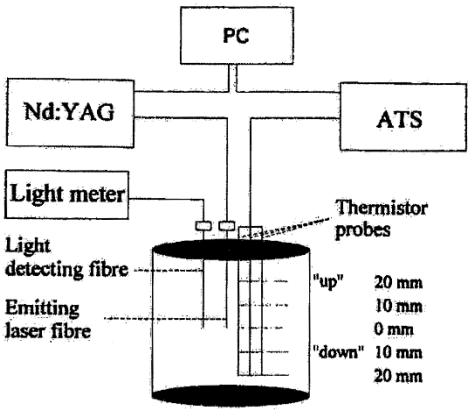

Figure 10. Schematic drawing of the laser thermotherapy system and the arrangement of the laser probe, the light detecting fibre, and the 5-point thermistor probes in the liver phantom. PC: personal computer; ATS: automatic thermotherapy system.

11.1 Interstitial laser thermotherapy: comparison between bare fibre and sapphire probe (37)

The aim of the study was to compare a bare laser fibre with a sapphire laser probe in terms of temperature distribution and light penetration.

The bare fibre will emit light only from the tip and in a forward direction whilst the sapphire probe with a frosted section of 5 mm will emit the laser light not only from the tip of the fibre but also from its lateral side, i.e. diffusing emission.

Calf liver was used as a phantom and the set-up of the device was as shown in Figure 11. The target temperature was 43°C in all experiments. The thermistor probes were placed parallel to the axis of the laser fibre and at 5, 10, and 15 mm distance from the laser fibre. Experiments were performed at laser output power between 1 and 4 W (n=8 in each energy group and fibre type). The temperature feed-back control was performed by using the middle thermistor located 10 mm laterally to the end of the laser fibre. Laser thermotherapy was delivered for 40 minutes. Light penetration was measured at a distance of 10 mm from the laser tip.

Carbonization, with rapid impairment of light penetration, was present in all but two experiments with the bare fibre while it was absent with the sapphire probe in all but 2 experiments at 1-2W, in half the experiments at 3W and in one experiment at 4W. Absence of carbonization was associated with excellent temperature control and a moderate decrease in the penetration of light (c.f. Figure 6). The time from start until target temperature varied with power in experiments without carbonization but not in experiments with carbonization. The temperature was higher below than above the laser tip.

In conclusion, it is easier to avoid carbonization and consequently impairment of light penetration and temperature control with a diffuser fibre than with a bare fibre. In addition, the temperature gradient was smaller with the diffuser fibre.

11.2 Temperature control and light penetration in a feedback interstitial laser thermotherapy system (38)

The aim of the study was to show the performance of a closed loop interstitial laser thermotherapy system using *ex vivo* and *in vivo* models.

The laser thermotherapy system consisted of a laser unit and a temperature feedback control unit interfaced with the laser as shown in Figure 11. The laser beam was delivered through a 600 µm flexible quartz fibre. In the liver phantom studies an artificial sapphire probe with a frosted section of 5 mm was connected to the tip of the fibre to diffuse the laser light not only from the tip of the fibre but also laterally along the frosted surface. The rat studies were performed at 2 W with a bare fibre and without blood inflow occlusion. The light detecting fibre was a 400 µm quartz fibre with a

spherical tip and placed 10 mm from the emitting laser fibre. Experiments were carried out either *ex vivo* on calf liver or *in vivo* on liver tumors in rat liver.

In the ex vivo studies, carbonization was absent in most experiments with laser output power of 1-2 W, in 50% at 3W and at 4-5 W it was present in most experiments. In experiments without carbonization there was a slow, continuous decrease in light transmission, while in experiments with carbonization there was a rapid fall in light transmission (c.f. Figure 6). The time from start of thermotherapy until the target temperature was reached varied with power when no carbonization occurred while the target temperature was reached at similar times when carbonization occurred. The in vivo experiments on liver tumors displayed similar findings as *ex vivo* experiments. In addition, microscopic examination showed necrotic tumor tissue.

In conclusion the studies demonstrated that a closed loop feedback laser thermotherapy system can produce stable and reproducible local hyperthermia with preservation of light penetration and that temperature control is better when carbonization is avoided, and light penetration is preserved.

11.3 Optical fiber solutions for laser ablation of tissue and immunostimulating interstitial laser thermotherapy – product development in the network of developers, industry and users (39)

The aim of the study was to define the most efficient laser fibre in terms of delivered energy without risk for the patient.

By merging the diffuser technology for cooled diffuser fibre with the technology for radial, non-cooled fibres a non-cooled diffuser fibre was designed. Validation was performed *ex vivo* in bovine cardiac muscle and *in vivo* in a porcine survival model.

For imILT a cooled diffuser fibre was unsuitable due to a substantial risk for the patient in terms of failed treatment or rupture of the cooling catheter, especially so in highly vascularized tissue. The non-cooled fibre was shown to safely induce ablations in highly vascularized tissues without device failures. In particular, the non-cooled diffuser fibre could produce larger ablations in highly vascularized tissue and tolerate higher power levels before carbonization as compared to the radial fibre.

The presented technical solution can increase the achievable ablation size and therefore hypothetically increase both the radicality and the immunological benefits of imILT.

11.4 Modelling and monitoring of tissue effects

Several studies have been performed to monitor the tissue effects following the ImILT treatment. The different studies are summarized in this chapter.

Table 3. Summary of studies evaluating tissue effects following the ImILT treatment

Study	Aim	Results
Energy delivery and monitoring in interstitial laser thermotherapy((40)	To show the feasibility of using EIT (Electrical impedance tomography) for non-invasive monitoring of the temperature distribution during laserthermia (imILT).	At 2-3 W there was a good control of the temperature, at 4 and 5 W there were large variations in the temperature readings but the feed-back system was able to control the temperature at the predetermined level regardless of laser power. Impedance initially follows the temperature but continuous to decrease at steady temperature. This might be due to irreversible tissue damage
Feedback interstitial diode laser (805 nm) thermotherapy system: ex vivo evaluation and mathematical modelling with one and four fibers (41)	To evaluate a prototype of the ImILT system with respect to temperature distribution, effectiveness of regulation, and ability to predict temperature distribution by computer simulation.	The stepwise power regulation system was efficient in maintaining a stable target temperature. The results indicate that the system can produce lesion volumes adequate for treating a relatively large tumor in a single session and that computer simulation may be useful for predicting temperature distribution
Finite element analysis for simplified thermal dose planning in interstitial laser thermotherapy (42)	To evaluate if diffusing laser fibres can be modelled as conductive heat sources without miscalculating coagulated volumes, and if finite element analysis, disregarding light transport, may be used for three-dimensional (3D) treatment planning in interstitial laser thermotherapy (ILT).	Coagulated volumes with a single diffusing or conductive applicator differed less than 1% at all studied irradiation times. In conclusion calculated and measured temperatures with four applicators agreed excellently.
Simplified treatment planning for interstitial laser thermotherapy by disregarding light transport: a numerical study (43)	To define the effect of light transport on the temperature distribution and the coagulated volume under conditions relevant to interstitial laser thermotherapy (ILT) of tumors in the human liver.	Numerical calculations showed that the influence of light transport on the coagulated volume was negligible in tissue with optical penetration depths below 3 - 4 mm at all studied irradiation times. The phantom experiment indicated good agreement with the calculated temperature distribution, both with a single diffusing laser fibre and with four fibres.

Another study evaluated if magnetic resonance imaging (MRI) could be used for thermometry during imILT (44). The temperature dependence of the proton resonance frequency was assessed in agarose gel with a high melting temperature (95°C) and in porcine liver in vitro at temperatures relevant to thermotherapy (25—80°C). Furthermore, an optically tissue-like agarose gel phantom was developed and evaluated for use in MRI. The phantom was used to visualize temperature distributions from a diffusing laser fibre by means of the proton resonance frequency shift method. An approximately linear relationship between proton resonance frequency shift and temperature change was found for agarose gel, whereas deviations from a linear relationship were observed for porcine liver. The optically tissue-like agarose gel allowed reliable MRI temperature monitoring, and the MR relaxation times (T1 and T2) and the optical properties were found to be independently alterable. Temperature distributions around a diffusing laser fibre, during irradiation and subsequent cooling, were assessed with high spatial resolution (voxel size = 4.3 mm^3) and with random uncertainties ranging from 0.3°C

to 1.4°C (1 SD) with a 40 s scan time. It was concluded that MRI may be used for temperature monitoring during imILT and should be advantageous based on the possibilities to real time temperature monitoring and monitoring of the temperature distribution in the entire tumor volume.

12 Concluding remarks summarizing preclinical results and clinical experience of imILT

12.1 Preclinical results

- The selection of type of tumor and the implantation site in the preclinical studies are clinically relevant factors for assessing the potential immunological effect following treatment with Laser Interstitial Thermal Therapy using imILT® protocol.
- The preclinical studies are well designed with a large number of animals and blinded assessment of the endpoints.
- In all preclinical studies assessing the efficacy of the ImILT protocol, equivalent or better results on the local control of the treated tumor as well as reduction in metastatic spread and reduced or eradicated secondary tumors (abscopal effect) compared to resection/sham ImILT was seen.
- The ImILT treatment was shown to result in a cellular immune response of tumor-infiltrating $CD8^+$ lymphocytes and produces an increased anti-tumor lymphocyte proliferative response in tumor-draining and systemic lymph nodes and spleen, and in increased HSP70 immunoreactivity in tumors and tumor-infiltrating macrophages.
- Although reduced, some blood flow and tissue perfusion are preserved at the tumor border during imILT treatment.
- imILT produces systemic immunologic effects both after radical and non-radical treatments.
- Temperature control is improved in non-carbonized tissue with preserved light penetration.
- A non-cooled diffuser fibre decreases the risk of carbonization.
- MRI may be used for real-time temperature feed-back control.

12.2 Clinical experience

- The recent clinical trials have been performed on patient groups that are terminally ill and/or without other treatment options.
- Adverse events due to the imILT treatment has to a large part been related to the serious conditions of the patients included in the study.
- Other adverse events are related to heating of ambient tissue and may be avoided using temperature feed-back control to protect sensitive tissues such as normal pancreatic tissue, major vessels, bile ducts, and bowel wall.
- imILT has induced abscopal effects in a few patients, further research is needed to identify the optimal indications, tumor properties and patient groups that would have the best chance for a benefit from the treatment.
- General surgical praxis to aim for treatment of the whole target tumor volume should be applied.
- In one study, treating patients with locally advanced pancreatic cancer, interim data showed that 3/5 patients had survived for > 15 months, where the median survival in the patient group are reported to be 6-11 months. The data will be updated once the study report has been finalized.
- In patients with breast cancer, imILT increased $CD8^+$ T cells within the tumor, $CD83^+$ mature dendritic cells at the tumor border and lowered the number of $CD25^+$ $Foxp3^+$ (T_{reg}) lymphocytes in regional lymph nodes. Thus, imILT induced changes in immunocompetent cells that are indicative of a favorable anti-tumor activity.

- The clinical data consist of a few patients and randomized studies with larger patient populations should be performed.

13 Discussion

As shown in the presented work, imILT is a special form of local tumor destruction, designed to induce changes in the treated tumor that favor anti-tumor activity on remaining cells and secondary tumors (abscopal effect).

imILT uses intermittent power and a master temperature probe for feedback control of laser power to ensure a stable temperature and treatment precision. Typically, the procedure aims at obtaining a temperature of 46˚C at a chosen distance from the laser tip for a duration of 30 min. As an example, when the master temperature probe is placed 3 mm outside the tumor border, a low but radical temperature zone (54-58 °C) is created at the tumor border (21). This gives a zone with temperatures that are below the coagulation threshold and this zone contains cells that maintain their cell structures while they are irreversibly damaged and eventually go into necrosis (8) (9) (10). In this zone, dying cells release uncoagulated proteins, including tumor antigens, into surrounding tissue and circulation, which is preserved.

Similar results have been published by others using local hyperthermia with laser. The common denominator in these studies has been the achievement of low temperatures around 55 °C at the tumor border (45) (46) (47) (48). This was accomplished by careful selection of laser power and time with the LITT method (45) (46). Similar temperatures (50-60 °C) at the tumor border were used by Dees et al who treated mouse melanoma and showed rejection immunity after laser destruction of the first tumor (47). Lin et al used low laser thermotherapy and found that tumor border temperatures <65 °C produced increased levels of CD3 lymphocytes in untreated distant tumors (48).

The TRANBERG® | Thermal Therapy System is based on that a target temperature of usually 46°C 3 mm outside the tumor is kept throughout a 30-minute treatment session. At this temperature level unfolding of proteins, DNA damage, and inability to DNA repair occurs and during the following cell death tumor antigens will be released in concert with DAMP molecules representing the phenomenon of immunogenic cell death. In most other hyperthermic treatments, like LITT, RFA and MWA, without the temperature feed-back regulation, temperatures above 60°C will occur also in the periphery of the tumor. These are temperatures that cause coagulative necrosis and coagulate and probably severely destroys tumor antigens. In addition, higher temperature will destroy the microcirculation in and around the tumor, thereby potentially reducing the possibilities for immunogenic material to reach the circulation. It should be emphasized though that complete destruction of the tumor by the laser light energy may not be needed to induce a productive immune response but is important for the local control. The imILT-induced immune response has the potential to affect non-treated tumors, i.e. to produce an abscopal effect. To accomplish this effect in patients with tumor induced immune suppression, imILT could potentially benefit from combination with, and act synergistically with, immune modulators and check point inhibitors like anti-CTLA-4 and anti-PD-1.

At 46°C the damage to the tumor cells cannot be immediately demonstrated (and if the treatment time is too short the damage will not be sufficient). However, as shown in several preclinical studies a treatment time of 30 minutes (but not 20 minutes or less) with a target temperature of 46°C will completely destroy the tumor, provided the feed-back temperature probe is just outside the tumor border. It should be remembered, however, that if the tumor has irregular borders the treatment may not reach the entire tumor.

There are several explanations for the improved immune-stimulating results with this kind of low temperature hyperthermia, as shown in the preclinical studies. The type of tumor cell death and the relatively low drop of tissue blood flow and perfusion should play important roles.

Treatment should aim at producing necrosis since it is more immunogenic than other forms of cell death (19) (20). Ideally, treatment should produce necrotic tumor cells without coagulating their proteins (antigens). This is difficult to achieve within the whole tumor. It can, however, be achieved at the tumor periphery, which is the important target where tumor cells are viable and growing and perfused with blood. Such an effect is produced with imILT (30) (26). Without a rapid growth and spread, the disease course could be delayed.

A requirement for both antigen presentation and the possibility for immune cells to gain access to the tumor is that the vasculature in the peripheral parts of the tumor is patent and allows exposure and trafficking.

Immune enhancing and tumor control do not need to be incompatible effects as shown by imILT that can be used for radical treatment concurrent with preserved blood flow and immunostimulating effects (21) (22) (30) (23) (25).

With respect to immunologic mechanisms, the preclinical and clinical data after the imILT treatment has identified changes in the immunogenic tumor microenvironment, which can explain the effect on spread and secondary tumors. Preclinical studies have shown an increase in HSP70, ED1 macrophages and CD8+ T cells following the ImILT protocol. In one pre-clinical study, the ImILT protocol resulted in an increased release of IFN-γ and IL-10 from tumor-draining lymph nodes and a decreased splenic release of IL-10. In a clinical study of breast cancer patients, imILT induced changes in immunocompetent cells in treated as compared to non-treated patients. Thus, imILT induced a significant increase in $CD8^+$ cytotoxic lymphocytes and mature $CD83^+$ dendritic cells at the tumor and a decrease in $CD25^+Foxp3^+$ T_{reg} lymphocytes in regional lymph nodes. These changes in the immunogenic tumor microenvironment is suggested to be related to the reduced secondary tumor burden (abscopal effect) and reduced spread.

The inflammatory milieu produced by local destruction induces DC activation, which facilitates antigen acquisition and processing as well as migration of DCs to draining lymph nodes where they interact with potential effector cells. Immunologic changes in tissue that have correlated best with clinical and preclinical success after various immunotherapies are: increased tissue levels of $CD8^+$ and mature DCs, lowered levels of $Foxp3^+$ (T_{reg}) cells and increased $CD8^+/Foxp3^+$ ratio (49) (50) (51). imILT has been shown to produce these changes in preclinical and clinical studies (25) (29) (32) (34).

imILT may increase anti-tumor immunity by creating a more inflamed tumor or convert a non-inflamed tumor into an inflamed tumors, including the creation of danger-associated molecular pattern signals (DAMPS) and proinflammatory cytokines (14) (52) (53) (54). One important DAMP is HSP70 which forms stable chaperone complexes with tumor antigens. These HSP-antigen complexes bind to danger signal receptors, Toll-like receptors 2 and 4. Our study (see chapter 9.4) showed that imILT increases HSP70 content in tumor cells and indicates that it induces uptake of HSP70 in tumor-infiltrating macrophages (24).

The fact that imILT can mediate systemic immunologic effects on its own indicates that it can induce changes in antigen presentation and the tumor microenvironment that favors anti-tumor immune activity. The strength and importance of these effects are substantiated by the abscopal effect and the reduced spread. It is also important that imILT produces systemic immunologic effects both after radical and non-radical treatments.

Thus, using the ImILT protocol has been shown to evoke a local control of the treated tumor, a powerful immunoreaction and an abscopal effect in a preclinical setting, where the ingoing parameters can be controlled. In a few cases in the clinical setting, an indication on a abscopal effect on secondary tumors has been seen. In one study, treating patients with locally advanced pancreatic cancer, interim data showed that 3/5 patients had survived for > 15 months, where the median survival in the patient group are reported to be 6-11 months. In the patients where no documented effect has been seen, it is often correlated to a suboptimal treatment of the tumor, not being able to complete the protocol due to the placement or size of the tumor. There are limited clinical data available, and additional clinical trials are planned to identify the tumor properties and patient groups that will have the optimal response of the treatment.

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