Small renal mass cryosurgery: Imaging and vascular changes
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The incidence of small renal masses has increased while the incidence of advance stage renal cancer has decreased over the recent years. Renal masses smaller than 4cm in diameter account for 48-60% of all diagnosed renal cancers. Overall, more than 50% of the patients are asymptomatic at the time of diagnosis. The increased use of body-imaging technologies, performed for a variety of reasons, results in an increased diagnosis of incidentally found renal cancer. When tumors are incidentally diagnosed they tend to be of lower stage and of smaller diameter compared to tumors found symptomatic at the time of diagnosis. Of all small renal masses (<4cm), approximately 20% is benign and 80% malignant. The majority is diagnosed in elderly patients sometimes bearing a high comorbidity rate that will influence treatment decisions. Clearly, these patients are considered candidates for active surveillance or ablative surgery. However, counseling this group of patients in the context of uncertain tumor pathology and biological potential remains a challenge. To predict whether a radiographically diagnosed small renal lesion is benign or malignant can be difficult. Despite several imaging methods used (gray-scale ultrasound, contrast enhanced ultrasound, contrast CT, and contrast MRI), some lesions have no specific classical discriminating features beside the fact they appear to be solid. Furthermore, tumor size and imaging alone are poor indicators for predicting the biological nature of the lesion. Possible other ways to avoid unnecessary, invasive treatment would be the use of diagnostic markers or to determine histology with image-guided core needle biopsies. However, the use of markers predicting the biological nature of renal masses ≤ 4 cm has not extensively been tested and validated. Principal guideline committees for the treatment of renal cancer advice that in doubt of the indication for surgical intervention one should perform image-guided core needle biopsies for histological examination prior to treatment. The rationale for this recommendation is to guide decision for surgery in indistinct small renal mass on radiological imaging and in patients with increased surgical risk. Despite there is evidence that computed-tomography-guided percutaneous biopsies can help to distinguish between benign and malignant tissue, this method is not routinely used. Hence, arguments such as sampling errors and possible track seeding are still used to favor the risk of surgery in ignorance of histological diagnosis. Although, Volpe et al reported that the complication-risk of image guided renal mass biopsies using coaxial techniques are low and rarely clinical significant. The overall estimated risk for tract seeding due to percutaneous biopsies is <0.01%. Today, laparoscopic and CT-guided renal tumor cryoablation are recognized as a viable alternative treatment to partial nephrectomy. Like all established treatment modalities also cryoablation will keep on undergoing transformations in order to simplify processes and to improve quality control. The two principal future directions of renal mass cryosurgery are focussing at enhancing its efficacy and the assessment of follow-up after treatment. This thesis discussed cryosurgical induced vascular changes in tissues and
alternative imaging techniques for the follow-up of cryoablated renal tumors. In order to understand how these findings relate to possible future directions one should critically comprehend the cryobiological processes.

Cryobiological principals; rationale for adjuvant therapy

Over the last 40 years, several studies are performed related to cryosurgery. Much of this work addressed the molecular basis of freezing effects in in-vitro experiments. Pathways of cryogenic cell death are studied and identified as direct and indirect mechanisms of tissue damage in in-vivo cryosurgery. These preliminary investigations formed the basis of today’s therapeutic cryosurgery, especially that of treating malignancies.

The biophysical cellular response to freezing and thawing is directly related to the behavior of water molecules in the intracellular and extracellular environment. Tissue response to freezing and thawing resulting in destruction is dependent on cell injury, tissue structure injury and functional impairment of the tissue compounds. The associated different cryobiological responses leading to cell death can be summarized in four pathways:

- immediate cell injury\(^{13, 14}\),
- cell injury as result of an initiated vascular response\(^{15-32}\),
- apoptosis\(^{33-35}\),
- immunological response\(^{36-43}\).

The primary injury mechanisms are immediate and vascular cell injury. The supplementary mechanisms as apoptosis and the immunological response are only cryobiological relevant in tissue at the ablation border area. There, where insufficiently low temperatures result in limited immediate tissue injury. Adjuvant therapies might be beneficial in achieving complete tissue destruction in this cryobiological sublethal area. Working mechanisms of adjuvant drug therapies will be related to one or more of the basic biological principles of cryosurgical induced cell injury.

**Immediate cell injury**

Direct cellular damage is a consequence of freezing. A low temperature leads to destruction of the structural framework of cells and thus results into immediate cell death. Direct cell death can occur in the freezing phase as well as in the thawing phase. The freezing is considered to have a stronger lethal effect on cells than thawing.

There are two different cryobiological responses, which are related to freezing rate, that lead to immediate cell death: slow freezing (~ 5 °C/min)
and fast freezing (~ 25 °C/min). A slow freezing rate results in dehydration injury of the cell and fast freezing causes intracellular ice formation. As the temperature gradually falls into the hypothermic parameters, it stresses the structure and function of cells. The solute concentration outside the cells begins to rise, causing dehydration of cells. This injures the cell by damaging the enzymatic processes that destabilizes the cell membrane. Giving sufficient time, this will eliminate most, but not all cells. Fast freezing results in the formation of ice in the intracellular environment. In this process, there is a lack of time for intracellular water to pass the membrane before freezing. The cytoplasm is strongly cooled and ice crystals are formed causing injury to organelles and membranes resulting in cell death.

During the thawing phase within the cells, large crystals form through fusing of smaller crystals that are refractory to all cell membranes. This leads to a second prolonged hypothermic situation with continued metabolic derangement also contributing to additional direct destruction of cells.

Based on these mechanisms contributing to immediate cell death, four parameters of cryotherapy related to the freezing process can be recognized: 1) cooling rate, 2) minimum temperature (low enough to ensure intracellular ice formation), 3) time held at minimum temperature and 4) thawing rate. Cooling rate is strongly dependent on thermal gradients within the tissue. Most cryosurgical research has been directed to minimum-temperature and aimed at establishing toxic temperatures ensuring killing tumor tissue. These toxic temperatures appeared to be cell-type dependant.

Vascular response

The cryo-induced vascular response played a central role in this thesis. A variety of earlier conducted studies showed that thermal gradients are strongly dependent not only of cell-type but also of vascular inhomogeneties. Beside the immediate cell injury, the amount of tissue damage is also dependant of the microenvironment in which the cryoablation takes place. This is of interest, especially at the border of the ice ball where the cooling rate is low and the temperature reached is not low enough to provoke immediate cell death. However, our research and other studies show that after a few weeks a sharp demarcation zone is noticed. This indicates that besides cryogenic induced immediate cell death the viability of cells must be dependent of adjunctive injury mechanisms such as the vascular pathway. Vascular injury is recognized as the second stage of cryogenic cell dead causing coagulative necrosis. Many investigators studied the effect of frostbite on blood vessels, showing that the primary component is circulatory stasis due to cellular anoxia. The vascular response to frostbite of human skin starts with homeostasis in the frozen tissue, surrounded by hyperemia. When the frozen tissue warms up again, a
hyperemic state sets in, followed by submission of blood flow and edema in the ablated area after which tissue necrosis and or repair through various mechanisms takes place \(^{20-22}\). These intrinsically vascular changes have been demonstrated in experiments with different animal and human soft tissues, applying varying temperatures (-20 °C to 5 °C) and thaw rates.

Cooling down of tissue results in vasoconstriction and, therefore, in a decrease of blood flow as a response. Prolonged slow freezing, more than 24 hours, cause vascular stasis starting with capillaries that become inactive. During fast freezing, the formation of ice involves the vascular anatomical structures and causes direct cellular damage to the endothelium. In addition to this cellular injury, ice propagates through the vessel, causing distension of the vessel lumen, thereby tears off the wall and endothelial clefts appear. These events lead to increased vascular permeability, edema formation, hemo-concentration, and cessation of blood flow, hypoxia and consequential tissue necrosis \(^{16}\).

Many investigators identify the period after thaw as being the most critical in determining the amount of tissue damage induced by vascular stasis. First, as the temperature climbs, circulation returns with vasodilatation. Two hours after the return of circulation defects of endothelial cell junctions give way to increased vascular permeability. It has been proposed that these events are a result of the release of vasoactive factors after thaw. Barker et al \(^{24}\) theorized that free radicals are formed as a result of the high-oxygen transport in the hyperperfused state. These free radicals cause endothelial damage by peroxidation of lipids in the endothelial membrane. Zook et al \(^{25}\) described an additional means of endothelial injury after thaw. They demonstrated that neutrophil adhere to the endothelium and release toxic enzymes needed to clean up dead cells. However, these toxic enzymes itself can damage the endothelium further \(^{23, 26-31}\). These studies suggest the involvement of endothelial-mediated injury and inflammation as underlying mechanisms in cryoinjury.

Multiple (mutual dependent) mechanisms are responsible for the vascular stasis and subsequent necrosis after freeze \(^{16}\). In previous reports damage to the endothelium, ischemia-reperfusion injury, inflammation and the resultant loss of microcirculatory support are considered essential in defining the limits of the cryolesion and trigger the induction of apoptosis and cellular necrosis \(^{16, 19-23}\).

Cessation of blood flow within the ablated area will start with the immediate destruction of microvessels \(^{31}\) and be complete by the vascular response that ultimately leads to cessation of blood flow in the lager vessels. Our study confirmed that it is expected that blood vessel transport has ceased and vessel destruction should be fully complete after 2 weeks. \(^{18}\). This stopped blood flow causes hypoxic stress of the tissue. Kimura et al \(^{32}\) showed in a cryoablated murine prostate cancer model that microvessel
density is decreased in the impact area of freezing and has a negative correlation with hypoxia. At the same site, existing hypoxia had a positive correlation with necrosis and apoptosis.

**Apoptosis**

Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms. PCD involves a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Research on apoptosis has increased significantly since the early 1990s. In addition to its importance as a biological phenomenon, one can identify defective apoptotic processes in an extensive range of diseases. Excessive apoptosis causes hypotrophy, such as in ischemic damage, whereas an insufficient number of apoptosis results in uncontrolled cell proliferation, such as cancer. Most tumor cell lines have a constitutive expression of anti-apoptotic proteins resulting in inhibition of apoptosis.

Freezing induced apoptosis is primarily present in the outer area of the cryogenic lesion. There, temperatures range between -0.5°C and about – 40°C, which is not always sufficient to kill all cells and both necrosis and apoptosis take place. An in-vitro study with human renal cancer cell line demonstrated that apoptosis peaked at six hours post-thaw. Even more, neo-adjuvant use of apoptotic initiating agents showed a significant increase in cell death at cryoablation. Therefore, promoting apoptosis with adjunctive therapy might complete destruction of cells and be therapeutically beneficial. In vivo experiments still need to determine to what extent apoptosis is a direct consequence of the freeze/thaw circle or is a secondary effect of dehydration and/or inflammation after and during cryosurgery.

**Immunological response**

Freezing induced immunological injury can be considered as the fourth mechanism causing cell death. Earlier data of clinical practice, report the regression of metastatic lesions after cryoablation of the primary tumor that suggests a potential systemic response to the local cryoinjury. However, the existence of a cryo-immunological response has been controversial since results from preclinical studies have been mixed. A review of the literature by Sabel, describes the existing evidence of both stimulatory and suppressive, immune responses after cryoablation.

Cryosurgical induced tissue damage induces a pro-inflammatory cytokine synthesis. Cytokines can be released from stromal cells and immune cells (IL-1β, IL-6, TNF-α) or tumor cells (IL-10, TGF-β) within the cryoablated
area. Antigen presenting cells, such as macrophages and dendritic cells, will start the acquired immune response by taking up antigen from apoptotic cells, necrotic cells, immune complexes, opsonized tumor cells, and heat shock proteins. These exogenous antigens are processed into peptides that bind with molecules of the major histocompatibility complex (MHC) class I and II, which then will be presented at the cell surface. Antigen-specific helper T-cells (CD4+), cytotoxic T-cells (CD8+), and regulatory T-cells (Treg cells) can be activated by recognizing and binding to the presented antigens. They stimulate the immune response by the secretion of cytokines, destroy tumor cells directly or lead to immune suppression, respectively. The MHC class I generates the cytotoxic response. However, exogenous antigens are presented on MHC class II molecules. Dendritic cells are better capable of cross-presentation (the process by which exogenous antigens enter the MHC class I pathways) than the macrophages and, thus, are more effective in promoting cytotoxic T-cell activity. Several factors can influence the modulation of the immune system either negatively or positively by cryoablation. Necrosis, the major component of cell destruction in the heart of the ablation area, leads to increased dendritic cell maturation and macrophage activation. Whereas, apoptosis can be recognized as a physiological process, which can lead to suppression of the immune response. Also, the mixture of cytokines that are released after cryoablation influences the nature of the initiated immune response being stimulatory or suppressive. Dendritic cells can provoke the cellular response and, therefore, play an eminent role in cryoablation. Since immature dendritic cells are bone marrow derived and are transported by the blood flow, the vascular response of the cryoablated area will influence the impact that these cells may have. At a quick-freeze rate (Δ 20 to Δ 50 °C/minute), intracellular ice occurs as deadly to the cell and necrosis results. In a mice model with established breast cancer, Sabel et al. have demonstrated the potential role of the immune response in relation to the freezing rate. Cryoablation using a high freeze rate resulted in a significant increase in tumor-specific T-cells, a reduction of pulmonary metastases and improved survival compared to surgical excision of the primary tumor. However, cryoablation using a low freeze rate resulted in an increase in regulatory T-cells, a significant increase in pulmonary metastases and decreased survival compared to surgical excision of the primary tumor.

Adjuvant therapy

Renal tumor size is a significant predictor for the risk of intraoperative complications including incomplete ablation performing cryosurgery. Therefore, in selected patients with larger renal tumors and with imperative reasons for choosing cryoablation as nephron-sparing treatment method, adjuvant therapy can be considered. Adjuvant therapy, can accentuate tissue destruction by modulating the response to known mechanisms of cryoinjury. Adjuvant therapy can focus at the promotion of cryogenic
injury, target volume reduction, and the combination of both. Thermophysical adjuvants can enhance the immediate cryogenic cell injury. Chemotherapeutic adjuvants act at the molecular level and induce apoptosis. Cytokines or vascular-based agents modulate the inflammatory response. Immunomodulating adjuvants stimulate immune cells to enhance tissue destruction.

Procedures that theoretically can support renal cancer cryosurgery are:

- Renal arterial embolization (RAE)
- Neo-angiogenic inhibiting drugs (NAID)
- Trans renal arterial apoptotic enhancing embolization (TRAAEE)

Common indications for RAE are symptomatic angiomyolipomas, palliation of unresectable renal cancer, hemorrhage, vascular lesions, malignant hypertension, and sequelae of end-stage renal disease. Reasons for RAE as adjuvant therapy to cryoablation can be reducing the rate of post-cryosurgical bleeding and increase the treatment efficacy by reducing tumor volume. Furthermore, lowering the percentage of Treg cells or reducing their activity can improve the anti-tumor immune situation and help reduce the chance for tumor recurrence and metastasis. In patients with intermediate and advanced renal cancer the effect of RAE versus RAE + cryoablation on the differentiation of Treg cells was studied by Li et al. Compared to RAE, RAE + cryoablation showed a wider range of tumor necrosis and a more significant drop in the percentage of Treg cells. So far, there are no studies conducted using RAE + cryoablation as a curative option for renal cancer.

When NAID-therapy is considered as neo-adjuvant therapy to cryoablation for renal cancer, a histological diagnosis is obligatory. The revised Response Evaluation Criteria in Solid Tumors can be used to assess the response to the adjuvant treatment of renal cancer. Kroon et al reported that, compared to tumors larger than 7 cm, smaller renal tumor size was related to more effective downsizing. They recognized that the potential benefit of neo-adjuvant treatment to downsize the primary tumor for nephron-sparing surgery including cryoablation may exist, particularly in tumors sized 5 to 7 cm.

Several in-vitro and in-vivo studies have shown the ability of chemotherapeutics to augment cell death at cryoablation. No study using cryo-chemo combination therapy has shown an augmentation up to the ice ball border. Limitations are the limited dose of the agent at the target tissue level and cancer specific sensitivity to the type of drug used. So far, systemic chemotherapy is not commonly used in the treatment of renal cancer. However, targeted adjuvant delivery to enhance the apoptotic sensitivity of only the target tissue can be performed by direct injection of the tissue, systemically using nano-sized carriers, and combined with trans-
arterial embolization. Trans-arterial chemoembolization (TACE) is frequently used as a therapy for liver tumours and involves the combination of a chemotherapeutic agent with a drug carrier that is delivered intra-arterially\textsuperscript{53}. The purpose of embolization is to reduce arterial inflow, diminish washout of the chemotherapeutic agent, and prolong contact time between cancer cells and the chemotherapeutic agents. So far, no studies have been published addressing trans-arterial embolization in combination with apoptotic agents as therapeutic method for the treatment of renal cancer. TNF-\(\alpha\) can act as an additional stressor to the microvasculature of the tissue and aid in the maturation of immune cells at the time of local tumor destruction during cryoablation\textsuperscript{50, 54}. Jiang et al showed that targeted delivery of TNF-\(\alpha\) using gold nanoparticles coated with the drug achieved a significant augmentation in tissue injury and greatly reduced systemic side effects\textsuperscript{50}.

Follow-up imaging of renal tumor cryosurgery

Within 5 years follow-up of all renal cancer patients treated with curative intent, 25% of the patients presents with metachronous metastases and an additional 20% with synchronous metastases\textsuperscript{1, 2}. However, the risk for metastatic development in patients with small renal cancers is small. Within 10 years follow-up 1-8.4\% will be diagnosed with metastases\textsuperscript{55, 56}. The biological potential of small renal cell carcinomas (RCC) seems to be mild. This reflects in the relative merit cancer specific survival of stage 1 disease\textsuperscript{57}. However, it is reported that lesions of 3-4 cm diameter have a significant higher risk for high grade and advanced-stage disease compared to smaller lesions\textsuperscript{58}. Klatte et al. reported that, following cryosurgery, the chance for local progression or recurrence is 8.5\%\textsuperscript{59}. They also found that the risk for local progression increased with 85\% per 1 cm increase of tumor size. The mean follow-up for cryosurgery in this study was 29 months. Although this follow-up time is short, local recurrence is rarely reported after 2 years\textsuperscript{60, 61}. The rate of reported distant metastases following renal cancer cryoablation is 1\%\textsuperscript{62}. In general, risk factors for renal cancer metastatic development are high nuclear grade and vascular invasion. However, determining vascular invasion and the accuracy of grading based on biopsy specimen is under discussion.

So far, principal guidelines have failed to provide evidence based follow-up schedule for T1a and T1b renal cancers\textsuperscript{7, 8}. The proposed algorithm for the follow-up of cryoablated RCC from the EAU guidelines stratifies for risk factors but fails to clarify what these risk factors are and how they are assessed. For example, they do not distinguish between histological subtypes. However, Leibovich et al. reported that in patients diagnosed with renal cancer, the histological subtype of clear cell carcinoma is an
independent predictor of progression of distant metastasis and cancer-specific survival.\(^6^3\)

The risk profile for the occurrence of cancer related incidents of cryoablaited renal tumors needs to be assessed. In the first instance, the risk for distant or local events needs to be assessed. Thereafter, the most cost-effective imaging modality with a significant sensitivity for detecting local and or distant disease should be selected. This can be a combination of different imaging modalities and may include diagnostic markers and histology obtained by image-guided core needle biopsies.

Pulmonary metastases are the most common metastasis of renal cancer. However, the risk for the development of distant metastases is approximately 1% and, therefore, a regular check-up including chest X-ray or CT-scan can be questioned. Furthermore, if pulmonary metastases occur, it can be discussed whether it is clinically relevant to detect these lesions before they become symptomatic. A regular CT-scan of the chest is probably clinically irrelevant, not cost-effective and exposes the patient to unnecessary radiation doses. Other common sites of metastatic disease from RCC include lymph nodes, liver and the skeletal system, which all are best evaluated with CT or MR imaging.

The risk for multifocal RCC is 5-8%.\(^6^4, 6^5\) Tsivian et al. reported that lesion size (2-4 cm), male gender, family history of renal cancer, histologic subtype, and grade were independently associated with occult multifocality.\(^6^4\) Histologic subtypes other than clear cell had an increased risk for occult multifocality of which the papillary subtype had the strongest association which conferred a seven-fold odds increase. Rare subtypes such as medullary and collecting duct RCC increased the odds by >10-fold.

The risk for local progression or recurrence following renal mass cryoablation is 8.5%.\(^5^9\) Our and other studies clarified that tumor diameter is related to the occurrence of incomplete ablation and local recurrence of cryoablation. Lehman et al reported that tumor size appeared to be a key metric for incomplete ablations following LCA.\(^6^6\) The majority of local recurrences will be detected in the first two years following cryoablation.\(^5^9\)

Multi-institutional long-term follow-up data can be used for the development of a nomogram that will aid to stratify the risk for treatment failure, new renal sites of RCC, and metastases. Based on the risk grade the follow-up can be categorized concerning how often checks should be carried out and what investigational tests are needed. However, under the influence of all kind of circumstances there always will be exceptions to the rule demanding a tailored approach.

In summary, the most clinical important and, thus, relevant events following renal mass cryosurgery are the development of a local recurrence or new
renal masses (multifocality). Therefore, it seems most relevant to focus at the detection of these events in the follow-up.

Incomplete ablation should be detected as soon as reasonable and first time imaging can be performed with a minimum of 2 weeks following ablation. So far, the best method to assess treatment failure is cross sectional imaging of the upper abdomen using contrast CT or MRI. MRI is equivalent to CT for evaluation of renal mass cryosurgery. However, CT has the advantage of widespread availability, more rapid examination time in comparison with MRI, and lower cost than MRI. In case at first time imaging the ablation is considered successful, than consecutive imaging is used to detect local recurrences and new renal masses. Local recurrences tend to occur in the first years following cryoablation and patients with an increased risk for local recurrence should be examined more frequently than patients with low risk. A local recurrence will meet two imaging characteristics, mass growth and vitality of tissue. Both can be used for the detection of a local recurrence.

The detection rate of new renal masses is related to the volume of the suspected mass. There is a temptation to lower the radiation exposure by using ultrasonography (US). However, US is less accurate than CT for revealing small renal masses and, thus, currently contrast CT is the first choice. Still, small solid lesions (<1.5 cm diameter) can be missed at diagnostic imaging. In the knowledge that the majority of small RCC are expected to grow with a mean of 2.8 mm diameter a year, one can possibly sustain with a yearly follow-up. Furthermore, one can discuss whether it is clinically relevant to diagnose new tumors < 2 cm diameter at follow-up of small renal masses treated with cryoablation.

The highest risk for a local recurrence is in the first years following ablation and the preferred way to detect a local recurrence is contrast CT or MR imaging. After three years follow-up the risk for local recurrence is low but new tumors can occur. After the time frame of three years following surgery, it can be suggested that imaging other than contrast CT would sustain in a reasonable detection rate of clinically relevant new tumors or local recurrence. Randomized control studies are needed to answer this question.

Conclusion to future prospects

Four cryobiological tissue response mechanisms to cryogenic ablation of tissues are mainly significant at the border of the ice-ball where they most likely result in only limited damage because of the insufficiently low temperatures. Adjuvant therapies might be beneficial in achieving complete tissue destruction in this zone. Working mechanisms of adjuvant drug
therapies will be related to the basic biological principles of cryosurgical induced cell injury. Currently, these adjuvant strategies remain experimental and need further study focusing at the choice of the adjuvant, the time interval between the adjuvant addition and cryotherapy, the delivery of the adjuvant to the tumor, and the dose of the adjuvant.

Risk factors for recurrence and metastases from small renal masses that underwent cryoablation still need further investigation. Long term follow-up and histological diagnosis are necessary in order to identify the risk factors and can help design the best method for the follow-up of a renal mass after cryosurgery.
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