Cryotherapy and immune checkpoint inhibitor therapy for the treatment of metastatic non-small cell lung cancer

KRIOTERAPIJA IR IMUNINĖS PATIKROS SLOPIKLIŲ TERAPIJA METASTAZAVUSIO NESMULKIŲJŲ LĄSTELIŲ PLAUČIŲ VĖŽIO GYDYMUI

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Summary. Lung cancer remains among the most frequently diagnosed and deadliest diseases in the world. While immune checkpoint inhibitors revolutionized the cancer treatment landscape, only a relatively small proportion of patients benefit from this treatment, likely due to the ability of cancer cells to evade or even exclude the immune system from tumors. Cryotherapy offers a possible way to counteract this process by inducing an inflammatory response and helping in cancer neoantigen presentation, increasing the effectiveness of immunotherapy. While preclinical studies seem to support this claim, further investigation is needed to conclude the benefits and risks of cryotherapy in combination with immune checkpoint inhibitors.
Keywords: lung cancer, cryotherapy, immunotherapy
Santrauka. Plaučių vėžys išlieka viena dažniausiai nustatomų ir mirtį lemiančių ligų pasaulyje. Nors imuninės patikros slopikliai sukėlė revoliuciją vėžio gydymo srityje, šis gydymas naudingas tik santykinai nedidelei pacientų daliai, tikėtina dėl vėžio ląstelių gebėjimo išvengti ar net išstumti imuninės sistemos ląsteles iš navikinio audinio. Krioterapija yra potencialus būdas neutralizuoti šį procesą dėl skatinamo uždegimo ir efektyvaus vėžio neoantigenų pateikimo imuninei sitemai, taip padidinant imunoterapijos veiksmingumą. Nors ikiklinikiniai duomenys rodo krioterapijos ir imuninės patikros slopiklių derinio efektyvumą, reikalingi detalesni tyrimai nustatyti galimas naudas ir rizikas pacientams.
Reikšminiai žodžiai: plaučių vėžys, krioterapija, imunoterapija.

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INTRODUCTION

Lung cancer is one of the most common oncological diseases in the world. According to the International Agency for Research on Cancer (IARC), more than 2.2 million cases were diagnosed in 2020 alone (11.4% of all new cancer cases), as well as almost 1.8 million deaths (18.0% of all cancer deaths) [1]. The close incidence and mortality rates of lung cancer are caused by patients often presenting with advanced disease, while according to a 2016 International Association for the Study of Cancer (IASLC) study the five-year survival rates for stages IIIA-IIIC non-small cell lung cancer (NSCLC) is 13–36%, and for stages IVA-IVB it is less than 10% [2, 3].

The most prevalent form of lung cancer is NSCLC, accounting for approximately 85% of all cases [3]. For years, the standard treatment for metastatic lung cancer was chemotherapy. Developing understanding of lung cancer etiopathogenesis led to new treatment options, such as tyrosine kinase inhibitors (TKIs) and immune checkpoint inhibitors (ICIs) [3, 4]. Unfortunately, these new treatments are still not reliable treatment options in all cases.

Most NSCLC tumors, harboring activating driver mutations and translocations, treated with TKIs or monoclonal antibodies are identified in only a small proportion of lung cancer patients – typically non-smokers, female patients and adenocarcinoma histological type [4]. Another disadvantage of these methods of treatment is that cancer cells inevitably acquire resistance. NSCLC patients without specific targetable genetic alterations are usually treated with immunotherapy as monotherapy or in combination with chemotherapy. The choice of ICI monotherapy or combination with chemotherapy as a first-line treatment currently depends on the expression of programmed cell death-ligand 1 (PD-L1) on lung cancer cells [3]. Unfortunately, phase 3 clinical trials show that patients, treated with ICI pembrolizumab monotherapy, achieve overall response in only 44.8% of cases and 47.6% with a pembrolizumab-chemotherapy combination [5, 6].

Immunotherapy has proven itself as a valuable tool in select cases of lung cancer, but for most patients this treatment is insufficient or only has a transient effect. Currently, identifying and eliminating the mechanisms of tumor resistance to immunotherapy is an area of active research. One promising direction in this field is cancer cell destruction using local ablative techniques, such as thermal ablation or cryotherapy, to release cancer cell contents as neoantigens for improved priming of the immune system and boosting the effectiveness of immunotherapy [7]. Bronchoscopic cryotherapy is already in use as a safe and effective option for the palliative treatment of airway stenosis, cough or hemoptysis in lung cancer patients [8]. The aim of this review is to provide evidence of possible systemic therapeutic effects this treatment method might have for NSCLC patients receiving immunotherapy.

Cancer immune landscape

Depending on the path of individual tumor pathogenesis the immune landscape of cancer may present in different ways. Each of them would present distinct characteristics, affecting prognosis and possible treatment modalities. In 2015 Teng et al presented a pragmatic classification of tumors, depending on tumor infiltrating lymphocyte counts and PD-L1 expression, to identify types for which ICI treatment would be most effective [9]. This classification was later refined to include tumor mutation burden (TMB), neoantigen burden and inflammation gene signature to separate the following subtypes [10]:

- Type 1 tumors exhibit high TMB and inflammation gene signature, indicating the existence of a suppressed, but still ongoing immune response. This microenvironment, commonly found in lung carcinomas, has the best probability of responding to ICIs.
- Type 2 tumors contain a low TMB, lack expression of an inflammatory gene signature and were earlier described as immune-desert or an immune cell-excluded phenotypes. The absence of an immune response represents impaired antigen presentation and results in poor outcomes when treated with ICIs. Therapies that could increase the amount of neoantigens in the microenvironment, like local ablative procedures, would potentially be of great benefit in this tumor type.
- Type 3 tumors have higher TMB than type 2 tumors, while still exhibiting an absence of inflammatory genes. This points to an exclusion of T-cells and Natural killer cells from the microenvironment by cancer cells, as well as other cells, such as cancer-associated fibroblasts. Local destruction of the tumor tissue may be able to ignite an acute inflammation, providing sufficient chemoattractants to counter this effect.
- Type 4 tumors contain a low TMB while maintaining high levels of inflammatory gene expression. The presence of these genes suggests some immune activity, although it most likely consists of immunosuppressive cells and favors tumor growth and metastasizing. The most likely ways to counteract these tumor types would be to either deplete these immunosuppressive cells or repolarize their phenotype to a proinflammatory state.

CRYOIMMUNOTHERAPY

Physical effects of cryotherapy

Cryotherapy, also known as cryosurgery or cryoablation, is a medical procedure that uses extreme cold for local destruction of abnormal tissue. It was first used as a cancer treatment in the 19th century, for palliation of breast, uterine and skin cancers, but the range of applications has been steadily expanding ever since [11]. After first being used for treating endobronchial tumors in 1968, bronchoscopic cryotherapy is now recommended as a safe and effective option for a variety of reasons, such as cryorecanalization, cryoextraction of foreign bodies or treatment of low-grade airway malignancies [12, 13]. Furthermore, transbronchial lung cryobiopsy is used for the diagnosis of interstitial lung diseases and pulmonary malignancies [14, 15].

The physical effects of cryotherapy on cancer cells have been studied extensively. During the freezing phase, extracellular water forms ice crystals, which in turn shear and damage cell membranes (Figure 1a). At lower rates of freezing, this causes a shift in osmotic pressure and intracellular water flow out of cells, causing their dehydration. Meanwhile at higher freezing rates intracellular water also forms ice crystals, resulting in lethal damage to organelles. Afterwards, a thawing phase occurs, during which smaller ice crystal fuse together, increasing mechanical damage. As these crystals thaw, water rapidly flows to now hypertonic intracellular space, causing cells to burst [16–18]. Furthermore cryotherapy causes local vasoconstriction and thrombosis via damage to endothelial cells, leading to edema, ischaemia and a secondary spike of necrosis days later [19, 20]. Further from the cryoprobe, disturbance of mitochondrial functions triggers apoptosis [21]. As connective tissue is particularly resistant to freezing injury, the architecture of the tissue remains for subsequent repair [22].

The effects of cryotherapy are most pronounced close to the cryoprobe and diminish going outward, although repeated freezing cycles and slow thawing phases increase the amount of extracellular fluids, expanding the cryoablation zone [18]. Depending on

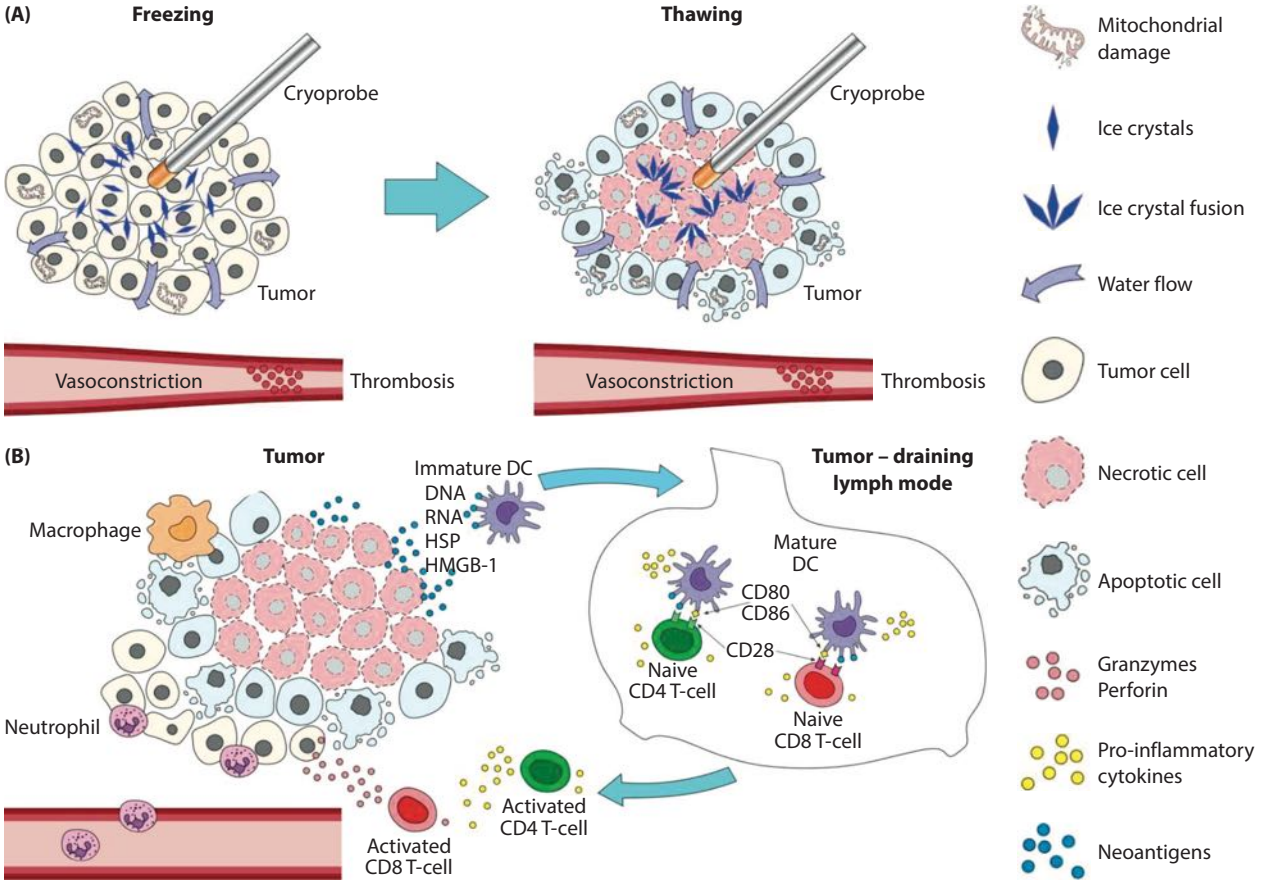

Figure 1. Effects of cryotherapy: a) Physical effects of cryotherapy; b) Immunologic effects of cryotherapy.

DNA – Deoxyribonucleic acid; RNA – Ribonucleic acid; HSP – Heat shock proteins; HMGB-1 – High mobility group box chromosomal protein 1; DC – Dendritic cell.

the distance from the cryoprobe and, subsequently, the temperature cancer cells are subjected to, their death occurs either by necrosis or apoptosis [18, 19]. As necrosis results in release of pro-inflammatory cytokines, resulting in immune activation, and apoptosis is immunosuppressive, the immunologic effects of cryotherapy stem from their balance [20, 23].

Local immunologic effects of cryotherapy

A growing amount of evidence suggests that cancer cell death after cryotherapy is also caused by an increase in proinflammatory cytokine levels and tumor specific T-cell activation [24–26]. Proteins, DNA and RNA released from cancer cells dying during cryotherapy remain in the patient's body as neoantigens and a sort of vaccination. Furthermore, "danger signals" such as uric acid or the chromosomal high mobility group box chromosomal protein 1 (HMGB-1) are released [20]. These molecules act as chemokines and cytokines for immune cells with tumor microenvironment (Figure 1b) [20, 26].

In murine cancer models, within hours after cryoablation surrounding blood vessels become congested, exhibiting endothelial cell activation and intravascular polymorphonuclear cell recruitment. Within days, polymorphonuclear leukocytes infiltrate the peritumoral area and reach their highest concentration [24]. Polymorphonuclear cells promote CD8 lymphocyte recruitment and activation by producing T-cell attracting chemokines (such as CCL3, CXCL9, and CXCL10), pro-inflammatory cytokines (IL-12, TNF-α, GM-CSF, and VEGF) and may also activate dendritic cells via cell to cell contact and through secretion of TNF-α [27]. Therefore, they are followed by infiltration and maturation of antigen-presenting cells – macrophages and dendritic cells into the cryoablated tissue [20, 24].

Mature dendritic cells transport neoantigens to the regional lymph nodes and present them to CD4 and CD8 T lymphocytes via Major histocompatibility complex (MHC) class II and I molecules respectively, activating lymphocytes specific for the presented neoantigens [28, 29]. In contrast to tumor cells, which may also present antigens through the MHC class I molecule, but often co-express immunosuppressive molecules, such as PD-L1, dendritic cells co-present stimulatory CD80 and CD86 proteins and secrete interleukins such as IL-12, important for Th1 immune responses [24, 28]. This way, naive CD8 lymphocytes receive proper signals of the antigen, co-stimulation, and inflammation during priming and initial activa-

tion [30]. Since peptide bonds are preserved during cryoablation, protein denaturation is reversible and cryoablation releases the most non-denatured proteins, compared to other local destructive therapies [31]. Subsequently, dendritic cells present neoantigens more efficiently in tumors treated with cryotherapy, compared to surgery, which is immunosuppressive due to subsequent tissue repair and suppressed inflammatory response, or radiofrequency ablation, which causes irreversible protein denaturation due to heat [28, 31, 32].

Neoantigens, released after cryotherapy prime the immune system solely against similar cancer cells to those destroyed. Kim et al performed cryoablation or radical excision on RENCA tumors generated in mice [33]. Once rechallenged with RENCA cell line, only 11.1 % of cryoablated mice developed tumors. In contrast, inoculation with a completely different CT26 colon carcinoma cell line led to 94.1% of these mice developing tumors, showing signs of antigen-specific immune response.

Systemic immune response following cryotherapy and the abscopal effect

Activated tumor-specific T-cells infiltrate the cryoablated tumor tissue to destroy cancer cells locally, while some may enter the systemic circulation and reach sites of distant metastases [34]. The subsequent reduction in the size of distant untreated metastases after the destruction of the primary tumor is called the abscopal effect (from latin 'ab' – away from, 'scopus' – target). Cases of cryotherapy inducing the abscopal effect in cancer patients have been reported since at least the 1970 [35, 36]. Conversely, later research showed that cryotherapy alone may not be counted upon to create a reliable and sustained effect [37].

Preclinical studies of solid murine tumors have shown the potential for the systemic effect of local cryotherapy. Sabel et al performed surgery or cryotherapy on 4T1 mammary carcinoma cells, injected and grown in mice [38]. Mice with primary tumors treated with cryoablation at a high freeze rate exhibited significantly fewer pulmonary metastases (4.89 nodules/mouse) when compared to controls (47.2 nodules/mouse) or surgical excision (9 nodules/mouse). Furthermore, Yakkala and colleagues performed cryoablation on in vivo murine B16F10 melanoma model, where cryoablation was shown to initiate local immune responses [39]. In addition to increased frequencies of CD8+ T-cells in the local tumor draining lymph node, an increase of CD4+ conventional T-cells in non-draining lymph nodes were observed as a sign of systemic immunity. After re-challenge with the same melanoma cells, signs of immunological memory were observed with restricted tumor growth and extended survival. Conversely, Waitz et al observed no significant changes in abscopal tumors in TRAMP-C2 murine model of prostate cancer treated with cryotherapy alone, suggesting that it may not always create a robust antitumor immune response, which would inhibit the growth of metastases and prevent disease recurrence [40].

It has also been reported that a certain extent of cryodestruction on tumor tissue may be best for inducing systemic antitumor response. Takhashi et al performed cryotherapy on mice with Lewis lung carcinoma cells inoculated subcutaneously into bilateral flanks [26]. Cryosurgery was administered in one, two or three freezing cycles on a tumor in one flank with contralateral tumor left untreated, followed by lipopolysaccharide injection 2 hours and 2 days later for immune stimulation. Immunohistochemistry showed that 20.74% of the tumor was destroyed with one cycle, 73.83% with two and 89.22% with three cycles. All three groups showed significantly slower growth of the contralateral tumor when compared to controls that only received lipopolysaccharide injection, although the effect was most pronounced after two cycles of cryotherapy and approximately three-fourths of the cryoablated tumor was destroyed. Whether the remaining cancer cells provide further stimulation for the immune system, or some other mechanisms are at play, remains unclear.

As mentioned before, both immunotherapy and cryotherapy can individually enhance the antitumor immunity, although this effect may not be universal. Further preclinical research, combining cryotherapy with immune checkpoint inhibitors, provided evidence of a possible synergy between these treatments. Combined cryotherapy and anti-CTLA-4 immunotherapy treatments have shown significant benefits in murine melanoma and prostate cancer models, with mice exhibiting increased counts of tumor-specific T-cells [29, 40]. These changes further translate to improved resistance against rechallenge with the same cancer cells. Similar results have been observed when combining cryotherapy with PD-L1/PD-1 axis inhibition. Mice, implanted with murine breast or renal carcinoma cells on bilateral flanks, showed signs of considerable abscopal effect once tumors on one side were treated with cryoablation and anti-PD-1 antibodies were administered [41, 42]. One study in particular showed that after cryoablation the proportion of PD-1 positive CD8 cells increases significantly, with malignant cells counteracting the antitumor response via IFN-γ induced upregulation of PD-L1 expression, tempering the systemic effect of cryotherapy [42]. The addition of PD-1 inhibitors neutralized these changes and provided a similar abscopal effect to that observed after SABR radiotherapy in PD-1 knockout mice [43]. It is reasonable to suspect that anti-CTLA-4 therapy would work similarly, maintaining

the cryotherapy induced anti-tumor immune response by preventing T-cell inhibition, although it remains to be seen which immunotherapy is more beneficial in combination with cryotherapy.

Clinical trials of cryotherapy and immune checkpoint inhibitors

Currently, no trials investigating the immune response and clinical outcomes in advanced NSCLC patients receiving cryotherapy and ICI therapy have been completed. In other cancer types, several studies have been conducted, showing the safety and efficacy of this combination.

In a pilot study of 19 women with stage I-II breast cancer were treated with preoperative tumor cryoablation, single-dose ipilimumab or both [44]. These treatments were safe and tolerable without delaying the planned surgery. Furthermore, combination therapy was associated with elevated Th1-type cytokines, as well as activation and proliferation of CD4 and CD8 T-cells. Another study of patients with metastatic renal cell carcinoma, randomized to receive tremelimumab or cryoablation plus tremelimumab, showed a more significant increase in T-cells and CD8 T-cells in the total tumor microenvironment upon treatment with cryo-tremelimumab combination therapy compared with tremelimumab monotherapy [45]. However, these changes did not translate to better patient outcomes, possibly due to a small sample size and mixed (clear and non-clear) tumor histologies, that are have different drivers of tumor development and responses to therapy.

Clinical trials, examining the efficacy of cryotherapy and ICIs in NSCLC settings have not presented their results yet and are detailed in Table 1.

An ongoing trial in Massachusetts General Hospital, United States [NCT03290677], aims to demonstrate the effectiveness and safety of cryoablation in reigniting a response to immune checkpoint inhibitors in stage IV lung cancer patients with disease progression. First, a biopsy sample is taken from an enlarging tumor to confirm a malignant growth and disease progression, after which percutaneous cryoablation on the same tumor is performed. The safety and feasibility of the procedure is determined based on observed adverse events. As a secondary outcome measure, the radiologic response rate is measured. Another trial at New York University, United States [NCT04049474], aims to evaluate the safety and feasibility of bronchoscopic cryoimmunotherapy on peripheral lung tumors in advanced NSCLC. Patients are allowed to receive concomitant chemotherapy, immunotherapy and/or radiation therapy, although no stratification by these treatment modalities is mentioned. The researchers are collecting peripheral blood samples from patients before the procedure, as well as 1 and 2 weeks after it, to assess for antitumor immune responses by multiplex flow cytometry and gene expression. These findings are planned to be correlated with pretreatment bronchoalveolar lavage specimens from the tumor environment. The patients are also set to be followed longitudinally for changes in tumor size and evaluated for progression-free and overall survival. Additional outcomes to be measured include the length of fluoroscopy exposure and the procedure itself and the ability of radial endobronchial ultrasound to identify peripheral lung tumors.

A multicenter CRYOMUNE trial in France [NCT04339218] aims to compare the one-year survival benefit of percutaneous cryoablation with ICI pembrolizumab and platinum-based chemotherapy over

Table 1. Recently ended and ongoing clinical studies, examining cryoimmunotherapy

NCT number	Condition	Cryotherapy procedure	Systemic treatment	Location	Current status	Completion date
NCT02469701	Stage IIIB or IV NSCLC	Cryoablation or thermal ablation	Nivolumab	Brown University USA	Terminated (Lack of accrual)	February 17, 2020 (Actual)
NCT03290677	Progressive metastatic lung cancer or melanoma	Percutaneous cryoablation	Immune checkpoint inhibitor therapy	Massachusetts General Hospital, USA	Recruiting	March 2025 (Estimated)
NCT04049474	Advanced inoperable NSCLC	Bronchoscopic cryotherapy	N/A	NYU Langone Health, USA	Recruiting	December 2024 (Estimated)
NCT04339218	Metastatic lung adenocarcinoma	Percutaneous cryoablation	Pembrolizumab + Pemetrexed + Carboplatin	Institut Bergonié, France	Recruiting	August 2023 (Estimated)
NCT04793815	Metastatic NSCLC	Bronchoscopic cryotherapy	Pembrolizumab monotherapy	Centre hospitalier de l'Université de Montréal, Canada	Completed	June 27, 2023 (Actual)
NCT06000358	Metastatic NSCLC	Bronchoscopic cryotherapy	Pembrolizumab monotherapy or combination with platinum based chemotherapy	Lithuanian University of Health Sciences, Lithuania	Recruiting	March 2026 (Estimated)

pembrolizumab and chemotherapy alone in metastatic lung adenocarcinoma patients. In addition, overall response rate, progression-free survival and adverse events are measured. Furthermore, peripheral blood samples are collected at baseline, day 1 of the 2nd cycle, and day 1 of the 3rd cycle of systemic treatment and progression, as well as stool samples (optional), likely for evaluation of immune responses and gut microbiome respectively. An interesting point is that patients are asked to provide samples of biopsy tissue at screening, at the time of the 3rd treatment cycle and disease progression. Pathological examination of biopsy samples at different time points before and during treatment could evaluate changes in tumor microenvironment, possibly tying them to the changes in peripheral blood samples and providing invaluable information for future studies, such as the search for biomarkers of disease response.

A recently initiated study at the Lithuanian University of Health Sciences, Lithuania [NCT06000358], aims to provide an analysis of synergy between bronchoscopic cryotherapy and immunotherapy in prospectively enrolled metastatic NSCLC patients. The study population encompases two main groups, frequently encountered in clinical practice – patients treated with pembrolizumab monotherapy or pembrolizumab and platinum based chemotherapy. The main goal of the study is to evaluate changes in T lymphocyte counts and function in peripheral venous blood, assessed via flow cytometry, RNA expression and cytokine panels before and during treatment. These results are planned to be correlated with clinical outcomes, including objective response rate, progression-free survival and overall survival. To accomplish study aims, funding has been generously provided by the Research Council of Lithuania and Future Biomedicine Fund.

Finally, the CRYOVATE trial has been recently completed in Montreal, Canada [NCT04793815], with results yet to be released. The aim of this study was to evaluate the safety and efficacy of bronchoscopic cryotherapy in patients with previously untreated advanced NSCLC, set to receive pembrolizumab monotherapy. The primary endpoint of the study was the overall response rate, with secondary endpoints of treatment-related adverse events, progression-free survival and overall survival. The project authors also aimed to collect biopsy samples before treatment, 4 weeks and 3 months during it and upon disease progression, to describe the pro-inflammatory changes associated with cryoactivation using immunohistochemistry, flow cytometry, RNA sequencing and cytokine panels.

CONCLUSION

The observed ability of cryotherapy to release neoantigens for the immune system provides an enticing new prospect in the fight against cancer. Currently the preferred method of performing cryotherapy for pulmonary tumors is percutaneous cryoablation, aiming for complete tumor destruction, which is related to a high risk of complications, such as pneumothorax or hemothorax. While bronchoscopic cryotherapy allows for peripheral tumors to be reached directly through the airway, minimizing this risk, only partial tumor destruction is usually achieved, mostly relegating it to a palliative role, preclinical research shows, that partial ablation may be sufficient for systemic antitumor effects. The upcoming studies will likely bring valuable information on various immunological effects of cryotherapy, either alone or supplementing ICIs in the setting of advanced lung cancer.

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