Arginase-1 peptide vaccine in patients with metastatic solid tumors

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Arginase-1 Peptide Vaccine in Patients with Metastatic Solid Tumors

A phase I study

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The study will be performed as described in this protocol and in accordance with regulatory requirements and Good Clinical Practice. It will be monitored by the GCP unit of the Copenhagen University Hospital and can be subject for external audition as quality assessment of the GCP unit. The investigator allows direct access to source data and documents including medical records of participants, for inspection by the relevant authorities, being the Danish Medicines Agency, GCP units and/or other health authorities.

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Abbreviations

AE	Adverse event
ALAT	P-alanine-aminotransferase
AML	Acute myeloid leukemia
ANC	Absolute Neutrophilic Count
APC	Antigen presenting cell
AR	Adverse reaction
ARG	Arginase
ARG1	Arginase-1
ASAT	P-aspartattransaminase
CCIT	Center for Cancer Immune Therapy
CNS	Central Nerve System
CR	Complete Response
CRF	Case Report Form
CRR	Complete Response Rate
CRP	C - reactive protein
CT	Computer tomography
CTCAE	Common Terminology Criteria for Adverse Events
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic cell
DTH	Delayed type hypersensitivity
ECOG	Eastern Cooperative Oncology Group
EFEK	Enhed for Eksperimentel Kræftbehandling
HLA	Human Leukocyte Antigen
HNSCC	Squamous cell carcinoma of the head and neck
ICS	Intracellular staining
IDO	Indolemamine-pyrrole 2, 3-dioxygenase
IFA	Incomplete Freund's adjuvant
IgG	Immunoglobulin G
IL-2	Interleukin 2
INR	International Normalized Ratio
KFE	Clinical experimental unit
LDH	Lactate-dehydrogenase
MDSC	Myeloid-derived Suppressor Cell
MHC	Major Histocompatibility complex
MR	Magnetic resonance
MSV	MilliSievert
MTD	Maximum tolerated dose

NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
NSCLC	Non-Small Cell lung cancer
OR	Objective response
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PERCIST	PET Response Criteria in Solid Tumors
PET	Positron Emissions Tomography
PFS	Progression free survival
RECIST	Response Evaluation Criteria in Solid Tumors
PR	Partial Response
SAE	Serious adverse event
SAR	serious adverse reaction
SD	Stabil Disease
S-HCG	serum-Choriogonadotropin
SKILs	Skin Infiltrating Lymphocytes
SOP	Standard operating procedure
SUSAR	Suspected unexpected serious adverse reaction
TAMC	Tumor Associated Myeloid Cell
TCR	T cell receptor
Treg	Regulatory T cells

The peptides mentioned in the protocol:

IO112	→ ARG 171-190;	AKDIVYIGLRDVDPGEHYIL	→	ARG1-18
	ARG 181-200;	DVDPGEHYILKTLGIKYFSM	→	ARG1-19
	ARG 191-210;	KTLGIKYFSMTEVDRLGIGK	→	ARG1-20

Objectives and endpoints

The primary objective is to assess the safety and tolerability of a peptide vaccine containing the enzyme arginase-1 (ARG1) with Montanide ISA 51 as adjuvant in patients with metastatic and treatment-refractory solid tumors. The primary endpoint is adverse events (AE) assessed by Common Terminology Criteria for Adverse Events (CTCAE) 4.0 and laboratory monitoring.

The secondary objective is to evaluate the immune response before, during and after treatment. The endpoint is responses of ARG1 specific T cells measured by ELISPOT and cytokine release assays.

The exploratory objective is to evaluate the clinical efficacy of the treatment. The endpoints are objective response (OR), progression free survival (PFS) and overall survival (OS).

Protocol synopsis

Background

Metastatic solid tumors are regarded incurable and fatal despite great progresses in medical oncology over the past decade. Treatment options are in end-effect palliative and given with the purpose of prolonging life. Standard therapies for metastatic solid tumors range from hormone suppression to aggressive chemotherapy regimens, but in the last couple of years immunotherapy has revolutionized several cancer treatments. This protocol describes a new approach in the treatment of patients with metastatic solid cancer by modulating the tumor microenvironment using a peptide vaccine.

The unregulated growth of cancer cells is only possible when the immune system does not launch an effective antineoplastic response. Cancer cells highjack the system of checks and balances in the immune system by exploiting the system of local adaptive immune resistance. One mechanism utilized by malignant cells is the overexpression of the immunosuppressive enzyme arginase-1 (ARG1).

ARG1 is an enzyme expressed by some cancer cell lines and different myeloid cells in the immunosuppressive tumor microenvironment. ARG1 catalyzes the conversion of the amino acid arginine to ornithine and urea. A depletion of arginine suppresses T cell function through the impairment of the T cell receptor (TCR)-complex resulting in immunosuppression in a cancer setting.

The theoretic background for a peptide vaccine against ARG1 is to boost the activity of ARG1-specific T cells to eliminate immune cells that express ARG1 as part of the local immunosuppressive microenvironment in cancerous tissues.

Center for Cancer Immune Therapy (CCIT) has identified spontaneous T cell reactivity against ARG1 peptides in peripheral blood mononuclear cells (PBMCs) of cancer patients and healthy donors. Thus boosting ARG1-specific T cells may directly modulate immune regulation [1].

Peptide vaccine safety

The potential peptide vaccine-related safety profile is linked to the presence of the target antigen in normal tissue and to the risk of acute anaphylactic reactions.

However, previous peptide vaccination trials have had very mild side effects, no matter which proteins have been targeted (see Risk evaluation). Research groups from CCIT have documented mild side effects testing the indolemamine 2,3-dioxygenase (IDO) peptide vaccine with no CTCAE grade 3 or 4 toxicities [2]. The ARG1 vaccine and IDO vaccine resemble in many ways. They are both self-proteins with a broad expression and they are lymphocyte suppressive enzymes with an overexpression in immunosuppressive cancer associated immune cells.

Moreover, a phase I trial treating cancer patients with an oral ARG1 inhibitor (CB-1158) presented data on drug related adverse effects in April, 2017. A total of 17 patients with advanced solid tumors were treated with CB-1158, and the drug was generally well tolerated with no drug-related serious adverse events[3]. Even though the ARG1 peptide vaccine and CB-1158 differ in both mechanism of action and administration they both target the same enzyme. Therefore, the AEs registered for patients treated with CB-1158 could possibly indicate what AEs to expect in the ARG1 trial.

<u>Montanide ISA 51</u>

Montanide ISA 51 can cause local discomfort at the insert site, but does usually not cause any systemic side effects with the dose used in this trial. (See "Product Resume Montanide ISA 51", appendix 11)

Target population

This protocol describes the treatment of patients with solid metastatic cancers. Despite many treatment advances in cancer therapy, most metastatic solid cancers are still incurable and most patients will at some point find themselves without viable treatment options. The majority of deaths are caused by metastatic solid cancers such as lung, colon, breast, and prostate cancer. Immunotherapy is an established and effective treatment for an increasing number of cancer patients including different metastatic solid cancers.

The treatment with an ARG1 peptide vaccine has a potential in all metastatic solid cancers with a high concentration of ARG1-expressing cells, such as myeloid-derived suppressor cells (MDSCs) known for their impairment anti-cancer immunity. These tumors include non-small cell lung cancer (NSCLC), colorectal cancer, urothelial cancer, breast cancer, ovarian cancer, malignant melanoma, and squamous cell carcinoma of the head and neck (HNSCC) [4,5]. We aim to test the

ARG1 vaccine in patients who have exhausted conventional treatment options. Hence, patients with progressive metastatic solid cancers of the types mentioned above in advances stages are eligible to enter this protocol.

Number of participants

The study is a phase I trial where 10 patients with metastatic solid tumors will be treated. If 3 or more patients experience grade 3-4 AE associated with the vaccination, the trial will be stopped.

Patient recruitment

The trial is expected to begin the inclusion in August 2018. Patients will be recruited from the Department of Oncology, Herlev & Gentofte University Hospital, but can also be referred from other centers in Denmark. The inclusion period is expected to extend over 12 months.

Treatment plan

Patients included in the trial will be treated with the ARG1 vaccine every third week for 48 weeks. Sixteen vaccines will be given in total.

At the end of vaccination, patients who are not excluded from the protocol because of progression will have follow up appointments after 3 and 6 months in the experimental unit (EFEK), Herlev Hospital.

Assement of safety

A systematic recording of AEs will take place during the course of treatment and follow up in accordance with the Common Terminology Criteria for Adverse Events (CTCAE). The AEs will be reported by the investigator, who will make a relatedness assessment. Recording will take place during patient attendance in the outpatient clinic or at the hospital ward at the Department of Oncology, Herlev Hospital.

Immunological evaluation

Blood samples

In order to monitor the immune response, ARG1 levels and immune related changes during treatment, project blood samples will be taken before, during and after treatment. Project blood samples will be taken at the follow up appointments after 3 and 6 months.

Tumor biopsy

At baseline and after the 4th vaccine, a tumor biopsy will be performed if the tumor is accessible. The objective is to evaluate the tumor immune microenvironment of each patient.

Immunohistochemistry and whole exome sequencing to assess initial mutational status will be conducted.

Delayed type hypersensitivity

Delayed type hypersensitivity (DTH) skin-test and punch biopsies from the DTH areas will be done after the 4th vaccination for the evaluation of skin infiltrating lymphocytes (SKILs). The SKILs are tested for specificity to the ARG1 peptide as a sign of induction of a functional immune response.

Clinical response evaluation

Clinical response evaluation will be performed with diagnostic imaging according to Response Evaluation Criteria In Solid Tumors (RECIST)[6], PET Response Criteria in Solid Tumors (PERCIST)[7]. Clinical examination before, during and after treatment (see the Treatment schedule).

Background

Metastatic solid cancer

Despite many and continuous advances in oncology, metastatic solid cancer is still regarded an incurable and fatal disease. Treatment options palliative and given with the purpose of prolonging life thereby often ameliorating suffering and disability. In Denmark 37.000 patients are diagnosed with cancer every year and more than 15.000 die from cancer-related causes [8]. The 4 most common and most deadly cancers forms are all solid cancers (breast, prostate, lung, and colon cancer) that readily metastasize. Combined, these 4 diagnoses alone amount to 55% of all new cancer diagnoses and 53% of all cancer-related mortality[9]. Standard therapies for these cancers range from hormone suppression to aggressive chemotherapy regimens. In the last couple of years immune therapy has revolutionized many cancer treatments. In solid tumors, malignant melanoma has pioneered the field but it is constantly expanded to other diagnoses. Immune therapy was recently approved for lung cancer[10] and colon cancer might be next[11]. The high prevalence and morbidity of metastatic solid cancers leaves room for therapeutic improvements.

Immune activation and regulation

In this section a very brief introduction is given to the adaptive immune system, which is one of the two major divisions of the immune system and is very important in the fight against cancer. Cells and molecules of the other division of the immune system, called the innate immune system, also play their important roles in cancer, but the vaccination therapy described in this protocol has its main effects in the adaptive immune system. The adaptive immune system has two branches comprised of the humoral and the cellular branch.

In the humoral branch of the adaptive immune system antibodies are the major effectors. Antibodies bind to molecules on their targets, whether the targets are bacteria, virus, fungi or malignant cells. Antibodies exert their function by physically blocking surface molecules on the target or by one of several ways mediating specific toxicity to the target. When a surface molecule is blocked by an antibody, the function of the surface molecule can be inhibited. Antibodies can kill targets by cross-linking molecules on the target, by activation of attack-molecules of the complement system to make holes in the target's membrane or by activating other immune cells to kill the target.

In the cellular branch of the adaptive immune system the foot soldiers are the T cells. Two subgroups of T cells are the CD8+ cytotoxic T cells (CTLs) and the CD4+ helper T cells. While the main role of CTLs is to directly kill cells that are virally infected or malignantly transformed, T-helper cells come in many subtypes and have many different roles in the immune system.
CTLs are very effective killer cells, and are probably therefore highly regulated. The most fundamental level of regulation comes from the principle that CTLs can only kill other cells, if the TCR of the CTL recognizes a "peptide-fingerprint" on the target cells. Peptides are small pieces of proteins. This fingerprint is a specific peptide epitope bound to a specific major histocompatibility-class I molecule together making a peptide:MHC-complex. All nucleated cells express these MHC-molecules that display to the immune system a random sample of what proteins are in the cell. If a cell is infected with a virus, viral peptides will be displayed. If a cell has turned malignant, cancer peptides will be displayed.

A T cell has many TCRs, but all TCRs on a T cell are identical. Therefore, a CTL is specific to one peptide-fingerprint. TCRs are made randomly, but whether a specific TCR is expressed on any T-cells circulating the body is regulated in the thymus. In the thymus, T cells are positively and negatively selected based on what their TCR recognizes and how strongly it is recognized. If the TCR binds nothing the T cell dies of neglect. If the T cells TCRs bind self-peptides too strongly, the T cell is eliminated.

After the cytotoxic T cell has left the thymus and circulates in the body, another level of regulation is added. This second level of regulation is based on the principle that CTLs must be primed by antigen presenting cells (APC), often a dendritic cell (DC), to be able to start killing target cells (Fig. 1). APCs eat antigens, chop up the antigens into peptides, and present them on MHC-molecules. Priming happens in lymphoid tissue when a CTL meets an APC that has taken up antigen epitopes that the CTL is specific to. The APC delivers a "signal one" to the T cell which is when the APC presents the peptide epitope on a MHC-molecule to the T cell. To be activated, the T cell needs a "signal 2" which it also gets from the DC because the DC expresses costimulatory proteins that activate the T cell. Once a CTL has been primed, it circulates the body is ready to engage target cells in the effector phase (Fig. 2). In the effector phase, CTLs kill target cells that on the surface have peptide:MHC-complexes that the CTL is specific to.

A third level of regulation is built into the priming and effector phases. Not only does the killing of a target cell depend of a “signal one” and a “signal two” in the priming phase combined with a complementary TCR-peptide:MCH match in the effector phase. What is further needed is the correct balance of so called co-stimulatory and co-inhibitory signals in both phases. In the effector phase one of the very potent inhibitory molecules is programmed death ligand 1 (PD-L1) on the target cell. If a CTL recognizes a peptide:MHC-complex but is inhibited by PD-L1 on the target cell interacting with its receptor PD-1 on the T cell, the T cell is left in an inactive state (Fig. 2A). The negative regulation in the effector phase is an important system to fine-tune the regulation of CTLs, shutting down self-reactive T cells that have slipped through the previous levels of regulation. The balance between stimulatory and inhibitory molecules acts to ensure that the T cells are activated when needed, but prevents T cells from causing damage to normal tissues by inhibiting the T cell response.

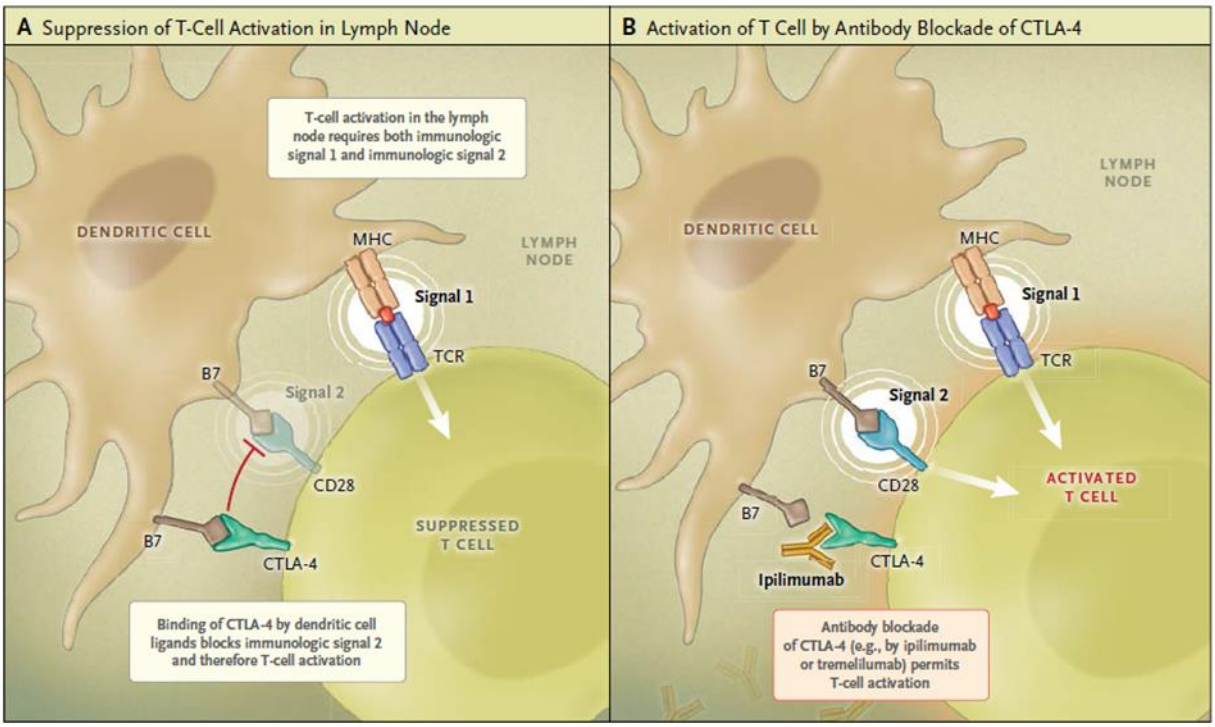

Figure 1: Immune checkpoints and checkpoint inhibitors, the priming phase. In the priming phase, an antigen presenting cell activates antigen-specific T cells by stimulating via both the MHC-TCR-pathway (signal one) and the co-stimulatory B7-CD28-pathway (signal two). In the priming phase, the immune responses can be inhibited if the T cells have upregulated the checkpoint molecule CTLA-4. CTLA-4 can be blocked by the checkpoint-inhibiting antibody Ipilimumab[12].

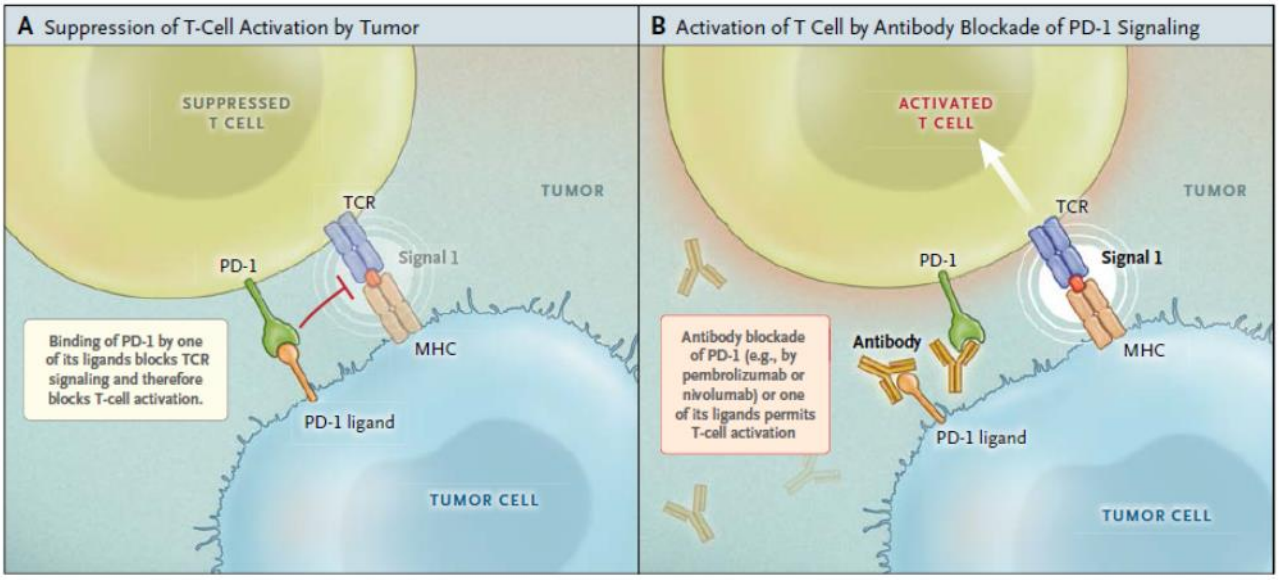

Figure 2: Immune checkpoints and checkpoint inhibitors, the effector phase. In the effector phase, antigen-specific T cells recognize target cells presenting the target peptide-MHC-molecule. In the effector phase, immune responses can be inhibited by the checkpoint molecules PD-1/PD-L1. Several antibodies blocking PD-1 or PD-L1 have been marked [12].

Cancer cells induce local tolerance

The unregulated growth of cancer cells is only possible when the immune system does not launch an effective antineoplastic response against the cancer. Impairment of the immune system is thus a hallmark of cancer [13]. Strikingly, the insufficient immunological activity in cancer patients seems not to be a general impairment of the immune system. Most cancer patients do not suffer from frequent infections, except when their immune system is very affected by treatment or when the cancer has reached a late state with profound impact on the body. Thus, the impairment of the immune system must be somewhat specific to the cancer cells and the local microenvironment around the tumor. This discrepancy between a near normal overall immune function and profound immunosuppression locally can be explained by the increase in immune regulatory molecules and immune regulatory cells in and around the tumor.

Cancer cells highjack the system of checks and balances of T cell activation by overexpressing inhibitory molecules such as PD-L1 and regulatory enzymes such as IDO. Furthermore, immunosuppressive regulatory cells also expressing inhibitory molecules like PD-L1 and ARG1 are recruited to the tumor. The expression of ARG1 by immunosuppressive regulatory cells inhibits T cells by depleting the microenvironment of the amino acid arginine crucial to T cell signaling and functions.

Combined this causes a very immunosuppressive local environment, skewing the normal balance between stimulating and inhibitory signals in the direction of immunosuppression in and around the tumor. The co-inhibitory molecules CTLA-4, PD-L1 and PD-1 are checkpoints in the immune system. One of the major pillars of the recent impressive advance of the field of immune-oncology

is the introduction of the so called immune checkpoint blocking monoclonal antibodies. Antibodies blocking the action of CTLA-4 in the priming phase or PD-1/PD-L1 in the effector phase block out these negative signals in the interaction of T cells with APC or target cells, respectively (see fig. 1B and 2B). Blocking the negative signals in the balance "releases the break" on T cells, allowing them to kill malignant cells. All in all, cancer cells have many mechanisms whereby they escape immune attack.

Mechanism of Action – vaccination therapy

All nucleated cells are characterized by a "peptide-fingerprint" (MHC:peptide complex) on the cell surface, which to the outside exposes a mirror of the protein content in the cell. Thus, immunosuppressive cells that overexpress ARG will exhibit ARG peptide:MHC complexes on the cell surface and can be recognized by T cells [14,15].

The principle of vaccination is to administer the target (the antigen) recognized by the immune system together with an immune enhancer, the adjuvant. The antigen can be composed in many different forms. Relevant examples in cancer vaccination are whole tumor cell vaccinations in which killed tumor cells are used as the source of antigen. Obviously, such an antigenic source comprises a huge number of proteins as potential antigens, many of which will in fact also be present in normal cells.

Administered in combination with a relevant adjuvant, cellular proteins derived from tumor cells will be taken up by APCs. The APCs will process the proteins, i.e. cut the proteins into peptides that are capable of binding to both class I and class II MHC molecules. MHC:peptide complexes expressed on the surface of APCs may elicit T cell responses specific for the individual peptides – and these T cells thus have the capacity to recognize cancer cells, which express an identical MHC:peptide molecule on the cell surface (Fig. 3).

At the other extreme, an antigen used for vaccination may simply be a peptide of 9-amino acids derived from a protein expressed by cancer cells. This form of antigen negates the need for antigen processing step by APC as the peptide is already in a form ready to be presented by the APC in the context of MHC. Such peptide antigen may activate T cells capable of recognizing the identical peptide sequence expressed on the surface of cancer cells in the context of MHC class I molecules. Both approaches have advantages and shortcomings.

Long peptides of approximately 15 to 20 amino acids have been used in therapeutic vaccination studies for more than a decade and can be viewed as an attempt to develop a strictly defined yet still broadly applicable target antigen (Fig. 4). A main advantage of the long peptide approach compared with the conventional short peptides is that such peptides can encompass epitopes binding with both MHC class I and II, i.e. both inducing CD4 T helper responses as well as CD8 cytotoxic T cell responses. Importantly, peptide binding to MHC class II is rather promiscuous, and any given peptide may be capable of binding to several different MHC class II molecules [16,17].

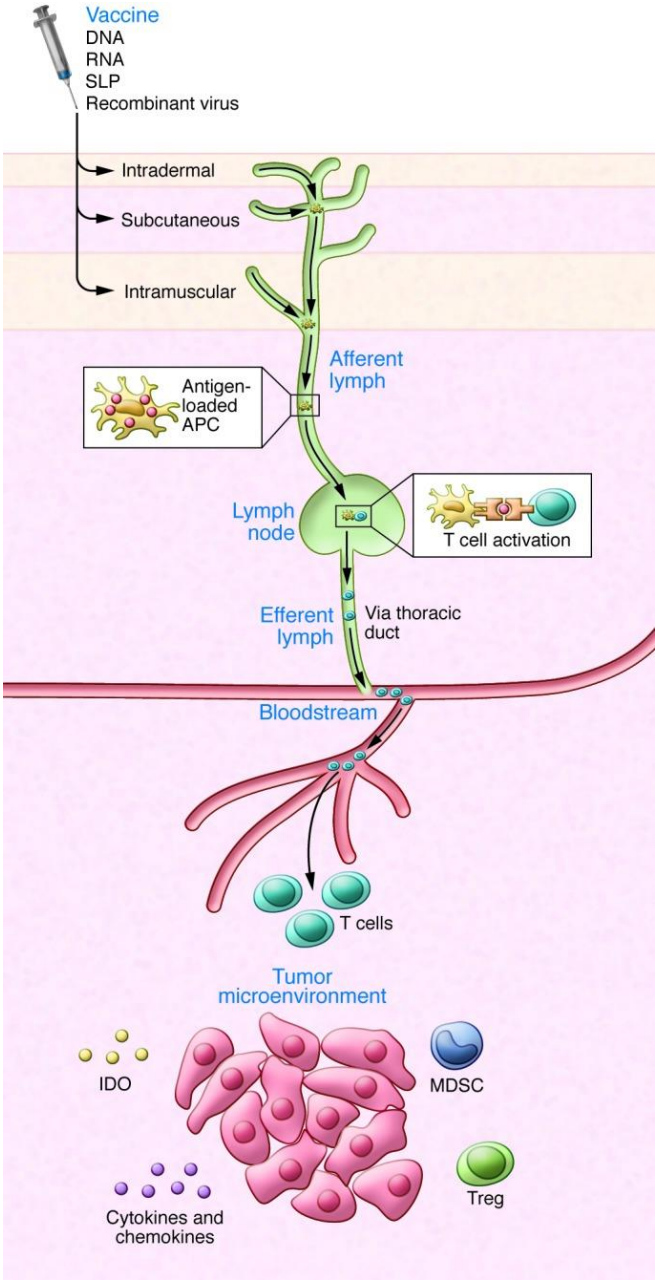

Figure 3: Principle of therapeutic cancer vaccination. APCs take up antigen at the injection site and migrate to a lymph node. During migration to the lymph node they mature to efficient antigen presenters, this is improved if a strong adjuvant provides a good "danger"-signal. In the lymph node, APCs meet T cells and prime them. When T cells are primed, they leave the lymph node to circulate the body patrolling for targets [16].

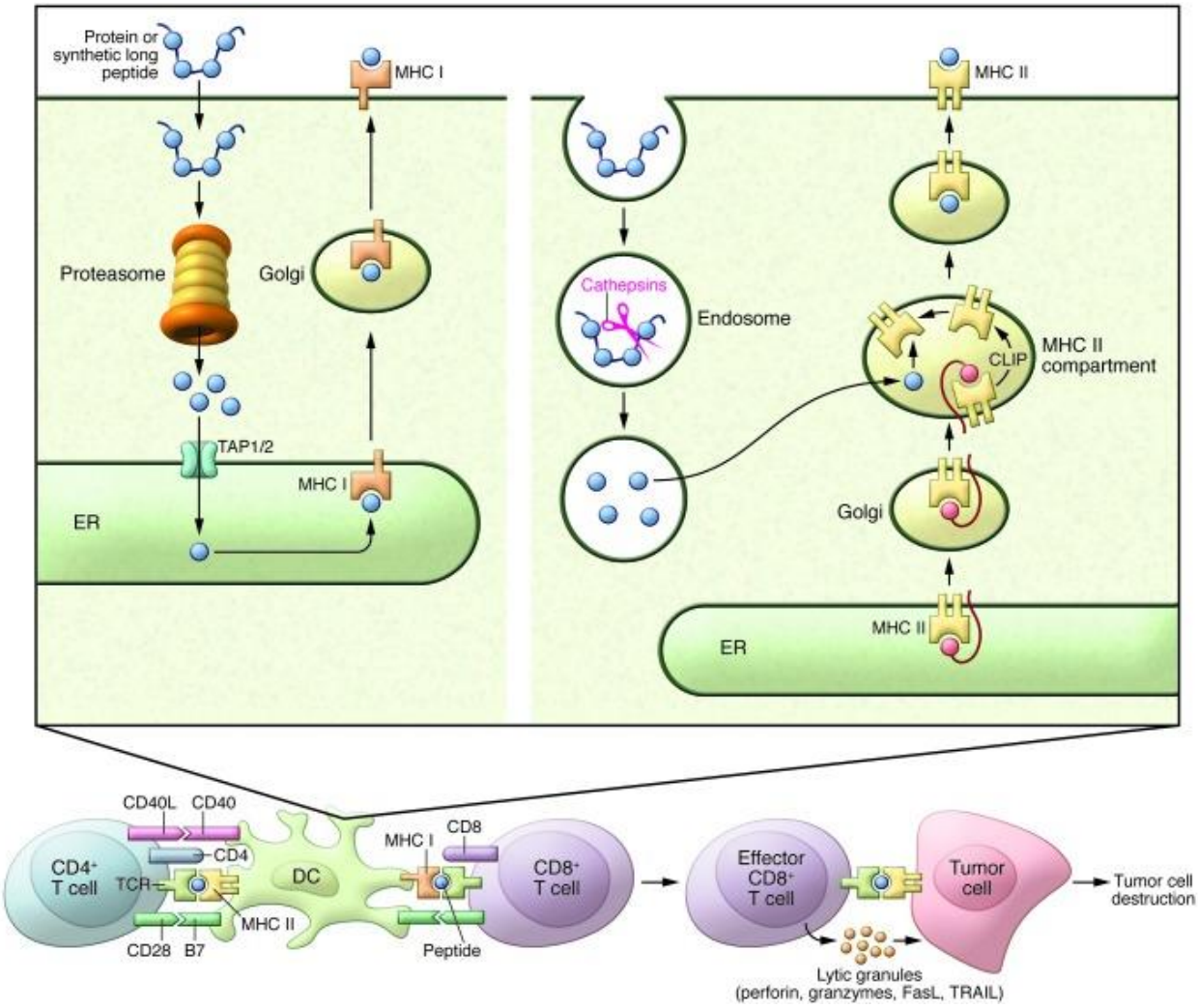

Figure 4: The processing of long peptides in APCs allows stimulation of both CTL and T-helper cells. In the right side of the figure, a long peptide is taken up via endocytosis, cut into pieces and presented on MHC-class II molecules. This allows it to be presented to CD4-positive T-helper cells. In the left side of the figure, a long peptide enters the cytosol. The jury is out as to how it enters the cytosol, but we know it happens. From the cytosol, it is processed like a normal protein from the cell. This normal way is by degradation in the proteasome, transportation into the ER and in the ER loading onto MHC-class I molecules. When a peptide is bound to a MHC-class I molecule, it is presented to CD8-positive CTLs. In this manner, a long peptide is not restricted to either class I or class II MHC-molecules. DC: dendritic cell. ER: endoplasmatic reticulum. TAP1/2: Transporter associated with Antigen Processing1/2 [16].

Arginase and its potential as a vaccination antigen

Arginine, the ARG substrate

Arginine is a conditionally essential amino acid obtained exogenously through diet and endogenously through de novo synthesis of cellular protein breakdown. In healthy adults the endogenously biosynthesis of arginine from citrulline satisfies metabolic requirements but dietary supplements are needed in various cases of metabolic stress and during growth [18,19]. Arginine serves as a precursor of creatine, citrulline, urea, ornithine, polyamines, proline, and glutamate[18,20]. The amino acid also functions as a substrate for the enzyme nitric oxide synthase (NOS) that converts arginine to nitric oxide (NO) and other nitrogen intermediates that are important for microbicidal activity (Fig. 5) [21].
Arginine plays a role in tissue repair and protein synthesis [22]. More relevant to this protocol, arginine plays an important role in mediating tumor immune regulation[23]. In a recent mouse model, it was demonstrated that activated T cells consume large amounts of arginine, and that an arginine supplement enhances T-cell survival and antitumor activity [24].

The ARG enzyme

Arginine is hydrolyzed to ornithine and urea by the enzyme ARG. In mammals, there are two existing ARG isoenzymes: ARG1 and ARG2. The isoenzymes catalyze the same biochemical reaction but they differ in cellular expression, location, and regulation [25,26] . The human ARG1 is a cytoplasmic enzyme mainly expressed in hepatocytes. It detoxifies ammonia by catalyzing the final step in urea cycle. ARG1 is also expressed in various extrahepatic tissues where it regulates arginine levels and provides ornithine for biosynthetic reactions [27]. The expression of ARG1 by some cancer cell lines and regulatory immune cells promote the development of an immunosuppressive environment [23,28]. The immunotolerogenic impact is partly due to the capability of some cells to secrete of ARG1 from intracellular granules into the microenvironment [29] (Fig. 10).

ARG2 is found in the outer mitochondrial membrane and in the mitochondrial matrix. It is predominantly expressed in the prostate, brain, kidney, small intestine, and pancreas. The physiological function of ARG2 is still poorly understood compared to that of ARG1 [20,23]. In cancer research, ARG2 expression has been documented in acute myeloid leukemia (AML) blasts, fibroblasts, and various solid cancers such as prostate cancer [30–32].

Arginase as an immune suppressor

The depletion of arginine by increased ARG expression has emerged as an effective immunosuppressive pathway for the mammalian immune system. The reduction of arginine from the microenvironment can result in impairment of lymphocyte responses to antigens and arginine-deprivation has now proved to be a strategy for cancer cells to induce immune tolerance[23,25]. This

effect of arginine-depletion on T cells was studied *in vitro* by Rodriguez *et al*. and it was demonstrated that an arginine deficiency directly affected T cell function through a down regulation the main signaling-transduction component of the TCR, the TCR ζ chain (CD3 ζ), in human T cells through mRNA instability. The impairment of T cell function was fully reversed by the replenishment of ARG[33]. At this time, T cell suppression have proved to be the main immunoregulatory mechanism of ARG on the tumor environment[34].

ARG producing cells

Cancer cells are capable of influencing the tumor microenvironment by hijacking local myeloid cells and trigger their differentiation into TAMs and MDSCs [35–37]. Both TAMs and MDSCs are highly immunosuppressive. They induce a favorable tumor environment, partly, by an ARG1-expression. TAMs and MDSCs are also capable of triggering the differentiation of regulatory T cells (Tregs) as well as inhibiting the proliferation and activation of natural killer cells (NK cells) and effector T cells[28,38].

In addition to ARG1 production induced by anti-inflammatory signals such as IL-4 or IL-13, MDSCs and TAMs also express the enzyme nitric oxide synthase (NOS) that catalyzes the production of NO and other reactive nitrogen intermediates from arginine when stimulated by pro-inflammatory cytokines such as INF-γ (Fig. 5)[34]. NO has the potential of showing both tumoricidial and tumor promoting effects depending on concentration, location, and timing, and a potential ARG1-induced inhibition of a tumorcidial NO-production by macrophages has been suggested as a strategy for cancer cell survival[19,21,23,39].

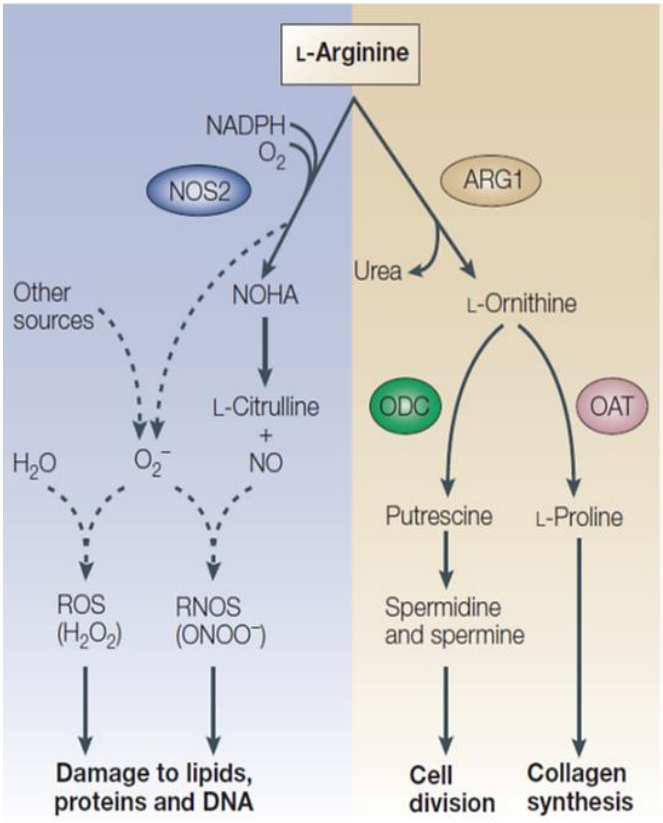

Figure 5: Schematic representation of ARG1- and nitric-oxide-synthase-2-dependent metabolic pathways. The activities of the enzymes ARG1 and nitricoxide synthase 2 (NOS2) are illustrated, together with the downstream pathways that are activated by arginine[34].

ARG1 as an immune modulator

The expression of ARG has been demonstrated in different cancer cell lines [34,40]. This protocol is focusing on the influence of the ARG1 on the tumor microenvironment, as several studies have demonstrated an ARG1 overexpression in the blood and/or tissue of patients with both lung [41] , ovarian [42], renal [43], breast [44,45], esophageal, and gastric cancer[46].

The overexpression of ARG1 by macrophages within the tumor environment has proved to favor tumor growth in murine lung carcinomas and human renal cell carcinomas[43,47].
It is therefore reasonable to believe that an ARG1 blockage will inhibit tumor growth, and this was in fact demonstrated in a murine trial where an injected ARG1-inhibitor reduced the tumor growth of lung carcinoma *in vivo*. In this study, C57BL/6 mice were injected with Lewis lung carcinoma (3LL) cells. The mice were devided into six grupous with six mice in each group. During the

following 18 days, the mice were injected s.c. with different substances and doses according to their group: 6 mice recived PBS, 6 mice recived 20 mg/kg of an ARG1-inhibitor, 6 mice recived 40 mg/kg of the ARG1-inhibitor, 6 mice recived 80 mg/kg of the ARG1-inhibitor, 6 mice recived the ARG1-inhibitor (80 mg/kg) + L-arginine (500mg/kg), and 6 mice recived L-arginine(500mg/kg). The mice injected with the ARG1-inhibitor and the mice injected with the ARG1-inhibitor + L-arginine had a significantly lower tumor growths than observed in the rest of the groups. The reponses were ARG-1 dose-dependent.
The ARG1-inihitor was also tested in mice lacking function T- and B cells (C57BL/6-Prkdc^{scid} mice) to investigate whether the ARG1-inhibitor-induced anti-tumor effect just described partly was mediated by an immune response. Sixteen mice were injected with Lewis lung carcinoma (3LL) cells, 8 mice were then injected s.c. with 80 mg/kg of the ARG1-inhibitor and 8 mice were then injected s.c. with PBS. The decrease in tumor growth seen in the C57BL/6 mice treated with the ARG1-inhibitor was not seen in the treated C57BL/6-Prkdc^{scid} mice indicating that the ARG1-inhibitor-mediated anti-tumor effect is partly lymphocyte dependend [47].
The role of ARG1 in the regulation of tumor induced immune tolerance makes it a promising therapeutic target. A phase I/II study evaluating an oral ARG inhibitor (CB-1158) as a single agent and in combination with Pembrolizumab is currently recruiting, *(ClinicalTrials.gov identifier (NCT number): NCT02903914)*(see Risk Evaluation).

The ARG1 peptide vaccine

The theoretic background for a vaccine against ARG1 is to boost the activity of ARG1-specific T cells (anti-ARG1 T cells) to attack cancer cells and immune cells that express ARG1 as part of the local immunosuppressive microenvironment in cancerous tissues.

CCIT therefore recently examined whether sequences of the ARG1 peptide served as a target for specific T cells to evaluate whether ARG1 would be a potential new vaccination antigen.
Naturally present peripheral ARG1-specific T cells were identified in both healthy donors and in patients with different cancer diagnoses [1].
To examine ARG1-specific cells *in vitro,* a screening of the entire ARG1 sequence was executed. Using the ARG1 peptide library and an IFNγ ELISPOT assay it was discovered that T cells recognized multiple ARG1 epitopes, including a hot spot region consisting of 50-amino acids that induced the most frequent responses ($ARG1_{161-210}$). (Fig. 6)

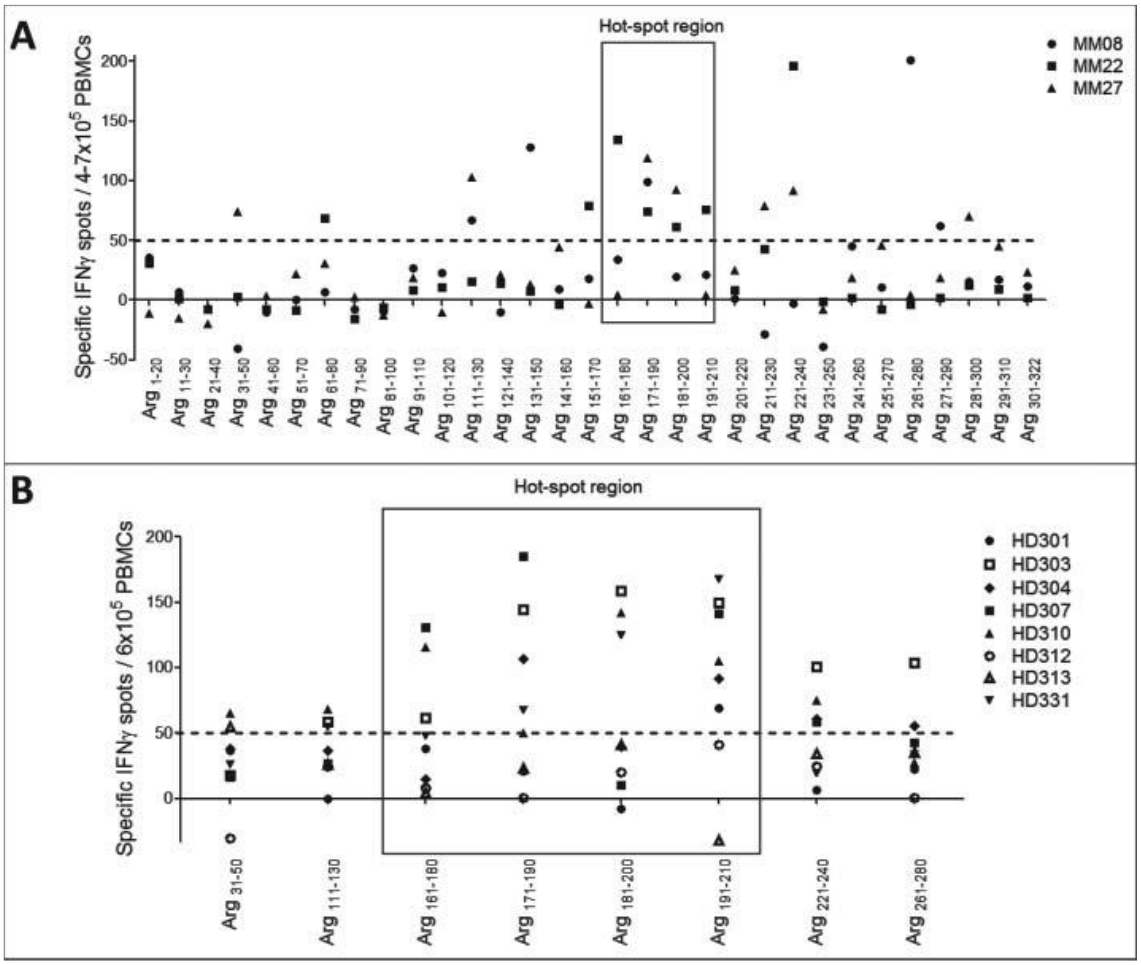

Figure 6. A) IFNγ ELISPOT screening of responses against ARG1 peptides in PBMCs from 3 melanoma patients. B) IFNγ ELISPOT screening of responses against eight selected ARG1 peptides in PBMCs from 8 healthy donors. Spot counts are given as a difference between averages of the wells stimulated with the peptide and control wells. Peptide and control stimulations were performed in duplicates or triplicates.

Both CD4+ and CD8+ T cell responses against an epitope from the hot-spot region ($ARG1_{161-180}$) were observed in two cancer patients, indicating that HLA class I and II epitopes are present in the ARG1 hot spot region (Fig. 7).

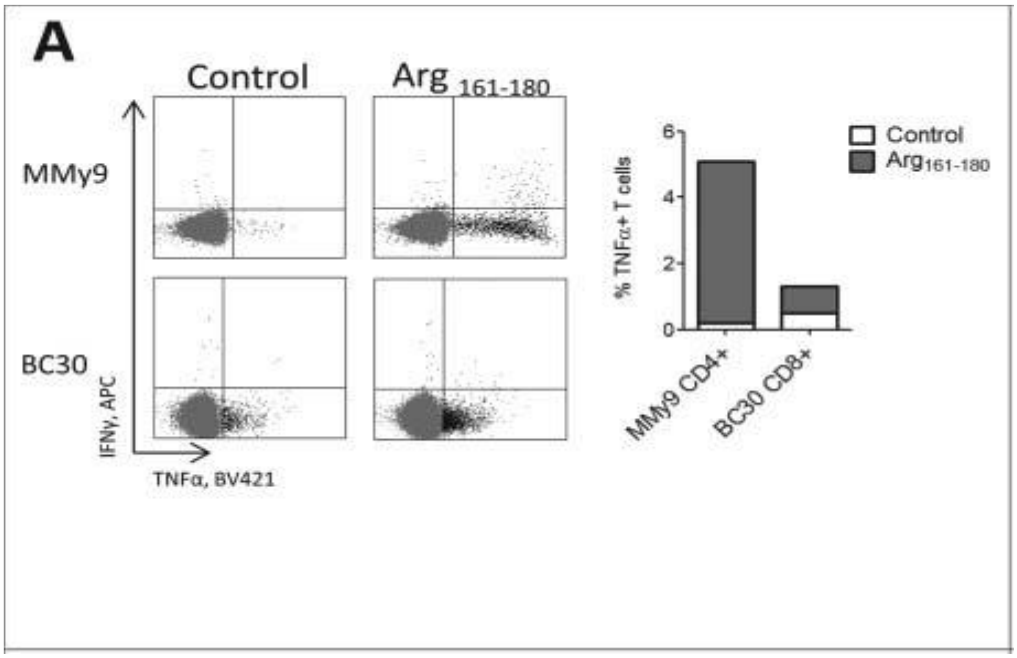

Figure 7. To the right: % of TNFα producing CD4+ and CD8+ T cells in intracellular staining of PBMCs from multiple myeloma (MMy9) and breast cancer (BC30) patients stimulated with Arg161–180 peptide and non-stimulated control. To the left: dot plots for Arg161–180 peptide stimulated.

To examine the presence of ARG1 specific T cells among tumor infiltrating lymphocytes (TILs), *In vitro* expanded TILs from melanoma patients were stimulated with the following epitopes from the hot-spot region: ARG1-17$_{161\text{-}180}$, ARG1-18$_{171\text{-}190}$, and ARG1-19$_{181\text{-}200}$. IFNγ was produced by CD4+ T cells in response to all three ARG-peptides in one of the TIL cultures (Fig 8).

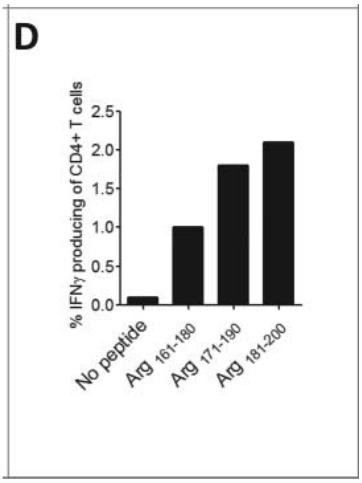

Figure 8 - IFNγ production by CD4+ T cells in *in vitro* expanded TILs from a melanoma patient in response to Arg161–180, Arg171–190, Arg181–200 peptides compared to a non-stimulated control.

Finally, autologous dendritic cells (DCs) and B cells were transfected with mRNA encoding ARG1 protein to assess the ability of ARG1 specific CD4+ T cells in recognizing ARG1 expressing immune cells. ARG1 specific CD4+ T cells isolated and expanded from two melanoma patients showed higher reactivity against DCs and B cells transfected with ARG1 mRNA compared to mock controls (Fig. 9).

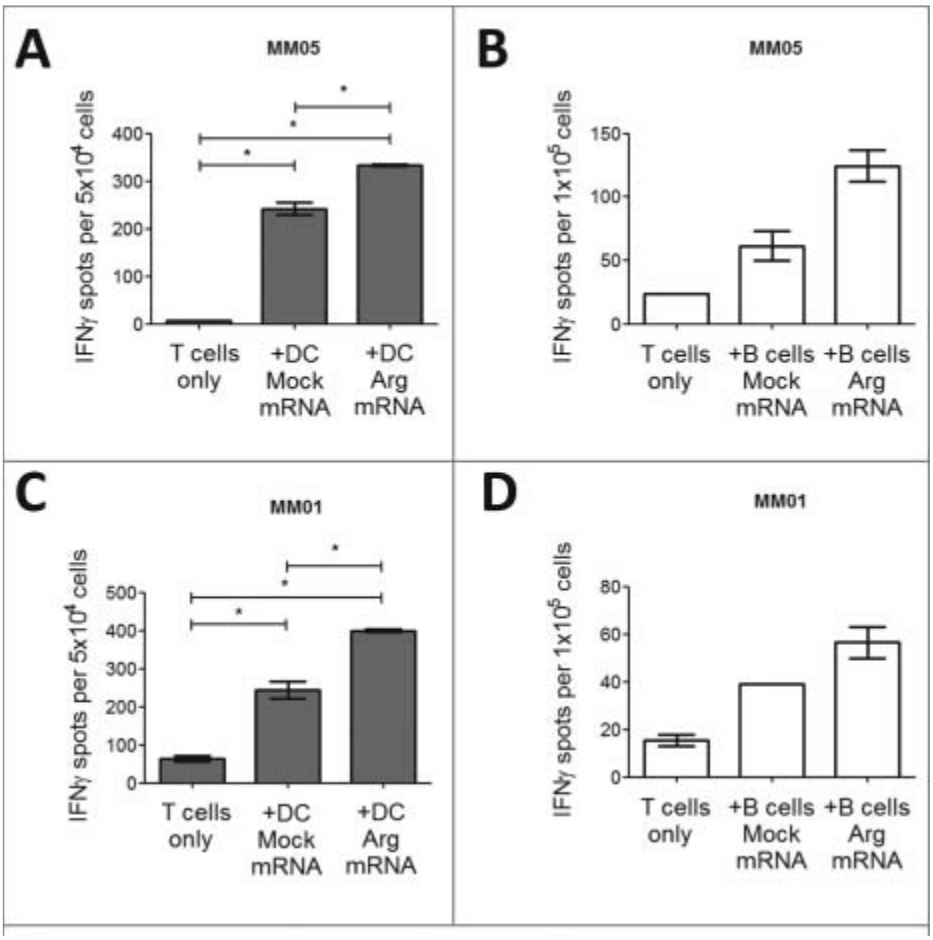

Figure 9. ARG1-specific T cells recognize ARG1-expressing immune cells. A),C): IFNγ response by ARG1 specific T cell cultures from two melanoma patients (MM01 and MM05) to autologous dendritic cells electroporated with irrelevant control mRNA (DC Mock) or ARG1 mRNA (DC Arg mRNA), effector to target 10:1. B), D) IFNγ response by ARG1-specific T cell cultures from two melanoma patients to autologous B cells electroporated with irrelevant control mRNA (B cells Mock mRNA) or ARG1 mRNA (B cells Arg mRNA), effector to target 2:1.

The patients from which ARG1-specific T cell cultures were generated were selected from a larger group of patients that were all screened for responses against ARG1 peptides.
In total, ARG1 specific T cell responses were screened in 25 cancer patients: 14 melanoma, 1 breast cancer, 1 renal cell carcinoma, 9 multiple myeloma. 13 of these patients showed a

statistically significant response against one or more of tested ARG1 peptides in IFNγ ELISPOT demonstrating that ARG1 specific T cells can be found in patients with different forms of cancer. Some of these responses were also further characterized using intracellular staining for IFNγ and TNFα in response to ARG1 peptide stimulation, to show that both CD4+ and CD8+ T cell responses are present among the ARG1 responding T cells [1].

Overall, ARG1-specific T cells are capable of recognizing ARG1-expressing cells,a vaccine that activates ARG1 specific T cells thereby attracting them to the tumor site and reducing ARG1 expressing cells such as MDCSs could be an effective new approach to reduce tumor burden (Fig. 10).The Th1 inflammatory response (IFNγ) induced by the ARG1-specific T cells could potentially induce IDO and/or PD-L1 expression in the immunoregulatory cells and cancer cells, thereby generating targets susceptible to PD1/PD-L1 immunotherapy.

The mechanism of action (MoA) of the ARG1-vaccine differ from the MoA of the ARG1 inhibitors presented in this protocol as the ARG1-18,19,20 vaccine will not directly inhibit ARG1. However, by sharing the same target-enzyme, both the ARG1-inhibitor and the ARG1-vaccine will inhibit ARG1 and increase arginine levels. The anti-tumor results of the ARG1-inhibitor described previously therefore indicate that the ARG1-18,19,20 vaccine could induce a potential anti-tumor response in the treated patients [47].

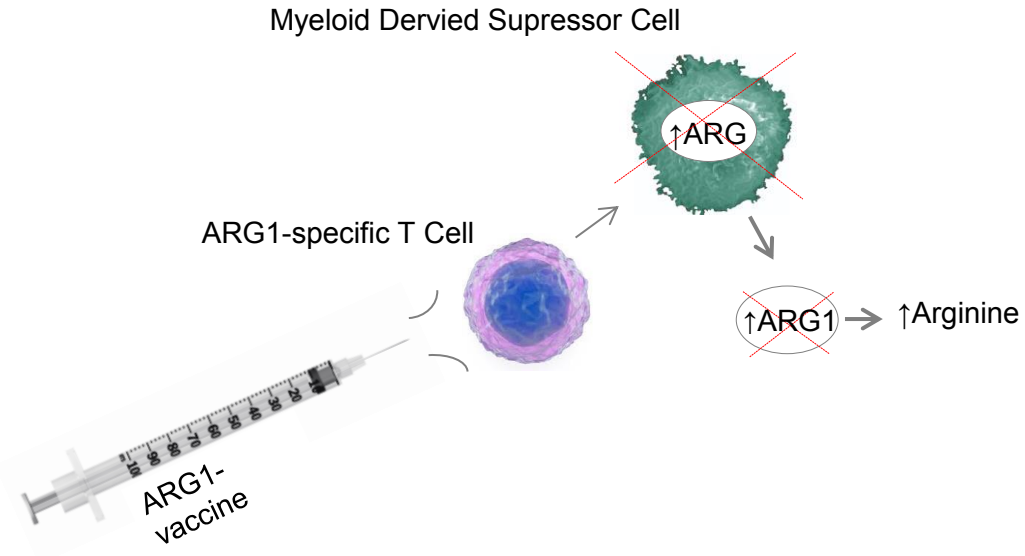

Figure 10. We in the CCIT hypothesize that the peptide vaccine consisting of ARG1 will boost the natural immunity mediated by ARG1 specific T cells as these T cells will accumulate at the tumor site thereby reducing ARG1 expressing regulatory immune cells (such as ARG1 secreting MDSCs). Consequently, we hypothesize that the ARG1 induced T cell suppression through arginine depletion will reverse.

Peptide vaccine safety

Like the PD-L1 and IDO peptide vaccines studied in CCIT, ARG1 specific T cells are not predicted to attack normal cells in non-cancerous tissues. It requires a high expression of a certain peptide in a cell to produce a high enough amount of peptide:MHC-complexes on the surface to overcome the threshold of being killed by peptide specific T cells. The affinity of T cell receptor to MHC:peptide complexes are 100-1000 fold lower than that between antibody and antigen. Consequently, many peptide:MHC-complexes have to be on the cell surface for the activation signal to be strong enough. Furthermore, the activation of peptide specific T cells is a slow process. Only by vaccinating several times, the amount of anti-peptide T cells will be sufficient. Finally, it is well described that "danger signals" are vital for the effector phase of an immune response. Thus, T cells preferentially engage in areas of inflammation with dying cells and other sources of "danger signals". These signals are present in tumors but not in normal, healthy tissue. This further explains the general lack of toxicity in anti-cancer vaccinations utilizing self-antigens [48,49].

Since the vaccine is based on a self-antigen, theoretically autoimmunity is a potential risk of the treatment. However, the peptide specific T cells are already present in unvaccinated healthy individual as well as cancer patients. Vaccinations with the peptides boost the activity of the peptide-specific T cells to engage regulatory cells and cancer cells and immune cells which express the proteins as part of the local immunosuppressive microenvironment in cancerous tissues.
As for the ARG1 vaccine, a potential concern is interfering with ARG1 function in the urea cycle, a central pathway in the liver for detoxifying ammonia generated from the breakdown of amino acids. However, pharmacological ARG-inhibition has been well-tolerated in numerous animal studies, including a rat model of hypertension in which the ARG-inhibitor nor-NOHA was injected over a period of 10 weeks [50,51].
Moreover, the oral ARG inhibitor (CB-1158) have been well-tolerated in a phase I trial *(ClinicalTrials.gov identifier (NCT number): NCT02903914)*(see Risk Evaluation).
All in all, it appears very unlikely that the ARG1-18,19,20 peptide vaccine will initiate a general overactivation of the immune system.

Preclinical development: We conducted an exploratory study with vaccination of mice with the humane sequence of ARG1-18,19,20. The mice did not present immune responses to the human sequence. Similar results were obtained in murine vaccine studies with human sequence of PD-L1. The murine organs have been reviewed by a pathologist without any sign of organ damage (See "Preclinical exploratory study of vaccination ARG1 peptides in C57Bl6 mice with Montanide ISA-51" appendix 09)

Adjuvant

A peptide antigen is administered with an immune enhancer known as an adjuvant. The adjuvant increases the uptake of the antigen by APCs, and mediates so-called "danger signals" to the APC.

When an APC takes up and presents antigen in the context of a danger signal, it will deliver co-stimulatory signals to T cells, which activates T cells that recognize the antigen.
The adjuvant to be used is Montanide ISA-51. Montanide is an incomplete Freund's adjuvant (IFA) which forms water in oil emulsion when mixed with an aqueous phase. It is commercially available from Seppic S.A, France, which maintains a Drug Master File. Montanide has been used in cancer vaccines in other research projects in more than 5000 patients corresponding to more than 50.000 injections subcutaneously or intramuscularly. IFA is used with success in combination with long peptides, where clinically relevant responses can be produced with IFA. For instance in a vaccine with a long HPV-16 peptide against vulva cancer [52–54]

Montanide has a well-documented safety profile. Frequently reported side effects after injection are flu-like symptoms (headache, chills, fever and nausea) while most common local reactions are granuloma, local pain, inflammation or erythema. These reactions are generally classified as mild to moderate and they are typically transient in nature.

Therapeutic peptide vaccines lack a suitable animal model for safety and dose testing
Therapeutic peptide cancer vaccines are a group of biopharmaceuticals that lack a useful non-human model for nonclinical safety evaluation according to the ICH S6(R1):

1. To identify initial safe dose and subsequent dose escalation schemes in humans
2. To identify potential target organs for toxicity and for the study of whether such toxicity is reversible; and
3. To identify safety parameters for clinical monitoring

According to the guideline it is recommended that safety evaluation is performed in a relevant species where the test material is pharmacologically active alternatively in transgenic animals or by use of homologous therapeutics.
To create an animal model with completely transgenic TCR is not possible. Furthermore, the amino acid sequences of ARG1, the target molecule in question, differ between humans and other animals, making the assessment of potential autoimmunity impossible using an animal model. Accordingly, it is not feasible to use a transgenic animal model to reproduce the pharmacological activity and toxicity that occurs in the human body because of the administration of peptide vaccines.
The numerous limitations on standard preclinical animal studies have been described in a consensus paper by a collaboration of international societies for cancer immune therapy [55]. This consensus paper underscores that "Even if mice were generated that expressed the appropriate HLA type and the human antigen sequences, such models might not adequately predict safety or autoimmune effects based on the diversity of the other components of antigen processing machinery involved." A clinical development paradigm for cancer vaccines has been proposed by a group of more than 50 experts from academia, regulatory bodies and the biotech/pharmaceutical

industry from Europe and the US FDA [56]. In this development paradigm, it is proposed to "…adopt a flexible approach, performing studies likely to be informative, while avoiding tests that contribute little valuable information" when performing preclinical safety testing of therapeutic cancer vaccines and the combination of cancer vaccines with other agents. Considerations for safety studies have been published[57]. The proposal is to focus on using in silico human expression data, and further that it is not feasible to replicate immune responses in animals since immune responses are dependent on human MHC-molecules.
Regarding identification of initial dose, there are likewise no suitable animal models. In the case of peptide vaccination, the dosage should be higher than an unknown threshold level, below which presentation by antigen-presenting cells will not be sufficient. Doses higher than the threshold dose will not increase effectivity, but also do not cause increased toxicity since peptides are rapidly degraded by local peptidases to amino acids. The dosages selected for the current study are based on experience.
In conclusion, studies in animals on safety, characterization of the pharmacokinetics, and determination of maximum tolerated dose (MTD) are not relevant in the case of therapeutic peptide vaccination.

Chioce of dosage

Cancer vaccines have emerged as a promising novel modality; however, identifying the maximum tolerated dose has been challenging. In contrast to cytotoxic agents, cancer vaccines modulate the immune system to target specific antigens on cancer cells and/or other immune cells. This mechanism of action is not expected to be directly dose related or to induce severe toxicity.

Most trials that have been conducted testing the safety and efficacy of therapeutic cancer vaccines in dose escalation studies have not provided any correlation between dose escalation and toxicities or immune responses (monitored as cellular response) [58].

In an analysis of clinical trial design suitable for therapeutic cancer vaccines, Rahma et al. found that the risk of serious toxicities with therapeutic cancer vaccines are extremely low and that toxicities do not correlate with dose levels. Furthermore, the conventional dose escalation design, with few exceptions, is regarded not suitable for cancer vaccines. Therefore, the main goal is to determine a dosage that can create an immune response and the method of defining that dose depends on whether the vaccine belongs to a class of vaccines that has been previously tested in humans. If the vaccine class (e.g, a peptide) has been administered in humans and found to be non-toxic and able to induce immune response, then it is proposed using a dosecomparable to the dose used in the previous trials[58].

Based on our previous clinical experience in the CCIT, administration of either 100 µg or 250 µg generates an intended immunological response measured by analyzing induced vaccine specific cellular response in the treated patients. Especially relevant in these settings is the finding that all the patients with stable disease (SD) who responded to treatment in a NSCLC trial exhibited a detectable vaccine specific T-cell response prior to vaccination (measured by IFNγ ELISPOT assay),

suggesting that the pre-existence of relatively high frequency anti-IDO T-cells in these patients correlate with the clinical efficacy [2,59].

To conclude, therapeutic cancer vaccines are generally expected to have a relatively safe profile and dose-independent efficacy. Based on the above findings, the peptide dosage of a 100 µg of each ARG1-18,19,20 peptide has been chosen for the trial, as this dosage is believed to be relevant to achieve both immunological and clinical responses.

Number of participants

The study is designed as a phase I trial. Ten patients with metastatic solid tumors will be treated. If 3 or more patients experience grade 3-4 AE in phase I associated with the vaccination, the trial will be stopped.

Patient recruitment

Patients will be recruited nationally and included at the Department of Oncology at Herlev Hospital. Patients with metastatic, incurable solid tumors (NSCLC, colorectal cancer, urothelial cancer, breast cancer, ovarian cancer, malignant melanoma, and HNSCC) can be included regardless of what earlier oncological treatment they have received; i.e. surgery, radiation, high-dose IL-2 or experimental treatment.
It is estimated that patient inclusion can be completed within 12 months.
Treating doctors at the departments of oncology can refer patients to the investigator. The investigator reads through the referral material and the patients are then invited to a talk about the protocol at the Department of Oncology at the experimental unit (EFEK).

Information and informed content retrieval

At the first appointment at the experimental unit, patients will be informed orally about the protocol by a doctor with experience in both oncology and immunotherapy under supervision of Professor Inge Marie Svane. The patient is always welcome to bring an assessor (relative, friend etc.) Written patient information in the form of ”Participant Information” will be handed out to the patient.
The conversation with the participant takes place in an undisturbed room and 60 minutes is allotted.
The information will be presented in an easily comprehensible language without the use of technical or value-laden terms. The consultation and written information will be in Danish, but in cases of foreign languages a certified translator will be provided.

Information given during the consultation will be based on the written participant information and include the following:

- Predictable risks, side effects, possible complications, disadvantages, as well as the possibility for unpredictable risks and complications
- Other treatment options including advantages and disadvantages, as well as the consequences of no treatment and supportive care
- Private and other confidential information contained in the medical record can be accessed both during and after the treatment. The medical record information will be reviewed by third party officials during the statutory quality assessment of the trial
- Other conditions which might have influence on the patient's decision on participation
- The possibility for the participant to withdraw their informed consent at any time after inclusion

If the participant wishes to participate, a new appointment will be booked where the consent is signed by the doctor and the participant. The new appointment and time of reflection will be within 4-7 days after the first appointment, but if the patient needs more time of reflection this can also be accommodated. If the patient wishes to sign the content at the first appointment this is also acceptable. If the patient fulfills all in- and exclusion criteria the patient will be included and receive treatment immediately after.

After inclusion, the participant will be informed,

- If additional risks, side effects, complications or other disadvantages are discovered during the trial
- If major changes are made to the protocol which might affect health or safety
- If other relevant health conditions are discovered during the trial so forth the participant has not expressed a wish not to receive such information
- About the results achieved and possible consequences to the patient so forth the participant has not expressed a wish not to receive such information
- If the trial is terminated before schedule, the participant will be informed of the reason

Information related to the participants will be protected under the legislation of processing of personal data and the health legislation, section 3 regarding patient's rights.
Patients will be receiving phone numbers to the "contact center" as well as the doctor on duty at the Department of Oncology. Patients are assigned a "contact doctor" and a "contact nurse" as well as instructions on how to reach these.

Study design

The study is a phase I study where 10 patients with metastatic solid tumors will be treated.

If 3 or more patients experience drug related grade 3-4 AE in phase I, the trial will be stopped; grade ≥ 3 for fatigue and skin related AEs. A minimum of 2 vaccines must be administered before inclusion of another patient for each of the 3 first patients included in the study. All patients will receive the same dose vaccine without dose escalation

Inclusion criteria

1. Age ≥ 18
2. The patient has metastatic solid tumors (NSCLC, colorectal cancer, urothelial cancer, breast cancer, ovarian cancer, malignant melanoma or HNSCC); progressive or recurrent disease on or following treatment with standard of care agents
3. At least one measurable parameter according to RECIST 1.1.
4. The patient has an ECOG performance status of 0 or 1
5. Life expectancy of at least 3 months
6. Prior PD1/PDL-1 allowed
7. The patient is a female of childbearing potential with negative pregnancy test
8. For fertile women*: Agreement to use contraceptive methods with a failure rate of < 1 % per year during the treatment period and for at least 12 weeks after the treatment. Safe contraceptive methods for women are birth control pills, intrauterine device, contraceptive injection, contraceptive implant, contraceptive patch or contraceptive vaginal ring.
9. For men: Agreement to use contraceptive measures and agreement to refrain from donating sperm
10. The patient has met the following hematological and biochemical criteria:

a. AST and ALT ≤2,5 X ULN or ≤5 X ULN with liver metastases
b. Serum total bilirubin ≤1,5 X ULN or direct bilirubin ≤ ULN for patient with total bilirubin level > 1,5 ULN
c. Serum creatinine ≤1,5 X ULN
d. ANC (Absolute Neutrophil Count) ≥1,000/mcL
e. Platelets ≥ 75,000 /mcL
f. Hemoglobin ≥ 9 g/dL eller ≥ 5.6 mmol/L

11. Mandatory provison of archival tissue and blood for biomarker testing at baseline
12. Mandatory provison of blood for biomarker testing during the study
13. Signed declaration of consent after oral and written information about the protocol

* A woman is considered of childbearing potential (WOCBP), i.e. fertile, following menarche and until becoming post-menopausal unless permanently sterile. Permanent sterilisation methods include hysterectomy, bilateral salpingectomy and bilateral oophorectomy. A postmenopausal state is defined as no menses for 12 months without an alternative medical cause[60]

Exclusion criteria

1. The patient has not recovered from surgery or is less than 4 weeks from major surgery
2. The patient has a history of life-threatening or severe immune related adverse events on treatment with another immunotherapy and is considered to be at risk of not recovering
3. The patient is expected to require any other form of systemic antineoplastic therapy or radiation therapy while receiving the treatment. However, radiation therapy treatment of non target lesion is allowed.
4. The patient has a history of severe clinical autoimmune disease

5. The patient has a history of pneumonitis, organ transplant, human immunodeficiency virus positive, active hepatitis B or hepatitis C
6. The patient requires systemic steroids for management of immune-related adverse events experienced on another immunotherapy
7. The patient has any condition that will interfere with patient compliance or safety (including but not limited to psychiatric or substance abuse disorders)
8. The patient is pregnant or breastfeeding
9. The patient is unable to voluntarily agree to participate by signed informed consent or assent
10. The patient has an active infection requiring systemic therapy
11. The patient has received a live virus vaccine within 30 days of planned start of therapy
12. Significant medical disorder according to investigator; e.g. severe asthma or chronic obstructive lung disease, dysregulated heart disease or dysregulated diabetes mellitus
13. Concurrent treatment with other experimental drugs
14. Concurrent treatment with Valproate or Xanthin Oxidase inhibitors
15. Known side effects to Montanide ISA-51
16. Any active autoimmune diseases e.g. autoimmune neutropenia, thrombocytopenia or hemolytic anemia, systemic lupus erythematosus, scleroderma, myasthenia gravis, autoimmune glomerulonephritis, autoimmune adrenal deficiency, autoimmune thyroiditis etc.
17. Severe allergy or anaphylactic reactions earlier in life

Evaluation before the start of treatment

The following parameters have to be performed within a month of the start of vaccinations (blood tests within two weeks):

- Medical history and clinical examination
- Performance status according to the ECOG scale
- Toxicity evaluation
- Concurrent medical intake
- Electrocardiogram

- Blood tests:
  - Hemoglobin, leukocyte differentiation count and platelets
  - Potassium, Sodium, Creatinine, albumin, uric acid, lactate dehydrogenase, Alkaline Phosphatase, Alanine transaminase (ALAT), amylase, bilirubin, ionized calcium, C Reactive Protein (CRP)
  - Hepatitis B, hepatitis C (IgG), HIV, HTLV-1(IgG), Epstein-Barr Virus EBV and Treponema.
- Pregnancy test: Women of childbearing potential must have a pregnancy test performed at the time of screening. This involves women who are not surgically sterilized or postmenopausal
- Diagnostic imaging: PET-CT, CT, or MRI scan of neck, thorax, abdomen, pelvis and optionally CNS and extremities depending on the cancer diagnosis.

Conduct of the study

Initial patient enrollment and treatment

A minimum of 2 treatments with the peptide vaccine have to be administered before inclusion of another patient for each of the first 3 patients included in the study.

1st patient	1st dose	2nd dose	3rd dose	4th dose	5th dose	6th dose	7th dose	9th dose	10th dose	
Safety evaluation			Go/no go for next patient							
2nd patient				1st dose	2nd dose	3rd dose	4th dose	5th dose	6th dose	
Safety evaluation						Go/no go for next patient				
3th patient							1st dose	2nd dose	3rd dose	
Safety evaluation before remaining patients' inclusion for remaining patients									Go/no go for remaining patients	
Dosing of remaining patients can start in parallel										x

Treatment plan and treatment schedule

Patients included in the protocol are treated with the ARG1-vaccine every third week for 48 weeks, whereupon no additional vaccinations will be given. In total, 16 vaccines will be administered. Each vaccine consists of 100 µg ARG1-18, 100 µg ARG1-19, and 100 µg ARG1-20 in 500 µl sterile water and 500 µl Montanide. In case of disease progression, patients are referred back to the oncology team, from which they were referred initially. Further treatment after disease progression will not be affected by participation in the study.Patients who receive all vaccines without progressive disease will have follow up appointments after 3 and 6 months in the experimental unit (EFEK) Herlev Hospital.

Detalied treatment and evaluation schedule

Treatment every third week Evaluation after every fourth vaccine						
Series no.	Screening	**1.**	**2.**	**3.**	**4.**	
Day:		1	1	1	1	21 days
Year: 20___						
Treatment						
ARG1-peptide vaccine		0	0	0	0	
Signed consent form	0					
Anamnesis	0					
Physical examination	0	0	0	0	0	
Hight and weight	0					
CTCAE 4.0	0	0	0	0	0	
ECOG Performance status	0	0	0	0	0	
BP, Puls, Tp.	0	0	0	0	0	
Mediaction intake	0	0	0	0	0	
Blood test						
Ipilimumab male/female*	0	0	0	0	0	
12 HER EKS		0				0
p-HCG (if relevant)	0					
MM1413/MM1414 screening*	0					
Tumor evaluation						
CT scan of the cerebrum	0					
CT/PET-CT/MRI scan	0					0
Other						
EKG	0					
Tumor biopsy	0					0
DTH						0

Series no.	**5.**	**6.**	**7.**	**8.**	
Day:	1	1	1	1	21 days
Year: 20___					
Treatment					
ARG1-peptide vaccine	0	0	0	0	
Signed consent form					
Anamnesis					
Physical examination	0	0	0	0	
Hight and weight	0				
CTCAE 4.0	0	0	0	0	
ECOG Performance status	0	0	0	0	
BP, Puls, Tp.	0	0	0	0	
Mediaction intake	0	0	0	0	
Blood test					
Ipilimumab male/female*	0	0	0	0	
12 HER EKS					0
p-HCG (if relevant)					
MM1413/MM1414 screening*					
Tumor evaluation					
CT scan of the cerebrum					
CT/PET-CT/MRI scan					0
Other					
EKG					
Tumor biopsy					
DTH					

Series no.	**9.**	**10.**	**11.**	**12.**	
Day:	1	1	1	1	21 days
Year: 20___					
Treatment					
ARG1-peptide vaccine	0	0	0	0	
Signed consent form					
Anamnesis					
Physical examination	0	0	0	0	
Hight and weight	0				
CTCAE 4.0	0	0	0	0	
ECOG Performance status	0	0	0	0	
BP, Puls, Tp.	0	0	0	0	
Mediaction intake	0	0	0	0	

Blood test					
Ipilimumab male/female*	0	0	0	0	
12 HER EKS					0
p-HCG (if relevant)					
MM1413/MM1414 screening*					
Tumor evaluation					
CT scan of the cerebrum					
CT/PET-CT/MRI scan					0
Other					
EKG					
Tumor biopsy					
DTH					

Series no.	**13.**	**14.**	**15.**	**16.**		**Evaluation**
Day:	1	1	1	1	21 days	1
Year: 20___						
Treatment						
ARG1-peptide vaccine	0	0	0	0		
Signed consent form						
Anamnesis						
Physical examination	0	0	0	0		0
Hight and weight	0					
CTCAE 4.0	0	0	0	0		0
ECOG Performance status	0	0	0	0		
BP, Puls, Tp.	0	0	0	0		
Mediaction intake	0	0	0	0		
Blood test						
Ipilimumab male/female*	0	0	0	0		
12 HER EKS					0	
p-HCG (if relevant)						
MM1413/MM1414 screening*						
Tumor evaluation						
CT scan of the cerebrum						
CT/PET-CT/MRI scan					0	
Other						
EKG						
Tumor biopsy						
DTH						

*Detalied blood test description

	MM1413/1414 Screening	Ipilimumab female	Ipilimumab male
Analysis	*B-Hæmoglobin*	*B-Hæmoglobin*	*B-Hæmoglobin*
	B-Trombocytter	*B-Trombocytter*	*B-Trombocytter*
	B-Leukocytter	*B-Leukocytter*	*B-Leukocytter*
	B-Leukocyttype	*B-Leukocyttype*	*B-Leukocyttype*
	P-Creatinin	*P-Creatinin*	*P-Creatinin*
	P-Natrium	*P-Natrium*	*P-Natrium*
	P-Kalium	*P-Kalium*	*P-Kalium*
	P-Calcium-ion	*P-Calcium-ion*	*P-Calcium-ion*
	P-Fosfat		
	P-Magnesium		
	P-ALAT	*P-ALAT*	*P-ALAT*
	P-ASAT	*P-ASAT*	*P-ASAT*
	P-Basisk Phosphatase	*P-Basisk Phosphatase*	*P-Basisk Phosphatase*
	P-Bilirubin	*P-Bilirubin*	*P-Bilirubin*
	P-Albumin	*P-Albumin*	*P-Albumin*
	P-LDH	*P-LDH*	*P-LDH*
	P-CRP	P-LH	P-testosteron
	P-APTT	P-FSH	
	P-Koag II-VII-X	P-Estradiol	
	P-Koag II-VII-X (INR)	P-Prolactin	
	P-TSH	*P-TSH*	*P-TSH*
	P-Frit T4	*P-Frit T4*	*P-Frit T4*
	P-Frit T3		
	HIVCOMBO	P-Carbamid	P-Carbamid
	P-HBVSAG	P-Hydrogencarbonat	P-Hydrogencarbonat
	P-HBVSAB	P-Albumin	P-Albumin
	P-HBVCAB	P-Urat	P-Urat
	P-HCVAB	P-Amylase	P-Amylase
	P-HTLVIGG	P-Glucose	P-Glucose
	P-EBV	P-Cortisol	P-Cortisol
	P-TREPONE		

Follow up

Patients who complete the treatment of vaccines for all 48 weeks will be followed in the specialized unit at EFEK 3 and 6 months after treatment.

Mont	**3**	**6**
Day:	1	1
Year: 20_____		
Toxicity evaluation and performance status	0	0
Clinical examination	0	0
Ipilimumab male/female	0	0
Project blood samples	0	0
Radiologic imaging with CT, MR or PET-CT scan according to cancer diagnosis.	0	0

Duration of treatment:

Treatment with the ARG1 peptide vaccine is maximum 48 weeks.
Treatment will be stopped if there is a confirmed disease progression according to the immune-related response criteria or by unacceptable toxicity

Concurrent treatment:
Vaccination with live virus vaccines are contraindicated during treatment and initiation treatment must be at least one month after any such vaccinations. Local palliative radiotherapy or surgery of solitary metastases can be performed during treatment.

Procedures regarding treatment
The vaccinations will be given at the Unit for Experimental Cancer Therapy Unit (EFEK) at the department of Oncology, Herlev and Gentofte University Hospital. EFEK is a unit specialized in conducting phase I first-in-human clinical trials. A 500 µl aqueous solution of 100µg ARG1-18, 100µg ARG1-19, and 100µg ARG1-20 peptide dissolved in sterile water is mixed to an emulsion with 500µl Montanide ISA-51 and given as a subcutaneous injection on the lateral side of the upper arm preceded by disinfection. The site of injection will alternate between the left and right arm for each vaccination.

During administration of the first 3 vaccines, the patient is observed for 30 minutes with measurements of pulse and blood pressure before and after the vaccine is given. The remainder of vaccines can be administered with observation of the patient for 15 minutes. Patients are observed for acute toxicities in the form of allergic reactions, including anaphylactic shock. Allergic reactions and/or anaphylactic shock will be treated per hospital guidelines.
The vaccines are ordered after contact to the project nurse at the clinical experimental unit (KFE) and to the manufacturing unit. (SOP: ”Bestilling og håndtering af ARG1-18,19,20 peptid vaccine til patienter i protokol AA1809”, appendix 14). When the vaccine is received, the “receipt of transport” is signed. (SOP: “Transport og udlevering af ARG1-18,19,20 peptid og Montanide, AA1809” appendix 15).

If the patient is prevented in attending the planned treatment day, the treatment can be moved to the following Tuesday or Thursday. If the treatment is postponed more than 6 weeks, patients will be excluded.

Criteria for treatment modifications

Delay of treatment

The treatment will be postponed if the patient experiences non-hematological AE grade ≥ 2, except fatigue and skin related AEs such as local reaction at the injection site. Otherwise the following criteria are applicable:

Blood samples	**Criteria**
Leukocytes	< 1,5 x 109 (grade 3)
Neutrophilic granulocytes	< 1,0 x 109 (grade 3)
Thrombocytes	< 50 x 109 (grade 3)
Hemoglobin	< 5 mmol/l (grade 3)
Creatinine	>3 x ULM (grade 3)
ASAT	>5 x ULN (grade 3)
ALAT	>5 x ULN (grade 3)
Bilirubin	>3 x ULN (grade 3)
Other AEs	
Skin-related AE	≥ grade 3
Fatigue AE	≥ grade 3
All other AE	≥ grade 2

The treatment will be postponed until CTCAE grade < 2.

Criteria for end of treatment

1. Unacceptable toxicity (see adverse events)
2. Disease progression
3. On patient request
4. Clinical judgement
5. Other anti cancer treatment. However, radiotherapy treatment of to non target lesions is allowed.
6. Pregnancy
7. Delayed treatment

Unacceptable toxicity:

If treatment related AE grade 3-4 is not reduced appropriately within 12 weeks, the treatment will be stopped and the patient is excluded. Shorter delays can occur multiple times with no consequences for the patient or the trial.

The patient will be excluded subject to the following toxicities:

Blood sample	**Criteria**
Leukocytes	< 1,0 x 109 (≥grade 4)

Neutrophilic granulocytes	< 0,5 x 109 (≥grade 4)
Thrombocytes	< 25 x 109 (≥grade 4)
Hemoglobin	< 4 mmol/l (≥grade 4)
Creatinine	>4,5 x ULM (≥grade 4)
ASAT*	>8 x ULN (≥grade 4)*
ALAT*	>8 x ULN (≥grade 4)*
Other Adverse Events	
Bronchospasms or hypersensitivity reactions	≥ grad 3
Skin related AE	≥ grad 4
All other AE	≥ grad 3
Any eye pain or reduced visual of grade 2 or above with no restitution within two weeks will lead to exclusion	

*ASAT/ALAT grade 3 with bilirubin > 1,5 x ULN
** Bilirubin > 3 x ULN with ASAT/ALAT > 2,5 x ULN

Disease progression: Treatment is terminated if disease progression occurs accorting to RECIST 1.1 at predefined evaluation time points.
Patient request: The treatment can be stopped at any time if the patient wishes so.
Clinical judgement: The treatment can be stopped because of medical conditions at any time if the investigator finds it in the patients' best interest.
Other treatment: Patients will be excluded at any time a new treatment with an experimental drug or other systemic anticancer treatment is initiated after inclusion in this protocol. The patient will be excluded if systemic treatment with corticosteroids is initiated unless it is on vital indication and in agreement with the physician responsible for the protocol. Supportive treatment is given on usual medical indication and is to be specified in the patient chart and flow sheets.
Pregnancy: A pregnancy test is to be performed before women of childbearing potential can be included in the study. Women of childbearing potential are to contact investigator immediately if pregnancy is suspected (absent or delayed menstruation etc.) at any point during the course of treatment and for 3 months after the end of treatment. A pregnancy test is performed if pregnancy is suspected and the participants can receive no more vaccination treatments and is terminated from the study if it turns out positive. A pregnant participant and the course of the pregnancy will be followed closely in the outpatient clinic.

Delayed treatment: The patient is terminated from the study if the treatment is delayed more than 6 weeks. It is the responsibility of the principle investigator to assess the cause of delay and define the time point for the start of the delay. A patient can be delayed several times during the cause of treatment without being excluded.

Early termination from the study

Participants, which are excluded from the study, will be referred back to the oncology team from which they came, for standard treatment. Patients that are excluded before the second vaccine will be replaced with new participants.

Concurrent treatment

- Supportive treatment is given based on clinical judgement and should be noted in the patient charge in accordance with existing practice
- Local palliative radiotherapy is allowed

Manufacture of the peptide vaccine

Peptide

For detailed description of peptide mixture production (see SOP "Fremstilling af ARG1-18,19,20 peptid til protokol AA1809" appendix 12.

The peptide sequence:

IO112	→	ARG 171-190;	AKDIVYIGLRDVDPGEHYIL	→ARG1-18
		ARG 181-200;	DVDPGEHYILKTLGIKYFSM	→ARG1-19
		ARG 191-210;	KTLGIKYFSMTEVDRLGIGK	→ARG1-20

Peptides will be provided with above 90 % purity from external producer. Before administration they will be dissolved in sterile water, mixed and sterile filtrated through a 0,22 µm (30 mm Ø) filter.

Producing the peptide vaccine

The final production of the peptide vaccine will take place under GMP approved laboratory conditions (approved by the Danish Medicines Agency) which quality assures a sterile therapeutic product. It will take place in JM702, Herlev and Gentofte Hospital, authority number: 24223.

Montanide ISA 51

Montanide ISA 51 is produced and sold by Seppic INC: www.seppic.com. France. Montanide is delivered sterile and ready for use in ampules of 3 ml. Montanide ISA 51/Vaccine adjuvant is based on vegetable oil. See product resume or link: http://www.seppic.com/human-health/vaccine-adjuvant/montanide-isa-51-@/1018/view-1042-seproduit.html

Preparing the vaccine
The peptide mixture and Montanide is delivered to the dept. of hematology shortly before use by a project nurse or laboratory staff from Hematologic Laboratory 54P4. The peptide mixture constitutes 500 µl consisting of 100 µg ARG1-18, 100 µg ARG1-19, 100 µg ARG1-20 dissolved in a watery solution. Montanide ISA-51 also constitutes 500 µl and just before injection, the two solutions are mixed through an I-connector Luer lock into a 1 ml syringe. The vaccine preparation is marked with patient name, personal security number, date and time of preparation.
For detailed description of the peptide preparation see SOP "Klargøring af ARG1-18,19,20 peptidvaccine til protokol AA1809."appendix 13. See also example of labels for the vaccine in "Eksempel på forsøgslægemiddeletiketter" appendix 06.

Microbiological control
Endotoxin and cultivation tests of the peptide preparations are performed at the Department of Clinical Microbiology, Herlev and Gentofte Hospital before use of the vaccines.

Evaluation

Data registration
The patients are given a patient number at inclusion in the study to secure patient anonymity. Clinical personal and selected persons in the laboratory will have access to patient information, but only in form of personal ID and name, to secure proper treatment.
The principal investigator has access to patient charts to obtain information regarding the cancer disease to be able to compare this information with the project specific analysis performed on cancer tissue and blood tests.
All relevant data is registered in case rapport form in collaboration with the clinical research unit (KFE). The principal investigator is responsible for manufacturing of the case report form (CRF) and data registration in the CRF after the course of treatment. CRFs will be reported to sponsor. Sponsor and principal investigator are responsible for data analysis on all included participants. Participant data and CRF will be kept for 5 years in accordance with current guidelines for storage of sensitive data. Completion of a final report will be done in collaboration between the members of the project group.

The analysis will include:
1. Adverse events registration
2. Immunological response
3. Clinical endpoints

Sensitive data and any other remaining samples will be anonymized at the end of the trial.

Following an approval from the Danish Data Protection Agency the Danish biotech company IO Biotech will gain access to pseudonymized trial data.

Monitoring

Monitoring of the trial will be done by the GCP unit at the University of Copenhagen. It is expected that the responsible GCP unit will contact and visit the principal investigator on a regular basis and request for access to monitoring of the different source documents in relation to the trial (Case Report Forms and other relevant documents).
Audit from the Danish Medicines Agency and other health authorities are likewise allowed on request. Monitor has access to laboratory test results and other participant information needed to verify the entries made in the Case Report Forms. The principal investigator (or his/her substitute) agrees to cooperate with the monitor so that potential problems discovered during the monitoring process will be solved.

Statistical considerations

The study is a phase I trial with the primary objective to evaluate safety of the combination therapy. Since the trial is a first in human study, a formal sample size calculation cannot be performed. From experience with similar studies, it is deemed appropriate to include 10 participants.
Ten participants are deemed sufficient to evaluate safety and considered safe if less than 20 % experience grade 3-4 AE.

All participants that are included and treated according to the protocol will be included in the statistical analyses. Participants who are excluded before receiving treatment will not be included in the statistical analyses:

- Where the inclusion criteria at baseline are not fulfilled
- Patients withdraw their consent
- Have started other treatment

For immunological responses, the 95 % confidential interval will be calculated for patient data and for response rates descriptive statistics will be used.

Effect parameters

Primary effect parameter

Primarily, safety and tolerability of the combination therapy is assessed. This is done by registration of all adverse events and possible unwanted events that occur in relation to the

treatment and in accordance with the CTCAE criteria. Data collection will take place in accordance with the data source list.

Secondary effect parameter

For the assessment of the immunological response, ARG1-specific T cells responses will be identified by the use of ELISPOT technology and antigen specific populations will be characterized for their cytokine secretion pattern.

We will further examine the effect of the vaccine in different subtypes of immune cells during therapy. Flow cytometry analysis will be performed prior to, during and after treatment. The numbers of different subtypes of immune cells will be registered and compared e.g. memory T cells, Treg, Natural Killer (NK) and MDSC.

Exploratory endpoints and objectives

The clinical effect of the treatment will be assessed by measure of tumor responses by standard radiologic imaging with CT, MR, PET-CT, or a MR scan according to cancer diagnosis. Before inclusion a recent scan must to be available for review. Before hospital admission baseline imaging will be performed. The evaluation will be evaluated according to RECIST 1.1 and PERCIST with the endpoints being objective response (OR), progression free survival (PFS) and overall survival (OS).

RECIST

Clinical evaluation will be done in accordance with RECIST 1.1 Guidelines[6] .

Complete response (CR): All lesions disappear.

Partial response (PR): Defined as ≥ 30% reduction in the sum of all measurable parameters' longest diameter.

Stabile disease: defined as < 30% reduction in the sum of all measurable parameters' longest diameter or a < 20% increase in the sum of all measurable parameters' longest diameter.

Progression: Defined as a > 20% increase in the sum of all measurable parameters' longest diameter or the appearance of new lesions.

CR and PR is to be verified by examination at a minimum of 4 weeks after documentation of the response at the earliest.

Immunologic monitoring

Project blood samples for immunological monitoring

110 ml blood will be sampled in Sodium-heparin prepared vials where mononuclear cells are isolated. 8 ml blood will be sampled in a dry medium for serum to be frozen. Plasma samples for gene analysis are made from 8 mL EDTA blood. Project blood samples will be taken 5 times the first year and thereafter at followup after three and six months.

Immune cells are isolated from the collected blood samples by use of leucosep/lymphopreb-technique and are frozen in a biobank for later analyses; immune assays on viable immune cells. Antigen specific immune reactivity will be tested by use of a panel of relevant immunological assays including ELISPOT, ELISA, proliferation assays, cytotoxic assays, intracellular staining (ICS), and multimeric staining of ARG1 specific CD8 T cells. Blood and serum samples are processed and stored at Center for Cancer Immune Therapy (CCIT).

Tumor biopsies

Biopsies from the available tumor lesions or involved lymph nodes will be performed before the 1st vaccine and after the 4th vaccine. Depending on the localization and accessibility, different types of biopsies will be performed. If the involved areas are not directly accessible, the biopsy will be conducted under sterile conditions via ultrasound.

Tumor biopsies will be used for explorative assays including:

• Immunoscore CR assay will be performed for quantification of CD3 and CD8 T cells in the core of the tumor for biopsy samples or in both the margin and the core of the tumor for resected tumors. This is done to asses "Immunoscores" predictive and prognostic value, which in turn will help select the patients who will benefit from the treatment and vice versa in the future.

• Immunoseek CR assay will be performed to detect CD8+ lymphocytes and PD-L1+ immune and tumor cells by a multiplexed IHC assay on the same FFPE slid if possible. Standardized Image analysis will allow quantifying cells by digital pathology with the support of a pathologist.

• IHC staining PD-1, will consist of positive PD-1 T cells quantification by digital pathology of a single FFPE biopsy slide.

• IHC staining ARG1-18,19,20 will consist of ARG1-18,19,20 protein quantification by digital pathology on a single FFPE biopsy slide.

Gene-based assays:

• Immunosign CR and Pan Cancer Immune profiling on NanoString platform will be used for testing patient sample biopsies for gene expression quantification of different immune genes.

• A whole exome sequencing performed on tumor samples and corresponding healthy tissues on pre-treated patient samples to assess initial mutational status for each patient.

The aim of the planned gene analyses is to learn more about;

1. Differences among patients in expression level of a panel of relevant normal genes in the tumor microenvironment which could influence the chance of benefit from treatment.
2. Expression of tumor/patient specific mutated genes which could influence the chance of benefit from treatment.

Analysis for identifying specific tumor gene expression signatures and mutations in the tumor cells, leading to patient-specific neo-antigens derived from these mutations will be performed.

These analyses will contribute to identification of patients that are most likely to respond to treatment.

Methods in gen-based assays

A whole exome sequencing will be performed on tumor samples and corresponding healthy tissues on pre-treated patient samples to assess initial mutational status for each patient.

This testing will be performed on Illumina NGS platform per batch of 14 samples.
Total genomic DNA will be extracted from fresh frozen biopsy or from 1 FFPE biopsy block to prepare 1 FFPE slide biopsies of 20µm. The best would be to have fresh frozen biopsies.
If a biopsy of healthy tissue cannot be provided then genomic DNA will be extracted from 5ml whole blood.

Data regarding potential disease causing genes will be generated as a byproduct of this analysis, but this data will not be used or explored further upon, since only data regarding tumor specific genes will be processed more closely. Therefore, we do not expect to obtain explicit knowledge regarding disease causing genes. Furthermore, 'Targeted sequencing' on a limited number of defined genes will be performed on the tumor tissue to obtain a 'immune profile' to determine which genes- and consequently which proteins- are expressed in the tumor tissue. Data will be handled according to national laws.

If by chance these analyses will discover known mutations with potential significant impact on patient's health, the case will be discussed with the Clinical Genetics Department, Rigshospitalet, unless the patient has chosen not to be informed as stated in the patient information. If the patient wishes genetic counseling it will be offered by specialized doctors, if mutations with potential significant impact on the patient's health are discovered. The following criteria will determine if further actions are indicated.

- There is a reasonable degree of possibility that a genetic disposition is present,
- There is solid documentation of a link between the genetic disposition and the development of disease,
- The tests used to determine the genetic disposition are well established,
- The disease in question can be prevented or treated, and
- The link between the genetic disposition and the development of disease has considerable importance for the patient.

If indicated, the patient will be contacted and asked for permission to referral to such Department for additional information and testing.

In case that a patient dies/is dead, or do not want information regarding significant health issues, a medical assessment, using the 5 above mentioned criteria, will determine whether relatives to the patient is to be informed, in accordance with Danish law (sundhedslovens § 43, stk. 2, nr.2.).

Delayed type hypersensitivity (DTH) & biopsy

Delayed type hypersensitivity will be tested after the 4th vaccination. The test consists of up to a total of 3 intradermal injections of either an aqueous solution of ARG1 or the negative control saline. After 48 hours, the diameter of the reaction is measured, and 6mm punch biopsies are taken from the injection sites. The biopsies are divided in two, half is frozen for later investigation, half is used to grow skin-infiltrating lymphocytes (SKILs). SKILs are tested for specificity to the ARG1 peptides as a sign of induction of a functional immune response. The patient can refuse to undergo the DTH-testing without exclusion of the trial.

Biobank and handling of biologic material

In connection with the current study, blood samples (110 ml/blood sample) and tumor biopsies will be stored in coded form at -150 °C in a research biobank at the CCIT in room PA102 until all analysis concerning the study is performed. Blood samples will be taken during the 48 week treatment period and thereafter at the followups after three- and six months. The material is hereafter transferred to a biobank for future biomedical research for up to 15 years if accepted by the Danish Data Protection Agency, after which the remaining material will be destroyed.

Analyses will be primarily performed at CCIT. However, some special analyses on tumor tissue or blood test samples will be performed at a partner institution after establishing a specific written agreement. All patient relevant information will be sent in an anonymous way. In case the patient's cells will be sent to partner institutions located abroad, these will be handled according to national laws and regulations of the specific nation where these have been sent. In such a case, all patient information will be communicated in coded form. A written Data Processing Agreement will be signed between the data controllers and the data processors abroad. If any gene analysis are to be performed abroad, the data processing agreement will include the 5 criteria (See immunologic monitoring) regarding the discovery of known mutations with potential significant impact on patient's health, as well as the requirement, that the partner abroad reports back to the primary project managers in Denmark so that relevant actions can be taken. If data processing is to be performed in a third-country, permission will be applied for at the Danish Data Protection Agency, or one of the agencies standard contracts will be used.

Samples that are not used in the study will be transferred to a biobank at CCIT for future biomedical research for up to 15 years and if accepted by the Danish Data Protection Agency. If additional studies in other research areas are to be performed on any samples obtained during the conduct of this study/samples are transferred to a new biobank, a request to do so will be

submitted to the Scientific Ethics Committee, Capital Region of Denmark, as per the 'Act of Processing of Personal Data §§ 5 and § 10, paragraph 2 and 3. After 15 years, any remaining tissue samples will be disposed of according to the local guidelines for destruction of biohazardous waste.
If a patient withdraws his/her informed consent, all biological material is to be destroyed if the patient wishes so.

Risk evaluation

Evaluation of potential risks associated with the peptide vaccines are based on clinical experiences from other cancer vaccination trials.

Acute reactions

Subcutaneous injections can in theory cause anaphylactic reactions as any other vaccine, but so far it has not occurred for cancer vaccines.
The kinetics of immune induction by cancer vaccines is slow, - that is weeks to months as compared to check point inhibitors.

Autoimmunity

There is a small theoretical risk of inducing autoimmunity when/if the vaccine eliminates the immunosuppressive arginase positive cells because arginase is a self-antigenpresent in various cell types in both unvaccinated healthy individuals as well as cancer patients.
However, all previous trials with peptide vaccinations have had very mild side effects, no matter which proteins have been targeted, as reviewed by Rahma and colleagues [58] .
The ARG1 vaccine is in many ways similar to the IDO vaccine tested at CCIT, and there are no apparent reasons the toxicity should be higher when vaccinating against ARG1. Both ARG1 and IDO are self-proteins with a broad expression. Both molecules are lymphocyte-suppressive they are all are overexpressed in cancer cells and immunosuppressive cancer-associated immune cells. The adverse events in our trials with IDO vaccination have been at as low a level as with other peptide vaccines; in the study by Iversen et al, no grade 3-5 toxicities were seen [2].
A phase I trial treating cancer patients with an oral ARG1 inhibitor (CB-1158) presented data on adverse effects in April, 2017. A total of 17 patients with advanced solid tumors have been treated with CB-1158, and the drug was generally well tolerated with no drug-related serious adverse events. Treatment related adverse events were limited to a single case of Grade 1 anemia, fatigue, increased ALT and myalgia. No Grade 3 treatment related adverse events were reported. Plasma levels of arginase were inhibited >90% in all patients, and in 10 of 11 patients plasma arginine increased 1.5 fold or more[3]. As mentioned earlier (see Assement of safety) the ARG1 peptide vaccine and CB-1158 differ in both mechinsm of action and administration way, making them very hard to compare. However, they both target the same enzyme and the AEs registered for patients trateted with CB-1158 could possibly indicate what AEs to expect in the ARG1 trial.

Tolerance
In contrast to autoimmunity there is also a theoretical risk of tolerance development leading to increased immune suppression and tumor tolerance and thus increased disease aggressiveness. However, this phenomenon is purely theoretical and the eventual risk is unknown.

Procedures and examinations
Patients included in the trial will have additional blood tests performed compared to patients receiving standard treatment. The amount of blood and the specific procedure when taking a blood sample are not associated with any significant risk. There is a minimal risk of bleeding and infection and pain and bruising can occur in the area.

Tumor biopsies will be taken by experienced doctors, and is not associated with any significant risk; again there is a small risk of bleeding and infection and pain and bruising might also accur in the area.

CT scans and PET-CT scans are not considered a risk based on the low dose radiation. Both full body CT and PET-CT scans give a radiation doses of around 15 milliSievert (mSv), which is about 5 times the annual background radiation[61,62]. The contrast used can very rarely cause allergic reactions and brief reduction in renal function; therefore patients are screened by standard procedures prior to the examination.

Monitoring of toxicity

Adverse events
Adverse events (AE) are defined as any undesirable experience occurring to a subject during a clinical trial, whether or not considered related to the investigational treatment. All AEs reported spontaneously by the subject or observed by the investigator or his staff will be recorded and described in the patient chart and the electronic Case Report Form (eCRF). The severity and consequences will be recorded for each AE. The severity and relation to the study medication will be assessed in accordance with the guidelines described in the following.
The investigator must attempt to identify all clinical and objective events from patients receiving treatment and determine their relation to the study medication. The investigator determines the relationship between AEs and treatment using the following guidelines:

Grading of adverse events
The severity of an AE refers to the intensity of the reaction.

Events are graded using CTCAE version 4.0. The following scale can be used if this grading is not applicable:

1 = light
2 = moderate
3 = severe
4 = life threatening
5 = lethal

Patients experiencing AEs will be monitored with the relevant clinical evaluations and laboratory investigations assessed by the attending physician. All AEs must be monitored until satisfactory restitution or stabilization. Results of the monitoring must be recorded in the patient chart and eCRF.
Abnormal laboratory tests are not to be recorded in the eCRF unless they have caused a clinical event, resulted in termination of the treatment or otherwise meet the criteria of a serious AE (see the following).

Serious adverse events
A serious adverse event (SAE) is to be reported to sponsor within 24 hours and is defined as any medical occurrence or effect that occurs at any dose:

- results in death;
- is life threatening (at the time of the event);
- requires hospitalization or prolongation of existing patients' hospitalization;
- results in persistent or significant disability or incapacity;
- leads to a congenital anomaly or birth defect;
- is a significant medical event

Adverse reactions
An adverse reaction (AR) can be expected if described in the IMPD or the relevant product summary, or unexpected if the grade or severity does not correlate with the product information in the before mentioned documents.
If the AR is unexpected, meets the criteria of a serious adverse reaction (SAR) and is found related to the investigational treatment, it is classified as a suspected unexpected serious adverse reaction (SUSAR). As this is a First in human trial, the SARs are difficult to classifiy as "expected" or "unexpected." Therefore, SARs will be reported as SUSARs.

Reporting of adverse events and adverse reactions
Investigator reports SAEs, SARs and SUSARs to sponsor within 24 hours. Sponsor reports SARs and SUSARs to the Danish Medicine Agencywithin 7 days if considered life threatening or fatal, and otherwise within 15 days. Consequences for the study must be reported. Sponsor submits a list annually that summarizes any SARs and SUSARs as well as a report regarding the study patients' safety to the Danish Medicine Agency and the Research Ethics Committee (investigator can report to the Research Ethics Committee as well).
Sponsor submits a final report to the Danish Medicine Agency at the end of the study, with a description of all SAEs, SARs and SUSARs.

The following is not to be reported:

- Deaths caused by the malignant disease or progression
- Hospitalizations or prolongation of current hospitalization caused by the malignant disease:
  - weight loss
  - fatigue
  - electrolyte derangement
  - pain management
  - anxiety
  - palliative hospitalization
  - stay at hospice or terminal care
  - progression of the underlying disease

- Hospitalizations or prolongation of current hospitalization if the sole reason for hospitalization or prolongation is:
  - fluid treatment or treatment of nausea
  - blood transfusion
  - platelet transfusion
  - febrile leucopenia/neutropenia
  - administration of investigational procedures
  - placement of a permanent intravenous catheter

These events are to be registered in the eCRF.

Known adverse reactions
Montanide ISA-51:
Montanide ISA-51 has been used in more than 150 clinical trials. Montanide has a well-documented safety-profile. Frequently reported side effects after injection are flu-like symptoms (headache, chills, fever and nausea) while most common local reactions are granuloma, local pain,

inflammation or erythema. These reactions are generally classified as mild to moderate and they are typically transient in nature.
Reference is made in attached drug information leaflet (appendix 11).

Arginase peptide:
The peptide has not been tested in humans, so no adverse reactions have been documented.

Handling adverse events

Autoimmune side effects will be handled according to a local guideline for handling side effects for checkpoint inhibitors based on recommendations by Weber et al[63]. This guideline is in Danish and named "Vurdering og håndtering af bivirkninger relateret til behandling med ipilimumab, nivolumab og pembrolizumab" and is available online and in print for the physician at duty. In the highly unlikely case that the vaccine induces a cytokine release syndrome, also known as a cytokine storm, the patient will be treated in accordance with the guideline on treatment of cytokine release syndrome recently published by Brudno et al[64].

Patients will be admitted at the Department of Oncology, Herlev Hospital, in case of occurrence of adverse events related to the investigational treatment that requires hospitalization.
The intensive care unit at Herlev Hospital will be informed of the study, since intensive care support could potentially be required if a treated patient develops anaphylactic shock. The information will be given prior to the start of patient inclusion in the protocol and treatment will not begin before the department management at the intensive care unit has confirmed that the information is received.

Insurance

Patients' participation in the study will be covered by the hospital's liability insurance.

Ethical aspects

This project will potentially add to existing knowledge in the field of immune therapy and help improve treatments and thus prognosis of prognosis of metastatic solid tumors.
There does not seem to be unacceptable risks or disadvantages regarding the planned treatment based on the current knowledge on peptide vaccines (see peptide vaccine safety).

Participation is voluntary and is preceded by oral and written information and the treatment will be stopped in case of unacceptable adverse reactions or if the patient wishes so at any time. The patient will receive treatment after the current guidelines at the department or referring department if he/she does not wish treatment according to the protocol. The study is therefore assessed as ethically proper.

The study follows the Helsinki agreement and the principal investigator is to obtain permission from the Danish Medicine Agencyand the Research Ethics Committee.

Reporting to the Danish Data Protection Agency

The study is reported to the Danish Data Protection Agency when approvals from the Danish Medicine Agency and the Research Ethics Committee are obtained. The law dealing with personal data will be respected. Information related to study patients is protected according to the law on personal data and the Act on Research Ethics Review of Health Research Projects.

End of study report

Sponsor will inform the Danish Medicine Agency and Research Ethics Committee within 90 days of study completion. The definition of study completion is 6 months after the last patients' treatment or after exclusion due to progression. If the study is prematurely terminated, the Danish Medicine Agency will be informed of the reason(s) for the termination. Sponsor will submit a final study report to the Danish Medicine Agency and the Research Ethics Committee with the study results including publications based on the study within a year of study completion.

Publications

Cathrine Lund Lorentzen, Mads Hald Andersen and Inge Marie Svane constitute the group responsible for the project.
Under the circumstance that the Vancouver rules are met, the group responsible for the project holds equal rights to the achieved results. The use and presentation of the data in any form, orally or written, can only take place with accept from everybody in the group. Positive, negative and inconclusive results will be reported in scientific journals. The order of authorship is determined by effort and the investigators will be co-authors on further publications derived from this study. The main analysis will be done when all patients have been treated which is expected to be during the year 2020/21.

Economy

The trial is fully financed. Funding has been acquired partly through the Department of Oncology and through IO Biotech ApS. IO Biotech is a biotech company established in the light of peptide vaccine patents from CCIT and "Region Hovedstaden". Lundbeck, Novo and Sunstone have invested in IO Biotech. The project is co-funded through a research agreement between the capital region and IO Biotech with 2.500.000 Danish crowns over 3 years. The money will be transferred to a research account with a doctor salary of 3 years and 700.000 Danish crowns for immune analyses for the vaccinated patients. IO Biotech also finances the vaccine products, while the remaining expenses are covered by CCIT and the department of oncology.

The project has been initiated by Mads Hald Andersen, Inge Marie Svane and Cathrine Lund Lorentzen

The ARG1 vaccine is developed by Mads Hald Andersen who is a part of the management at the CCIT. By Danish law on public inventions at public institutions, the capital region holds the patent, which is licensed for commercialization through the industrial partner IO Biotech. In the case of a sale of the patent, by law the income will be distributed with 1/3 for the inventor, 1/3 for CCIT, 1/6th to Herlev and Gentofte Hospital and 1/6th to Tectra, the technology transfer unit of the capital region.

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