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SERMENT DE GALIEN

En présence des Maitres de la Faculté, je fais le serment :

- D'honorer ceux qui m'ont instruit(e) dans les préceptes de mon art et de leur témoigner ma reconnaissance en restant fidèle aux principes qui m'ont été enseignés et d'actualiser mes connaissances

- D'exercer, dans l'intérêt de la santé publique, ma profession avec conscience et de respecter non seulement la législation en vigueur, mais aussi les règles de Déontologie, de l'honneur, de la probité et du désintéressement ;

- De ne jamais oublier ma responsabilité et mes devoirs envers la personne humaine et sa dignité

- En aucun cas, je ne consentirai à utiliser mes connaissances et mon état pour corrompre les mœurs et favoriser des actes criminels.

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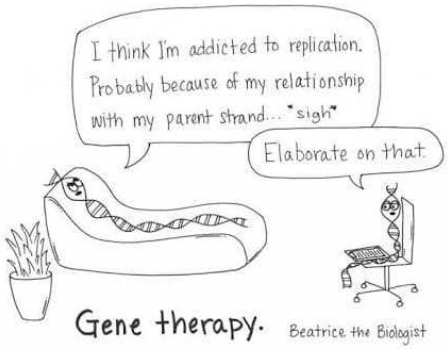

PLAN

Liste d'abréviations

2D / 3D	2 Dimensional / 3 Dimensional	EGF	Epidermal growth factor
5-FC	5-Fluorocytosine	EIAV	Equine infectious anaemia virus
5-FU	5-Fluorouracil	EMA	European medicines agency
AAV	Adeno-associated virus	EMT	Epithelial-mesenchymal transition
AcMNPV	Autographa californica multiple nucleopolyhedrovirus	EpCAM	Epithelial cell adhesion molecule
Ad	Adenovirus	EPR	Enhanced permeation retention
ADCC	Antibody-dependent cell-mediated cytotoxicity	ESC	Embryonic stem cell
ADME	Administration, Distribution, Metabolism, Elimination	EU	European union
ALL	Acute lymphoblastic leukaemia	FDA	Food and drug administration
AMM	Autorisation de mise sur le marché	FIV	Feline immunodeficiency virus
APC	Antigen presenting cell	G phase	Gap phase
apoE	Apoprotein E	GalNac	N-acetylgalactosamine
ASO	Antisense oligonucleotide	GCP	Good clinical practice
ATCC	American type culture collection	GCV	Ganciclovir
ATMP	Advanced therapy medicinal product	GEM / GDA	Genetically-engineered mouse / GEM-derived allograft
ATP	Adenosine triphosphate	GLP	Good laboratory practice
ATRA	Tretinoin	GM-CSF	Granulocyte/macrophage-colony stimulating factor
BCR-ABL	Breakpoint cluster region - Abelson	GMO	Genetically modified organism
BiKE	Bi-specific killer engager	GTMP	Gene therapy medicinal product
bp	Base pair	GVHD	Graft-versus-host disease
BPDS	Biolistic particle delivery system	H-1PV	Parvovirus H1
BV	Baculovirus	HDAd	Helper-dependant adenovirus
CA 19-9	Carbohydrate antigen 19-9	HER2	Human epidermal growth factor receptor 2
CAGR	Compound annual growth rate	HEV	High endothelial venule
CAR-T	Chimeric antigen receptor -T cell	HPV	Human papillomavirus
cATMP	Combined ATMP	HSCT	Haematopoietic stem cell transplantation
CD--	Cluster of differenciation	HSV	Human simplex virus
CDP	Cyclodextrin polymer	hTERT	Human telomerase reverse transcriptase
CEA	Carcinoembryonic antigen	HV	Helper virus
CHMP	Committee for medicinal products for human use	I phase	Interphase phase
CML	Chronic myeloid leukaemia	ICH	International conference of harmonisation
CMV	Cytomegalovirus	ID	Intradermal
CRISPR-CAS9	Clustered regularly interspaced short palindromic repeat-CAS9	IFN	Interferon
CRS	Cytokine release storm	IFP	Interstitial fluid pressure
CTC	Circulating tumour cell	IGF	Insuilin-like growth factor
CTCAE	Common terminology criteria for adverse effects	IL	Interleukin
ctDNA	Circulating tumour DNA	IM	Intramuscular
CTIS	Clinical trials information system	IRES	Internal ribosome entry site
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4	iRNA	Interference RNA
CTV	Cancer terminator virus	IT	Intratumoral
DAMP	Damage-associated molecular pattern	ITR	Inverted terminal repeat
DC	Dendritic cell	IV	Intravenous
DCK / UMK	Deoxycytidine kinase /Uridine monophosphate kinase	KHz	Kilo-Hertz
DLT	Dose-limiting toxicity	KRAS	Kirsten rat sarcoma
DNA	Deoxyribonucleotide	LH-RH	Luteinizing hormone – releasing hormone

DPC	Dynamic polyconjugates	LNGFR	Low-affinity nerve growth factor receptor
dsDNA	Double stranded DNA	LNP	Lipid nanoparticle
EE1A1	Eukaryotic translation elongation factor 1 alpha 1	PSCA	Prostate stem cell antigen
LTR	Long terminal repeat	RP2A	Highest dose with acceptable toxicity
M phase	Mitosis phase	rAAV	Recombinant AAV
MAA	Market authorisation application	RECIST	Response evaluation criteria in solid tumours
MCTS	Multicellular tumour spheroid	RISC	RNA-induced silencing complex
mda	Melanoma differenciation associated gene	RNA	Ribonucleic acid
miRNA	Micro-RNA	ROR2	Receptor tyrosine kinase-like orphan receptor 2
MCNP	Membrane/core nanoparticles	RSV	Respiratory syncytial virus
MRI	Magnetic resonance imagery	RXR	Retinoid x receptor
mRNA	Messenger RNA	S phase	Synthesis phase
MSC	Mesenchymal stem cell	SBRT	Stereotactic Body Radiation Therapy
MTD	Maximum tolerated dose	SC	Subcutaneous
MUC-1	Mucin-1	SCID	Severe combined immunodeficiency
MVA	Modified vaccinia ankara virus	sCTMP	Somatic cell therapy medical products
MYXV	Myxoma virus	sgRNA	Single guide RNA
N/A	Not Applicable	shRNA	Short hairpin RNA
NEK2	NIMA Related Kinase 2	siRNA	Small-interfering RNA
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells	SME	Small and medium-sized enterprises
NG	Nanoghost	SNALP	Stable nucleic acid-lipid particles
NGS	Next generation sequencing	ssDNA	Single stranded DNA
NIH	National institute of health	sst2	Somatostatin receptor subtype 2
NK	Natural killer cell	SV40	Simian virus 40
NKT	Natural killer T cell	TAA/TSA	Tumour-associated antigen / Tumour-specific antigen
NOD / SCID	Nonobese diabetic	TALE / TALEN	Transcription activator-like effectors / Nucelase
OS	Overall survival	TEPs	Tissue Engineered Products
OV	Oncolytiv virus	Tf	Transferrin
PAMP	Pathogen-associated molecular pattern	TIR	Terminal inverted repeat
PC	Pancreatic cancer	TLR	Toll-like receptor
PCR	Polymerase chain reaction	TME	Tumour microenvironment
PD-1	Programmed cell death protein 1	TNF	Tumour necrosis factor
PDAC	Pancreatic ductal adenocarcinoma	TriKE	Tri-specific killer engager
PDGF	Platelet-derived growth factor	UBC	Ubiquitin C
PD-L1	Programmed cell death ligand 1	USA	United states of america
PDX	Patient-derived xenograft	UTMD	Ultrasound targeted microbubble destruction
PDX-1	Pancreatic and duodenal homeobox 1	VEGF	Vascular endothelial growth factor
PEG	Poly(ethylene-glycol	VSV	Vesicular stomatitis virus
PEI	Polyethylenimine	WHO	World health organisation
PET	Positron emission topography	WMA	World medical association
PFS	Progression-free survival	yCD	Yeast cytosine deaminase
POC	Proof of concept	ZFP / ZFN	Zinc finger protein / Nuclease
PRR	Pattern recognition receptor		
PSA	Prostate specific antigen		
lncRNA	Long non-coding RNA		

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I. INTRODUCTION

Le cancer reste aujourd'hui un problème de santé au niveau mondial, étant la deuxième cause de mortalité après les maladies cardiovasculaires. Avec les progrès technologiques, la caractérisation de l'initiation, la progression et la formation de métastases liées à la cancérogénèse évoluent également. Parallèlement, les recherches scientifiques évoluent afin d'améliorer les possibilités de diagnostiques précoces et de thérapeutiques efficaces. Parmi ces avancées, dans l'ère de la génétique, se situe les médicaments de thérapie innovante et plus particulièrement, les produits de thérapie génique.

La thérapie génique constitue des traitements à base de matériels génétiques et de moyens de transferts diverses et variées afin de trouver la meilleure construction thérapeutique pour combattre le cancer visé. De multiples transgènes et vecteurs ont été développés et continuent à progresser pour permettre d'adapter au mieux le traitement à la pathologie suivant l'actualité de la médecine personnalisée. Le cancer, décrit par *Hanahan* [1] comme étant une maladie multifactorielle, les outils utilisés pour y remédier doivent l'être aussi, plaçant la thérapie génique dans un arsenal de traitements à combiner dans les protocoles anti-cancer, telles que la chirurgie, la radiothérapie, la chimiothérapie et les thérapies ciblées et immunes.

Cela dit, beaucoup d'incertitudes et de questions se posent autour des médicaments de thérapies innovantes notamment sur l'emploi et le devenir de ces thérapies lors de l'usage humain. C'est pourquoi les autorités administratives comme l'agence Européenne du médicament (EMA) travaillent en lien avec les états membres, telles que la France, afin de permettre le meilleur accès, l'efficacité et la sécurité de ces traitements chez les patients qui en ont besoin.

La recherche de thérapies innovantes comme la thérapie génique est d'autant plus indispensables pour affronter les cancers mal pris en charges par les traitements actuels. Ceci est notamment le cas du cancer du pancréas, marqué par un taux de mortalité s'approchant à celui de l'incidence et qui est estimé à être le 2^{e} cancer le plus

mortel dans les pays occidentaux d'ici 2030. En analysant les essais cliniques en cours pour les médicaments de thérapie génique dans l'indication du cancer du pancréas, il serait possible d'élucider l'application des techniques de thérapie génique développées à cette pathologie. Ceci pourrait nous indiquer non seulement les méthodes privilégiées dans ce cancer mais pourrait aussi mettre à jour les potentiels traitements à arriver prochainement sur le marché mondial.

II. CURRENT SITUATION IN CANCER, TREATMENTS AND GTMPS

Cancer epidemiology

Cancer is a worldwide major health problem being the second most common cause of death, behind cardiovascular diseases. According to the World Health Organization (WHO),[2] there were over 18 million new cases of people suffering from cancer worldwide with a mortality of 9.5 million deaths in both sexes and all types of cancer. This incidence is estimated to grow to 29.5 million people in 2040 with 16.4 million cases of cancer-associated deaths.

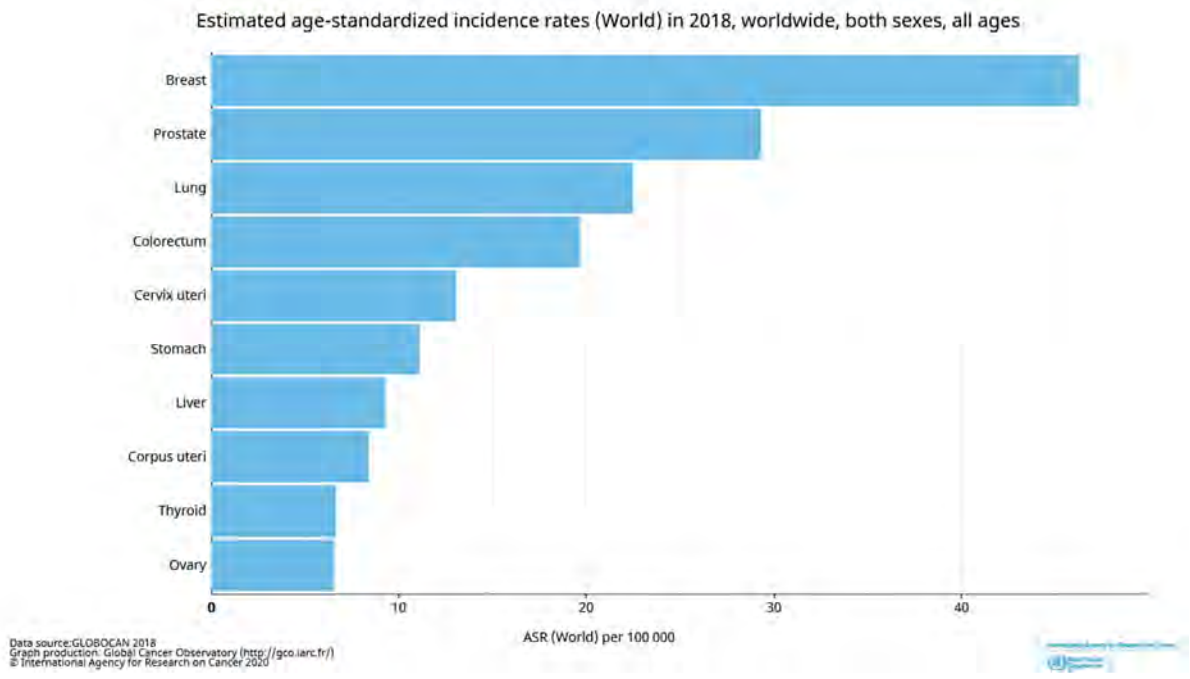


Figure 1: Graph of highest world incidence rates divided in cancer types [2]

The American Cancer Society depict the most common cancers in 2019 to be prostate, lung and colorectal cancers accounting for 42% of all cancers in men, with prostate cancer being nearly one fifth of all new diagnoses.[3]

In Western countries, the cancer incidence rate has reduced by approximately 2% per year over the last ten years in men, all cancer types confused. Unexpectedly, this drop is the result of a decrease in prostate specific antigen (PSA) testing - previously used as a routine screen for prostate cancer - and therefore avoiding over-diagnosis and overtreatment.

In parallel, incidence rates in women has remained generally stable over the last decades. Certain cancers in women such as lung cancer and colorectal cancer have seen a reduction in incidence explained by the unravelling of risk factors, like reduced/ceased tobacco uptake and increased precocious diagnoses through colonoscopy, respectively. On the other hand, breast cancer cases are on the rise, possibly linked to the increase in obesity in Western countries.

Liver cancer has the fastest grossing incidence rate in both men and women with a majority of cases considered as preventable through modifying risk factors (obesity, alcohol consumption, cigarette smoking and hepatitis B and C viruses).

For the most common cancers (apart from uterine cervix and corpus cancers), survival has improved over the last fifty years. For some, such as breast and prostate cancer, this is partly due to a reduction of lead time bias through the evolution of detection practice. Progress in other cancers is reflected by the progress in treatment protocols. This is indeed the case for hematopoietic and lymphoid malignancies and the discovery of targeted therapies. For example, chronic myeloid leukaemia has seen an increase in 5-year survival rates from 22% in the mid-1970s to 69% for patients diagnosed between 2008 and 2014, with nearly normal life expectancies for people treated by tyrosine kinase inhibitors.

Contrary to most cancers, lung and pancreatic cancers have had slower advances, mainly because a majority of cases are diagnosed at a distant stage in the disease.

After a peak of cancer mortality in 1991 in developed countries, there has been a steady decrease of about 1.5% per year in cancer-associated deaths. This decline has

been steeper in men than in women resulting in a larger number of averted deaths in the male population. The reduction in cancer mortality is a result of a reduction of identified risk factors (e.g. smoking) in addition to early detection and treatment, and advances in therapeutic options.

Between 2012 and 2016, however, cancer associated deaths rose for cancers of the liver, pancreas, uterine corps, brain and nervous system, soft tissue as well as the oral cavity and pharynx amalgamated to the Human Papillomavirus (HPV).[4]

Disparities in cancer incidence and mortality rates occur according to socioeconomic statuses, race and ethnicities and geographical locations. Furthermore, variations exist according to a patient's age. Indeed, leukaemia is the most common childhood cancer accounting for 28% of all cases. Even though overall child cancer incidence has been on the rise, death rates have continued to decrease with survival rates varying significantly between cancer types and age at diagnosis. Remission rates in childhood acute lymphocytic leukaemia have increased to 100%, this improvement being due to the optimization of chemotherapies rather than the development of new therapies.

Although socioeconomic inequalities in cancer control could be reduced through an increase of access to basic health care and interventions in developing countries, cancer remains the second leading cause of deaths in Western countries, behind heart disease.

Cancer is a disease linked to an uncontrolled proliferation of certain cells in the organism, presenting multiple abnormal characteristics, named “Hallmarks of cancer” and which are continuously being updated. These include – but are not restricted to – an unlimited capacity of division, eluding growth suppressors, continued proliferation, resistance to cell death, the capacity of neo-angiogenesis and capacity of invasion, metabolism rewiring, altered signalling pathways and metastasis.[1][5]

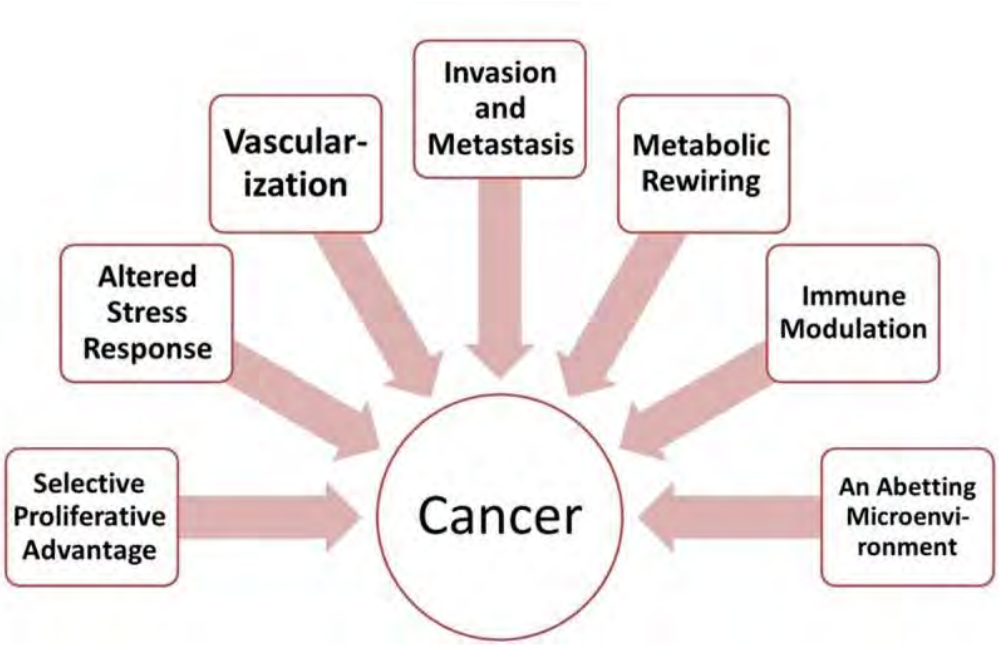


Figure 2: The hallmarks of cancer revisited [5]

Multiple therapeutic strategies exist when faced with cancer. Here is a brief overview of the main therapeutic options:

Surgery

Surgery can be curative or preventive as well as used in diagnosis (biopsies) or exploratory surgery (laparotomy or laparoscopy).[6] It is the oldest treatment for cancer

and remains today the best therapeutic option in certain cases. There are, however, risks accompanying surgical procedures, including the shedding of cancer cells into the circulation increasing the risk for metastases[7] as well as associated morbidity.

Radiotherapy

Radiotherapy uses external ionising radiation to destroy the tumour. The term brachytherapy is used to designate radioactive implants surgically placed inside a tumour. Used in the treatment of certain cancers such as tongue, pharynx, breast or prostate, brachytherapy allows the delivery of high doses of radiation while ineffective on the environing healthy tissue.[8]

Hormonotherapy

Hormonotherapy is used to correct hormonal imbalances found in certain hormone-dependant cancers, for example by reducing oestrogen expression in breast cancer,[9] to stop either intrinsic cancer cell proliferation and/or growth factor secretion to blunt autocrine loops. This is done by using either oestrogen antagonists such as Tamoxifen or by reducing oestrogen synthesis through anti-aromatases. Another hormone-dependant cancer such as prostate cancer can be treated with androgen receptor blockers or LH-RH (luteinizing hormone – releasing hormone) agonists which reduce testosterone synthesis.[10]

Immunotherapy

Immunotherapy consists in reinforcing the patient's immune system with immunostimulants (interferon alpha or interleukin 2 for example). More recently immunotherapy consists in drugs against PD1 (programme cell death 1), PD-L1 (programmed death ligand 1) or CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), which are inhibitory immune checkpoints overexpressed in certain cancers, such as melanoma, lung cancer or Hodgkin's lymphoma amongst others.[11]

Chemotherapy

Deoxyribonucleic acid (DNA) is normally found in the cell's nucleus. It is there that replication of DNA is undertaken during cell division. The cell cycle or cell division cycle is divided in two main stages: interphase (I) and mitosis (M), with interphase further divided into G1, S and G2 phases (*fig. 3*). In certain cases, as is often the case with neurones, cells leave the division cycle and enter a resting quiescent or G0 phase. DNA is in majority replicated during S (synthesis) stage and the centrosomes, nucleus and daughter cells are separated during mitosis (M). The gap (G) phases ensure the correct flow of the cell cycle, with checkpoints regulated by cyclins and cyclin-dependant kinases (CDKs) determining cycle progression.[12]

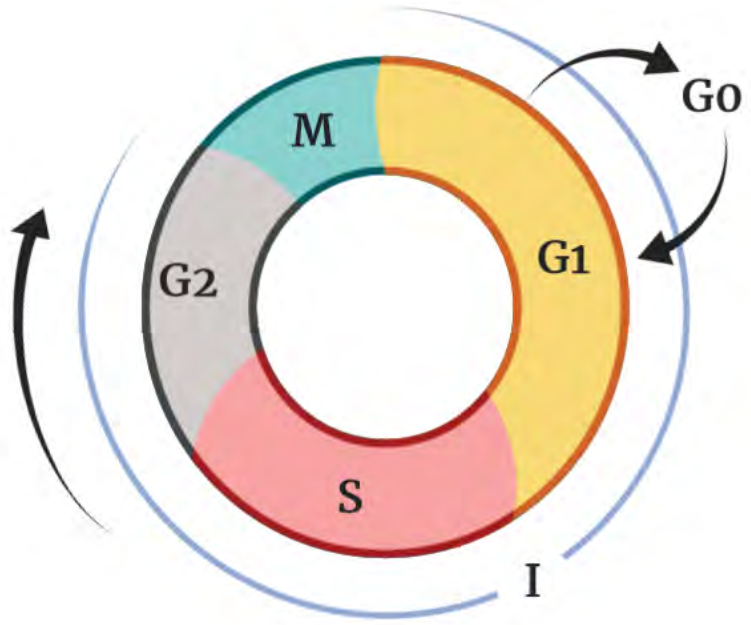


Figure 3: Phases of the cell division cycle

It is also in the nucleus that transcription takes place, where genes are transcribed into endpoint messenger ribonucleic acid (mRNA), later translated into functional proteins in the cytoplasm. Replication and transcription happen through a list of steps, each one composed of specific reagents (nucleic acids), enzymes and helper proteins ensuring a job correctly done. It is these genetic structures or processes that are targeted by chemotherapy which can be further categorised as cytotoxic or targeted therapies.[13]

- Cytotoxics

Cytotoxic chemotherapies are non-selective of cancer cells. They block dividing cells by acting directly on DNA, through modifications of physicochemical properties, or indirectly by inhibiting enzymes essential to replication and transcription, or by interacting with the mitotic spindle. There are six types of cytotoxic chemotherapies. Some, such as alkylating agents, topoisomerase inhibitors and antitumour antibiotics are cell-cycle independent. Others are cell-cycle dependent, for example antimetabolites are S-phase dependent and antimitotics are M-phase dependent.[14]

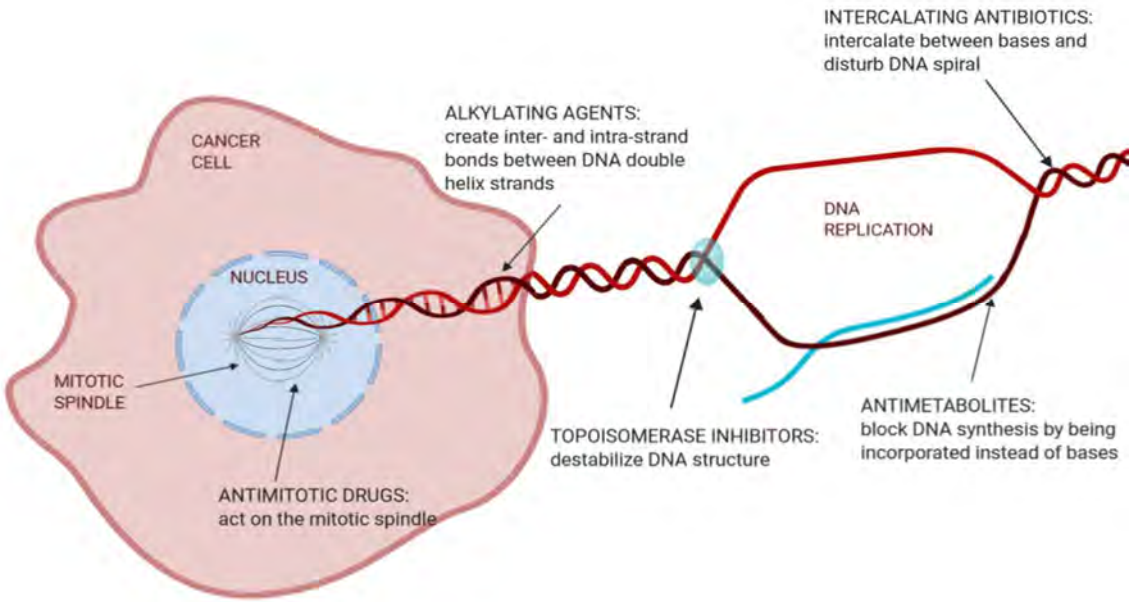


Figure 4: Schematic representation of cytotoxic chemotherapy agents

Antimetabolites stop one or more steps of DNA synthesis and are subdivided into antifolates and nucleoside analogues.[15] Antifolates (Methotrexate, Raltitrexed) inhibit dihydrofolate reductase, an enzyme essential in purine and pyrimidine base synthesis, leading to a lack of building blocks necessary during DNA replication therefore blocking it. Nucleoside analogues (Mercaptopurine, Fluorouracil, etc) present a structural analogy to DNA or RNA nucleosides and their incorporation leads to inhibition of replication/transcription or protein synthesis.

Alkylating agents attach an electrophile alkyl group to the guanine base found in DNA through covalent bonding. This will inhibit replication and transcription of the DNA strand as well as form free radicals causing DNA strand breaks.[15] Alkylating agents can be further characterized in mustard gases (Cyclophosphamide, Chlorambucil, etc, derived from the Yperite mustard gas used during World War I), metal salts (Carboplatin, Cisplatin, etc) and nitrosoureas (Carmustine (BCNU), Lomustin (CCNU), etc).

Antitumour antibiotics are derived from natural molecules derived from the fungus Streptomyces. Different types of antitumour antibiotics act in different ways. Intercalating agents such as Anthracyclines (Daunorubicin, Doxorubicin, etc.) have a plan structure and are capable of intercalating between the two DNA strands and therefore block DNA polymerase progression. This inhibits replication and transcription. Furthermore, they are also capable of inducing DNA breaks through free radicals and inhibiting topoisomerase II.[15] Bleomycin uses oxygen, iron and a reducing agent with a thiol group to create free radicals which will then provoke breaks in DNA strands. Mitomycin is another antitumour antibiotic but with a mechanism of action similar to that of alkylating agents.[16]

Topoisomerase inhibitors hinder enzymes that stabilise transitory DNA strand breaks during replication.[15] Blocking these causes definitive breaks in the strands and inhibition of DNA replication. This class is divided in inhibitors of Topoisomerase I (Irinotecan, Topotecan) derived from Camptothecin and inhibitors of Topoisomerase II (Etoposide) derived from podophyllotoxin.

Antimitotic drugs, along with topoisomerase inhibitors, are plant alkaloids and act on cells that are in the mitotic phase of the cell cycle. The vinca alkaloids are derived from the periwinkle plant (*Catharanthus rosea*)[16] and bind to β-tubulin, preventing their polymerisation with α-tubulin into microtubules – cells are thus unable to enter metaphase of mitosis. Taxanes are made from the bark of the Yew tree (Taxus)[16] and bind to microtubules, preventing their depolymerisation stiffening cells and blocking them in metaphase.

Miscellaneous antineoplastics are made up of chemotherapy drugs that are unique. Hydroxyurea is a hydroxycarbamide that inhibits ribonucleotide reductase, depriving the formation of deoxyribonucleotides.[14] Mitotane is an adrenocortical steroid inhibitor.[17] Asparaginase and Pegaspargase are enzymes that hydrolase the amino acid asparagine therefore inhibiting protein synthesis in cancer cells by depriving them of asparagine.[18] Retinoids are differentiation inductors.[19] These include Tretinoin (ATRA) and Isotretinoin which are active metabolites of vitamin A implicated in cell growth and differentiation, and Bexarotene an agonist of retinoid x receptor (RXR) receptors that regulate transcription factors implicated in cell growth and differentiation.

- Targeted therapies

Targeted therapies act of specific mechanisms found in cancer cells and were developed after the discovery of mutated, overexpressed genes in carcinogenic cells. Targeted therapies are cell cycle independent and have a better specificity to tumour cells than cytotoxic agents, they are further divided in protein kinase inhibitors and monoclonal antibodies.

Protein kinases are enzymes that catalyse the transfer of a phosphate group from an ATP molecule onto an amino acid of a transmembrane or intracellular protein. Depending on the amino acid phosphorylated, we distinguish tyrosine kinases and serine-threonine kinases. These phosphorylations are implicated in signal transductions leading to cell divisions and repressing apoptosis. Protein kinases are deregulated in multiple cancers. Some are associated to growth factor receptors, such as VEGF (vascular endothelial growth factor), EGF (epidermal growth factor) or PDGF (platelet derived growth factor) whereas others result from chromosomic translocation or genetic mutation – BCR-ABL (break point cluster region – Abelson) found in chronic myeloid leukaemia for example. **Protein kinase inhibitors** are specific to these enzymes and perturb their signal transmission, mostly acting upstream of cytotoxic agents, and targeting only the cancer cells expressing these protein kinases.[20]

Monoclonal antibodies are immunoglobulins specifically directed against proteins presented on the surface of malignant cells. The first historical example is Trastuzumab (Herceptin, Genentech)[21] which was later discovered to recognise the

HER2 protein overexpressed in certain breast cancers. Other examples include monoclonal antibodies against the VEGF or EGF receptors. Some can be used as vectors carrying cytotoxic molecules, increasing the specificity of target of these drugs.

Whilst chemotherapies can be considered as nonspecific ‘weapons of proliferating cells mass destruction’, recent advances in molecular characterisation of tumours has progressed towards more targeted and ‘specialised’ medicines such as targeted therapies and immunotherapies. Repositioning the genetic nature of tumours through gene therapy is the next step in the ladder of anti-cancer protocols and is hoped to be effective where these current treatments are limited.

Definitions: what exactly is gene therapy?

Advanced Therapy Medicinal Products (ATMPs) are innovative therapies developed from tissues, cells or genes and are currently being studied as medicine in a wide range of human diseases. ATMPs can be divided into four categories: Gene Therapy Medicinal Products (GTMPs), somatic Cell Therapy Medical Products (sCTMPs), Tissue Engineered Products (TEPs) and combined ATMPs (cATMPs). The latter incorporates medical devices as part of the medicine.

Advanced Therapy Medicinal products are defined in Article 2 of European Regulation EC 1394/2007[22] as “having properties for treating or preventing diseases in human beings, or that they may be used in or administered to human beings with a view to restoring, correcting or modifying physiological functions by exerting principally a pharmacological, immunological or metabolic action”.

Gene Therapy Medicinal Products have been defined in Annexe 1 of Directive 2001/83/EC[23] of the European Medicines Agency (EMA) as a biological medicinal product having the following characteristics:

- Contains an active substance containing or consisting of a recombinant nucleic acid for human use or administration with the aim of regulating, repairing, replacing, adding or deleting a genetic sequence.
- The recombinant nucleic acid sequence or the product of its genetic expression relates directly to its therapeutic, prophylactic or diagnostic effect.

GTMP include both gene therapy products and cell-based gene therapy (for example tumour vaccines and CAR-T cells) products but exclude vaccines against infectious diseases (*Annexe 1*).

Some GTMP products indicated in oncology have the '**orphan medicine**' indication. Orphan medicines are defined by the EMA as those used for diagnosis, prognosis or treatment of a life-threatening or chronically debilitating condition that is rare (less than five out of ten thousand affected in the European Union) or where the medicine is unlikely to generate sufficient profit to justify research and development costs. Of the gene-based therapeutics that are EMA market-approved we can find **Kymriah** and **Yescarta** with this label.[24]

Rules and regulations

ATMP regulation in the European Union governed by Regulation EC 1394/2007, initially based on Regulation EC 726/2004,[25] is implemented since 2008 and dictates the obligation of a marketing authorisation prior to the marketing of ATMPs. A specialised committee within the EMA named Committee for Advanced Therapies (CAT) is tasked in the evaluation of ATMPs presented for market authorisation application (MAA) and prepares a draft opinion. The Committee for Medicinal Products for Human Use (CHMP) takes this opinion into account before delivering their final views and authorisation. If the gene product contains a genetically modified organism (GMO), the market authorisation submitted to the EMA should also contain an environmental risk assessment. Since the establishment of this European regulation, the EMA approved 10 out of 19 submitted ATMP products between 2009 and 2018. [26]

The initiative of the European Commission services and EMA is to collaborate with the member states (*Annexe 2*) in order to facilitate the development and authorisation of these products within the European Union (EU) in benefit of the patients. Schemes have been set up in order to support these EU developments (e.g. priority medicines, PRIME) as well as the setting up of small and medium-sized enterprises (SMEs) and academia. Member states, under ATMP regulation and in certain conditions, are also permitted to allow the use of advanced therapies not yet authorised by the Commission, as so-called 'hospital exemptions'. These actions are expected to facilitate the best treatment course with opportunities to novel therapeutics for patients as well as promote innovative development, investment and competitiveness within the EU.

The global market for cancer gene therapy was over 650 million dollars in 2018 and is estimated to increase with a compound annual growth rate (CAGR) of 22.8%, predicting a global market share of over 2.5 billion dollars in 2025.[27]

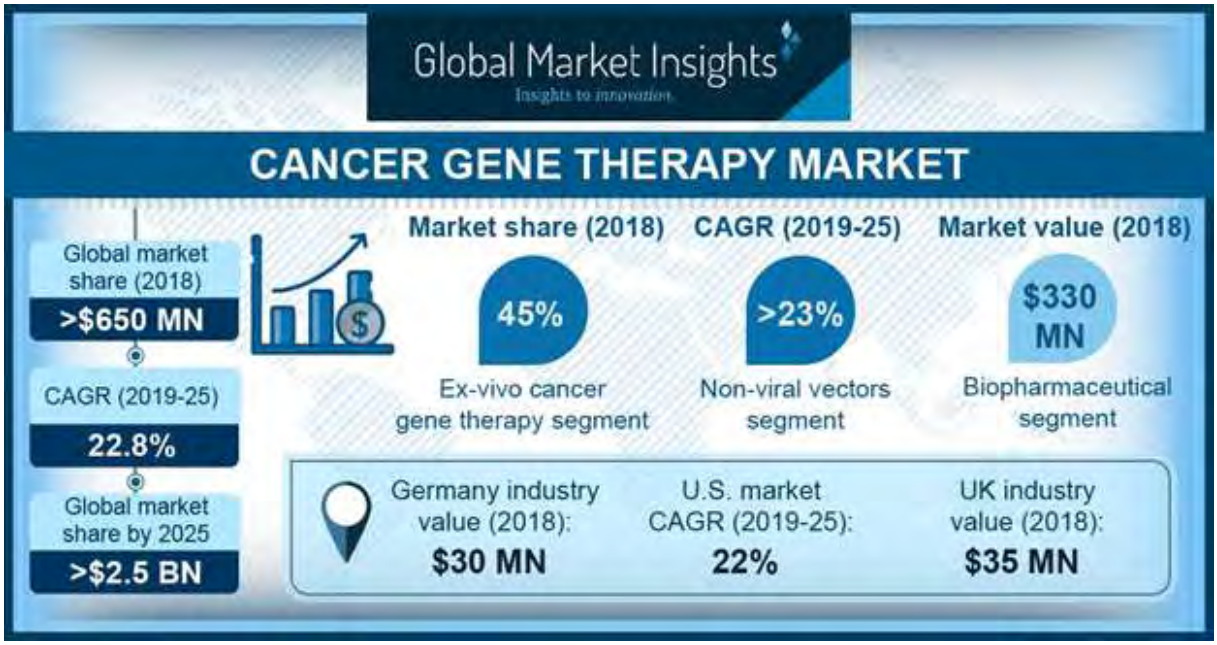


Figure 5: Growth of the gene therapy market worldwide [27]

The EMA regulations are in conjecture with the guidelines of GTMP and European pharmacopoeia set out by the international conference of harmonisation (ICH). These rules and regulations are to ensure the safety and efficacy of these innovative treatments, from the proof of concept in the research lab, to clinical trials in patients and even after during pharmacovigilance period.

A brief history

The identification and cauterization of tumoral masses goes back to Ancient Egypt (Edwin Smith papyrus 1600BC) although it was Hippocrates (460-370BC) who described them as *karkinos* (*carcinos*), and Galen (130-210AD) who further separated this term for malignant tumours from *oncos*, meaning swelling in Greek, describing all tumours.[28]

It was only in 1809, after removal of an ovarian tumour (without anaesthesia), that surgery became the first effective anti-cancer treatment, all the more effective after development of anaesthesia (1846) and antisepsis (1867). It was at the end of the 19th century that metastatic dissemination surfaced; around the same time was the discovery of x-rays by Roentgen (1895) and radium by Pierre and Marie Curie (1898), paving the way for radiation therapy.[29]

During the beginning of the 20th century, Paul Ehrlich conceptualised the use of chemicals for the treatment of cancer, debuting the era of chemotherapy, broadened in the 1940s by employing nitrogen mustard – previously used as poison gas in World War I – and folic acid antagonists in lymphoma and leukaemia respectively. Chemotherapy in cancer was further developed in the 1960s and 70s by combination therapies and neo- and adjuvant uses with surgery and radiation.

The paradigm shift towards targeted therapies arose in 2006 with Imatinib aimed at a molecular abnormality of chronic myeloid leukaemia (CML). Immunotherapy has also become a field of interest, with antibodies such as Rituximab (anti-CD20) being approved for medical use since 1997.[29] More recent investigations in oncologic immunotherapy has centred on cellular immunity, especially with the possibility of genetically engineering immune cells through gene therapy.

Oswald Avery exposed DNA in 1944 as being the media of cellular information and not proteins as previously thought. This work led to the discovery of the structure of DNA by Watson and Crick in 1953, followed by the 'breaking of the genetic code' and central biology dogma (DNA to RNA to protein) in 1961 by Nirenberg and colleagues. The following discoveries including restriction sites and enzymes such as nucleases and reverse transcriptase ultimately led to sequencing of the human genome in 2000.[29]

Gene therapy was first conceptualised in 1972, with Martin Cline successfully transferring a functioning gene into a mouse for the first time.[30] He conducted the first attempt of human gene transfer in 1980 for β-thalassemia treatment and although he claims a positive gene activity six months after the trial, it is considered as unsuccessful since his data was neither published nor verified and against NIH (National Institute of Health) guidelines.[31]

In 1990, the FDA approved the first US-based gene therapy experiment developed for patients suffering from severe combined immunodeficiency disorder (SCID). The defective gene was successfully replaced although only transiently so regular injections were needed. The first cancer gene therapy clinical trial was approved by the FDA in 1994 and consisted in a subcutaneous injection of autologous cancer cells transfected with combined antisense/triple helix technologies in an anti-gene anti-IGF-I vaccine to investigate the immune response in treated patients with malignant glioblastoma tumours expressing IGF-I.[32] This anti-IGF-I vaccine resulted in clinical and radiographic improvements with no treatment-related toxicities other than deep venous thrombosis in several patients, prompting to further develop and improve this approach for clinical use.[33]

Over the years there have been several reported deaths during gene therapy trials. The first and most symbolic is without question that of Jesse Gelsinger who, in 1999, suffered an immune rejection response to the adenoviral vector carrying the gene to correct an ornithine transcarbamylase deficiency, an X-linked genetic disease of the liver.[34] In this particular case Jesse could have received life-long enzyme replacement therapy for his non-threatening life disease, gene therapy was therefore inappropriately prescribed. The risks associated to this type of gene therapy procedure emphasize the need for caution when choosing the best treatment plan adapted to the patient. Following was five cases of leukaemia in children receiving gene therapy treatment ultimately curing SCID-X1 by replacing the interleukin-2 receptor γ chain gene using a retroviral vector, as well as the death of one of the children.[35][36] This was due to reactivation of endogenous LMO2 by the gene delivery vector that contained live viral promoters, and propelled massive reconsideration of the viral genome used in gene therapy strategies. Nonetheless, these results have fuelled the controversy that arose in concert with the development of gene therapy products.

Gendicine[37] was the first gene-based drug approved for clinical use in humans in 2003 by the Chinese State Food and Drug Administration: a recombinant adenovirus coding for the wildtype p53 gene (Ad-p53), used in synergy with chemo and radiotherapy for the treatment of head and neck squamous cell carcinoma. Due to the lack of transparency, Gendicine was never used outside China.

Next the oncolytic adenovirus **Oncorine** (H101)[38] was approved in 2005 - also by the Chinese State Food and Drug Administration - in combination with chemotherapy as a

treatment of late stage refractory nasopharyngeal cancer. Similarly to Gendicine, Oncorine is only used in China.

Other noteworthy first gene therapy products include:

Neovasculgen[39] approved in Russia in 2011 for the treatment of peripheral artery disease;

Glybera (Alipogene tiparvovec)[24] was the first treatment to be approved for clinical use in either Europe or the United States for lipoprotein lipase deficiency (LPLD). Gaining infamy as the "million-dollar drug" (cost per treatment – the most expensive medicine in the world between 2012 and 2015), Glybera was removed from the market;

Imlygic (talimogene laherparepvec)[24] a genetically modified herpes virus (HSV-1) as an oncolytic virus for inoperable melanoma approved by the FDA and EMA in 2015.

There are currently twenty-three approved clinically-used human gene therapy or human cell-based gene therapy products, six of which are used in oncology (*Table 1*). [40]

Product name	Indication	Developing industry	Authority approved	Composition
Gendicine	Head and neck squamous cell carcinoma	Shenzhen SiBionoGeneTec	CFDA - 2003	Recombinant adenovirus encoding p53 (rAd-p53)
Imlygic (Tamilogene laherparepvec)	Inoperable melanoma	BioVex	USA FDA (2015) EMA (2016)	Genetically modified HSV-1
Kymriah (Tisagenlecleucel CTL019)	B-cell acute lymphoblastic leukaemia (ALL)	Novartis	FDA (2017) EMA (2018)	Chimeric CAR-T targeting CD-19 – Lentiviral vector
H101 (Oncorine)	late stage refractory nasopharyngeal cancer	Shanghai Sunway Biotech	CFDA (2005)	Recombinant human adenovirus type 5 (rAd5-H101)
Rexin-G	Metastatic solid tumours	Epeius Biotech	Philippine FDA (2007) FDA (2010)	Retroviral vector with cytocidal cyclin G1 construct (Mx-dnG1)
Yescarta (Axicabtagene ciloleucel)	Large B-cell lymphoma	Kite Pharma	FDA (2017) EMA (2018)	Chimeric CAR-T targeting CD-19 – Retroviral vector
Zalmoxis	Allogenic T cells encoding HSV-TK and ΔLNGFR	MolMed	EMA (2016 rem. 2019)	Haematopoietic stem cell transplantation (HSCT) graft versus host disease (GVHD)

Table 1: Approved gene and cell-based gene therapy products for oncology

Zalmoxis is a product generated to overcome the effects of T cell depletion following haematopoietic stem cell transplantation (HSCT) in the prevention of graft versus host disease (GVHD). It is composed of allogenic T cells expressing a shortened human low-affinity nerve growth factor receptor (ΔLNGFR) and modulated genetically to express HSV-TK (thymidine kinase enzyme). As of October 2019, it was withdrawn from the market due to no benefit offered on disease-free survival.[41]

Similarly, **Rigvir** (ECHO-7) an echovirus-based oncolytic virus approved in Latvia in 2004, was removed from the market in 2019 due to incorrect viral doses.[42]

Translational gene therapy – a dynamic process

Being an innovative field in full development,[43] gene therapy techniques and clinical applications are continuously evolving. Similarly, the work needed to bring forth a new therapeutic involves a dynamic production line, from conception in the lab with *in vitro* and preclinical studies to clinical studies in medical establishments and government approval (FDA, EMA or AMM for France).

This work has the intention of regrouping the advances in gene therapy both at a fundamental level and a clinical point of view. Furthermore, by acknowledging the translational aspect of drug development, it is possible to expose the 'demand and supply' balance for gene therapy in oncology: how biomolecular techniques have evolved due to clinical needs and inversely how advances in cancer diagnostics and molecular biology have engendered the need for state-of-the-art therapeutics. The analysis of ongoing clinical trials will focus on pancreatic cancer which, despite conventional treatments, still has a bleak outcome and therefore the potential of benefiting immensely from gene therapy medicines.

III. GENE THERAPY FUNDAMENTALS: BUILDING A GTMP

As described previously, gene therapy entails the transfer of genetic material within patients' cells in order to treat or reduce a disease. Gene therapy products can be grossly divided into two categories: viral and non-viral (including non-vectorised)-based therapies depending on the delivery method used.

The genetic material in question is in the form of either genes, gene segments or oligonucleotides and can be used for *in vivo* or *ex vivo* treatment approaches - *in vivo* treatments involving the administration of the gene therapy directly to the patient whereas *ex vivo* applications involve collecting and treating target cells before introducing them back to the patient.

In oncology, gene therapy has been used to create cancer vaccines, target viruses to cancer cells for lysis and death, decrease the blood supply to the tumour and introduce genes into the cancer cells that cause death or restore normal cellular phenotype. The targeted cells range from cancerous to non-cancerous cells, immune cells and stem cells.

The delivery method and transgene/therapeutic agent(s) have to be carefully selected and engineered depending on the target organ, cell or molecule and, in general, of the cancer intended to treat. For this, a variety of gene therapy applications and vectors exist and are continually being developed.

The transfer of genetic material directly into a host cell is defined as ‘transfection’, whereas its incorporation in viral and bacterial vectors is named ‘transduction’. The transfection efficiency of genetic material using non-viral approaches is lower than that with viral gene-therapy, although there is an advantage in safety and a greater ease in genetic engineering when dealing with non-viral procedures.

Genetic material/the building blocks

DNA, mRNA, short double-stranded RNA such as interference RNA (iRNA) and antisense oligonucleotides (ASO) used in gene-based therapeutics, are molecules quickly degraded *in vivo* by endonucleases. Indeed, plasmid DNA intravenously injected into mice is estimated to have a half-life of approximately ten minutes.[44] Moreover these exogenous molecules need to evade the immune system as well as to avoid renal and liver clearance and unintended interactions.

Delivery of exogenous DNA requires an additional step when compared to other genetic material since it involves entry into the nucleus, an obstacle which is significant in the development of DNA-based therapies. Furthermore, due to their larger size and risk of mutagenesis, DNA-based therapies give rise to more obstacles when considering drug delivery and safety.

Single-stranded mRNA molecules are less stable than DNA, but have the advantage of being less immunogenic and non-mutagenic, since mRNA does not require nuclear localisation and therefore does not risk genomic integration.

Interference RNA include small-interfering RNA (siRNA) and micro RNA (miRNA) which are ribonucleotides of around 20bp long and involved in gene silencing. siRNA are double-stranded whereas miRNA have a short hairpin structure but both are non-coding RNA structures involved in the interference pathway (*fig. 6*), neutralising targeted mRNA and therefore interfering with the expression of specific genes between transcription and translation processes. miRNAs also play a role in post-translation

regulation of gene expression. Levels of miRNA are affected by antagomirs or miRNA sponges[45] – short-stranded oligonucleotides that inhibit miRNAs – or through miRNA replacement therapy by introduction of synthetic miRNAs or miRNA mimics.

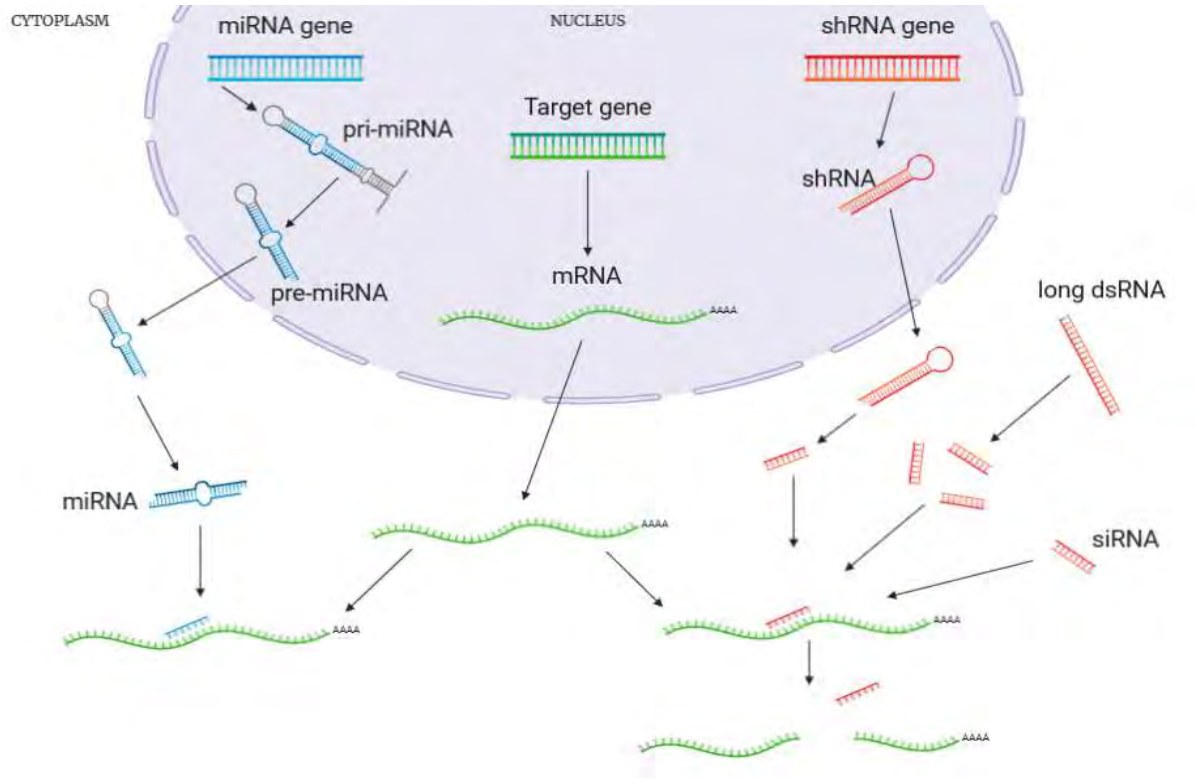


Figure 6: The RNA interference (iRNA) pathway

Due to their implication in multiple gene expression and biological processes, maintaining a balance is delicate. Indeed, in 2016 a phase I trial investigating a liposome-encapsulated miR34 (tumour suppressor from the P53 pathway) mimic was shut down due to multiple immune-related severe adverse events (cytokine release syndrome (CRS) or 'cytokine storm').[46]

Suicide gene

The concept of suicide gene is the introduction of a transgene into the target cell that, through various mechanisms, will induce cell death.[47]

A basic example of a suicide gene is by metabolising a previously indolent chemical, such as the cytosine deaminase gene from *Escherichia coli* that converts 5-

fluorocytosine (5-FC) into 5-Fluorouracil (5-FU) which is then taken up by cellular enzymes and transformed into three cytotoxic antimetabolites which block thymidine and DNA synthesis and create errors during protein synthesis.

Another extensively investigated system is the herpes simplex virus' thymidine kinase gene (HSV-TK), which converts ganciclovir (GCV) to ganciclovir monophosphate, which is further converted to ganciclovir triphosphate by the cancer cells' enzymes. Ganciclovir monophosphate is found to delay proliferation processes in cancer cells, provoking apoptosis.

Suicide gene products can be made from sequences transcribed by particular promoters. Examples include the H19 RNA oncofoetal gene or the human telomerase reverse transcriptase (hTERT),[48] which can be found abnormally expressed in certain tumours. Blocking the promoters by suicide genes leads to the death of the cancer cells that overexpress them.

Other suicide gene approaches include monoclonal antibodies or toxins such as the *Corynebacterium diphtheria* toxin-A chain (DTA-H19).[49] Applications of suicide gene therapy are being explored, either alone or in combination with radiotherapy and chemotherapy.

Gene silencing

Gene silencing is the regulation at the transcriptional or translational level of gene expression. This can be done from an epigenetic point of view, through regulation of the level of DNA methylation of promoters,[50] or can be achieved via the RNA-induced silencing complex (RISC) responsible for mRNA dismantling and known as the interference pathway.

Delivery of siRNA or miRNA against a particular gene, an oncogene or cancer-related gene for example, will target the mRNA molecule of this gene and lead to its degradation or block protein synthesis. Endogenous miRNA levels can also be acted on, by the introduction of miRNA-duplexes to compensate under-expressed miRNAs or by using siRNA complementary to the seed sequence of the oncomir (over-expressed tumour-promoting miRNAs).

There are however, several issues in gene silencing, such as off-target effects and high toxicity through immunostimulation.

Gene editing and gene repair

Gene-editing techniques provide the possibility of precise correction or modification of genetic sequences allowing for gene correction or transgene insertion by insertion, deletion, integration or sequence substitution. Although difficulties have risen with their use in clinical contexts,[51] such as somatic silencing of gene products, gene editing systems are at present exploited to create genetically-engineered models (e.g. cellular, organoids, mice and rabbits) and to develop related drugs and research tools. The first clinical trial using the gene editing tool CRISPR-CAS9 in cancer therapy debuted in 2016 in China, involving the knock-out or programmed cell death protein-1 (PD-1) in non-small cell lung cancer.[52] The latest news have revealed no toxicity-related side effects and engineered T-cells detected up to nine months after infusion.[53]

Zinc-finger proteins (ZFP) and transcription activator-like effectors (TALEs) are systems that target unique genetic sequences through their customisable domains and fuse with nucleases (ZFNs and TALENs). Nucleases have been used to create knockout cell lines, for example knockdown of the E6 oncoprotein in HPV+ cell lines, although their use in therapeutic – which requires mass reduction in gene activity – require optimisation to increase their editing activity. More recently, CRISPR-CAS9 systems have dominated gene editing since it does not need protein engineering for specific DNA binding.[54]

The CRISPR (clustered regularly interspaced short palindromic repeat)-CAS9 recognises and induces double-strand breaks. The most common CRISPR-Cas9 system used is from the bacteria *Streptococcus pyogenes* (spCas9) with CAS9 a nuclease guided by a single guide RNA (sgRNA) directed towards a specific DNA sequence. This system is quicker and simpler than ZFNs and TALENs and has the potential of engineering multiple sites simultaneously. Furthermore, it has also been used in high-throughput screening of oncogenic mutations.

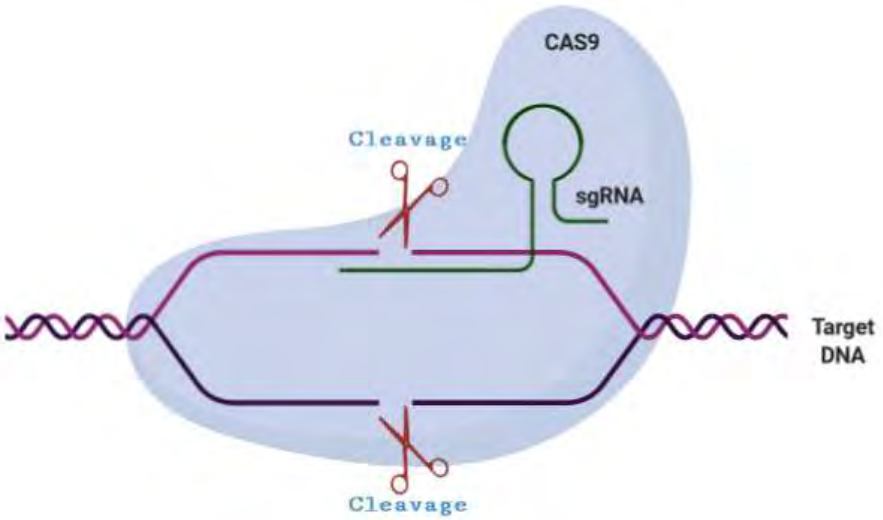


Figure 7: The CRISPR-CAS9 gene editing system

Breaks induced by these systems can be taken up by endogenous repair mechanisms to create newly edited dsDNA. Nevertheless, an issue in using these gene engineering systems is the cleavage in off-target sites, risking unwanted mutagenesis. To this regard, techniques in tweaking the system to make it safer for clinical use (nanoblade delivery of Cas9-sgRNA ribonucleoprotein[55] for example), as well as developing multiple target sites and gene networks are actively being studied.[56] The CRISPR-CAS9 gene editing tool is currently used in the development of chimeric antigen receptor (CAR) cells.

Immunomodulation

In an era where immunotherapies are being actively researched, the idea of using gene therapy to help implement it is appealing. Immunomodulation gene therapy consists in enhancing the patient's immune system to act against tumours. This can be approached via several methods, involving either or both the humoral and/or adaptive immune system.

Tumour vaccines consist in presenting tumour-associated antigens to the immune system thus triggering an adaptive immune response specifically targeted against the tumour. A gene-based tumour vaccine incorporates genes coding for proteins that are

overexpressed in cancer, differentiation antigens or tumour-specific epitopes or neoepitopes.[57] Next generation sequencing (NGS) has been a crucial tool in the prediction of neoepitopes in personalised cancer therapy.

The chimeric antigen receptor (CAR)-T cells therapy (*fig. 8*) involves obtaining T cells from a patient and genetically engineering them *ex vivo* to produce antigens against neoepitopes. An interesting technique especially in haematological neoplasms, tisagenlecleucel (**Kymriah**) and axicabtagene ciloleucel (**Yescarta**) are EMA-approved therapies against B-cell acute lymphoblastic leukaemia (ALL) and large B-cell lymphoma respectively. Limitations of CAR-T cells include the associated cost of production and the risks of under- or over-stimulation of the immune response depending on the neoepitopes expressed.

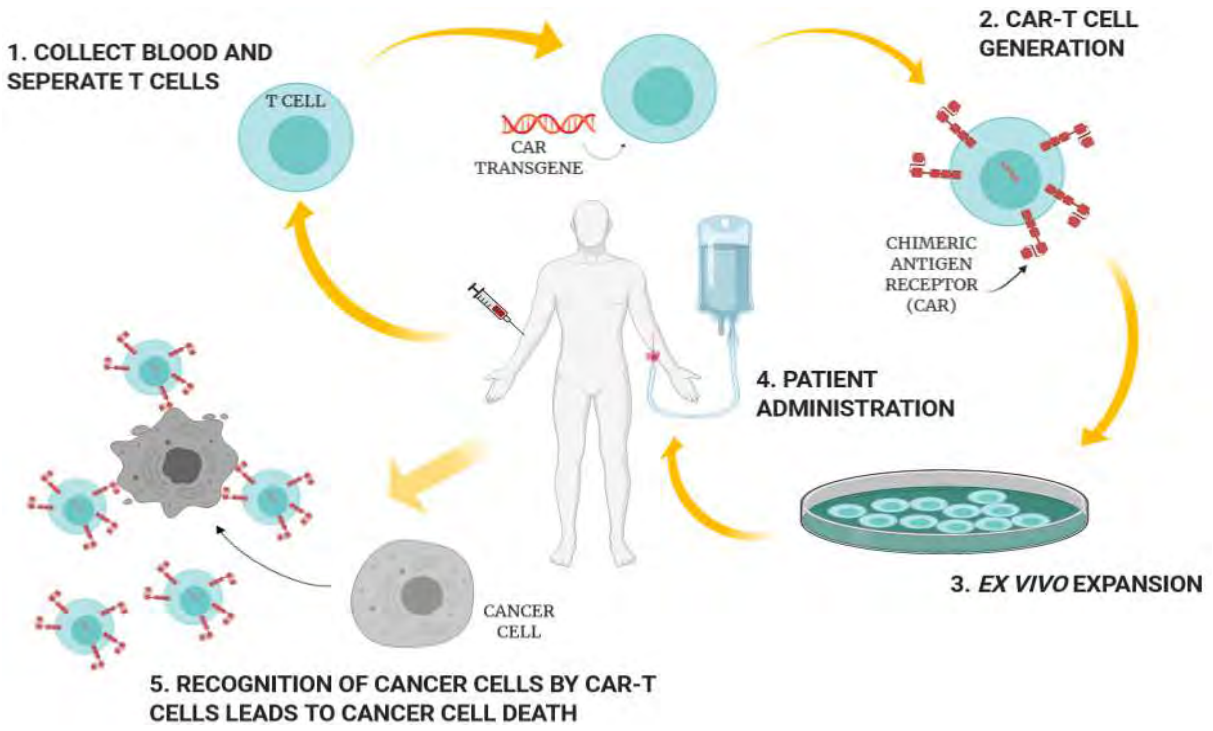


Figure 8: Representation of CAR-T cell therapy

Other immune cells are also being investigated to express genetically-engineered CARs, including gamma-delta (γδ) T cells, natural killer (NK), and natural killer T (NKT) cells.[58] γδ T cells are a subset of T lymphocytes that also possess an NK receptor (NKG2D) and cytotoxic activities in cancer cells that are not yet completely elucidated. These cells could be engineered to express CAR-T cell products all the while

maintaining their unique tumour infiltrating and killing capacities. Similarly to T lymphocytes, arming cytotoxic NK cells to target specific antigens (CAR-NK) has shown promising results in preclinical phases and in numerous clinical trials targeting both haematological and solid cancers. Moreover, bi- and tri-specific killer engagers (BiKEs and TriKEs) contain a single variable portion of an antibody linked to one or two variable portions respectively from other antibodies of different specificity.[59] These increase cell potency and persistence of CAR-NK cells while maintaining their specificity. NKT cells are activated by both antigen-dependent and antigen-independent mechanisms. In addition, they are capable of secreting a wide variety of regulatory cytokines, activation antigen presenting cells (DCs) and cytotoxic cells (CD8+ T cells and NK), contributing to their appeal as CAR-expressing immune cells.

T cells can also be engineered to express other types of receptors, for example the dominant negative TGFβ receptor, a mutated form which abrogates the negative signalling cascade following TGFβ ligation, or chemokine receptor-engineered T cells to improve tumour localisation.

Cytokine levels can also be acted upon by using cytokine gene therapy. This is used for anti-tumour cytokines such as interleukin (IL)-2, IL-4, IL-6, IL-12, IL-24, interferon (IFN)-α/β/γ and tumour necrosis factor (TNF)-α/β. The toxicity encountered after cytokine systemic administration has rethought their use towards combined antitumour strategies.[60]

Mechanical methods

- Biolistic particle delivery system (BPDS)

Gene gun or biolistic particle delivery system uses heavy metallic particles precipitated with naked DNA. These microparticles are propelled into the target cell by a high-voltage electric spark or helium discharge.[61] The DNA is then gradually released in the cell. Particle bombardment is used mainly in genetic immunization, particularly targeting the skin. Nevertheless, other uses including genetic vaccination, immunomodulation and suicide gene therapy in cancer treatments are being investigated as well as other target organs, such as the liver and the brain.

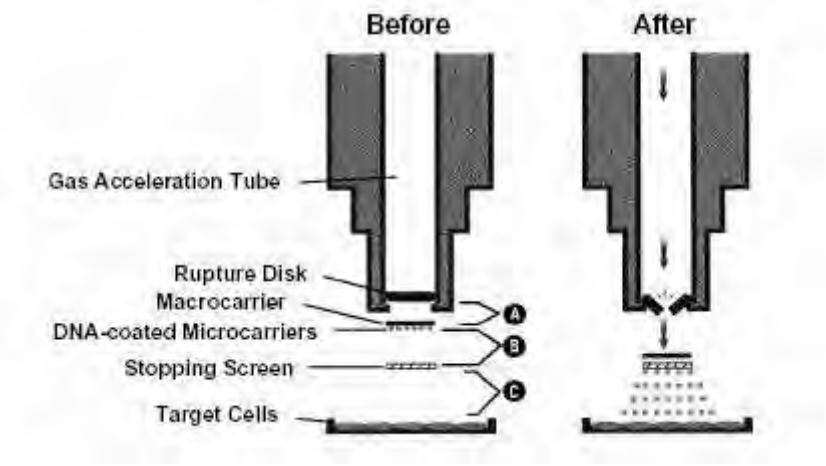


Figure 9: The Biolistic bombardment process, Bio-Rad [62]

- Microinjection

Microinjection directly introduces genetic material in the cytoplasm or nucleus of a single cell during a microsurgical procedure and uses a glass needle, a precision positioning device and a microinjector. Injections are usually performed under microscopic control and are commonly employed to produce transgenic animals.[61] Although it is an extremely efficient technique which allows nuclear delivery of naked

DNA while bypassing cytoplasmic barriers, it is an extremely laborious procedure (cells injected one by one) and impractical for *in vivo* gene delivery.

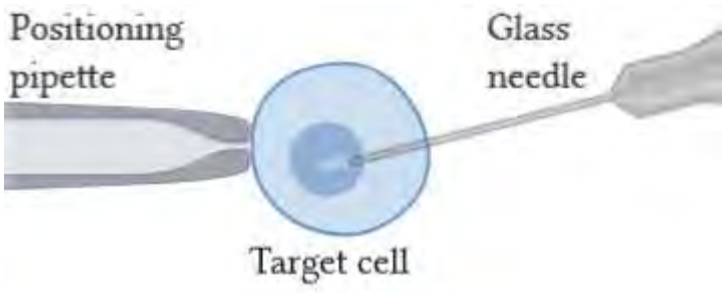


Figure 10: Illustration of microinjection

Physical methods

- Electroporation

Electroporation creates transient pores in the cell membrane through electrical pulses using a pulse generator and applicator (electrodes). The cell thus becomes highly permeable enabling the uptake of genetic material present in the surrounding medium. The exact mechanism of pore formation and DNA uptake is still under investigation.[61]

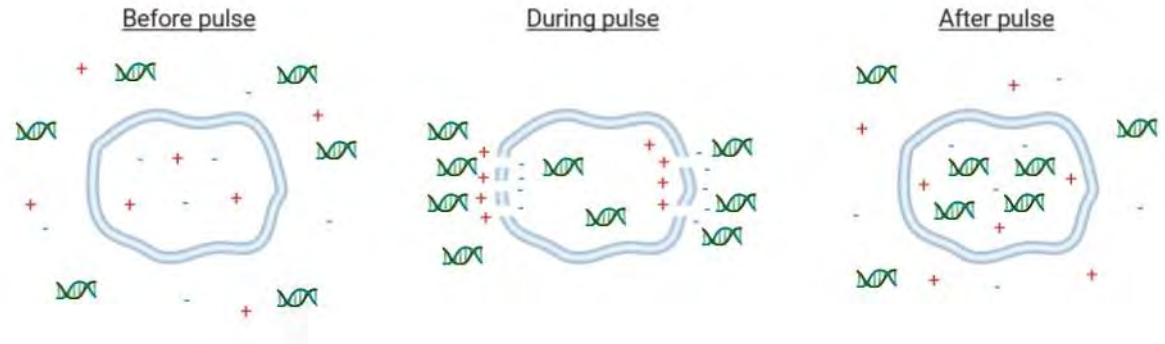


Figure 11: Representation of electroporation

Nevertheless, physical factors contribute to the efficiency of gene delivery such as pulse duration and electric field strength, as well as biological factors including cell size, DNA concentration and conformation. For example, short pulses of high electric

field pulse are needed for delivery of small anti-cancer drugs, whereas longer pulses of lower electric field strength are optimal for gene transfer.

- Hydrodynamic injection

Hydrodynamic delivery is the use of hydrodynamic pressure induced by a high-speed injection of a large volume of fluid in capillaries, increasing permeability of endothelial and parenchymal cells. This translates in an efficient delivery to parenchymal cells, mainly to the liver, of DNA, RNA and, more recently, siRNA molecules. An example is the hydrodynamic tail vein injection of plasmid DNA in rodents.

Hydrodynamic injections have also been used to amplify efficiency of viral vector transductions.[63]

- Sonoporation

Sonoporation uses low intensity ultrasounds (~20 KHz) to increase cell permeability.[61] Contrast agents exist to increase or stabilise the cavitation mechanism believed to be the result of sonoporation. Cavitation is the rapid change in pressure in liquids leading to the formation of cavities or bubbles. After collapse of these active bubbles, the energy released in shock waves is responsible in pore-formation of the adjacent cell membrane.

Several factors influence gene transfer through ultrasound such as transducer frequency, acoustic pressure, pulse duration, exposure duration as well as the ultrasound contrast agent used.

- Magnetofection

Magnetofection associates magnetic nanoparticles of iron oxide coated with a polymer, and the genetic material complexed with either its transfection agent or vector (viral or non-viral). The nanoparticles are delivered to the cell surface and are “pulled” into the cells by a magnetic field, followed by the release of genetic material and the hope that the nanoparticles won’t influence cellular function.[61]

Magnetofection enhances transfection, optimising the procedure in primary cells for example. This technique has been successfully used to deliver antisense

oligonucleotides both *in vitro* and *in vivo* and is being developed in *ex vivo* models for tissue engineering, tumour vaccines and to enhance *in vivo* gene delivery.

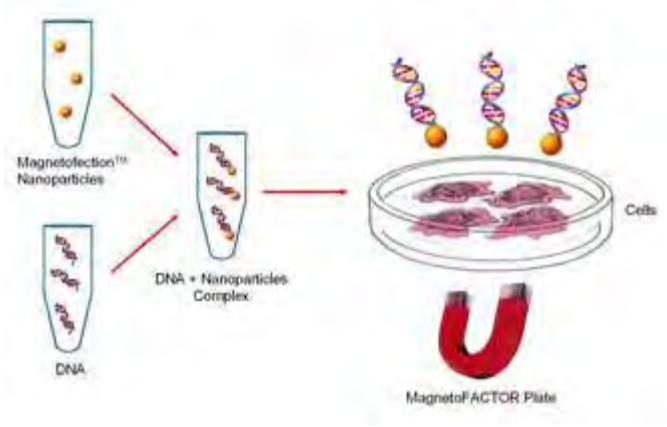


Figure 12: Magnetofection procedure, Chemicell

Chemical/structural modification

DNA used in therapy is often expressed in plasmids which are easy to construct and amplify. In addition, plasmids are episomal and non-integrating to the host genome, decreasing the risk of insertional mutagenesis found with other delivery methods, such as certain viral vectors.

Plasmid construction necessitates an enhancer-promoter sequence[64] which will depend on the level and duration of expression. Viral enhancers and promoters such as cytomegalovirus (CMV), respiratory syncytial virus (RSV) and simian virus 40 (SV40) usually have a transient expression, whereas mammalian promoters such as the human ubiquitin C (UBC) and the eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) promoters offer more constitutive gene expressions.[44]

The size and conformation of the transgene also plays on gene expression efficiency. For example, small covalently closed circular plasmids tend to give higher transgene expressions than a larger or linear construct. Chromatin attaching sequences or transposition systems based on recombinases (PiggyBac, Sleeping Beauty)[44] have been developed to promote long-term expression, although their safety has not as yet been completely elucidated. These transposon systems are currently used in CAR-T cell engineering, notably for B-cell malignancies.

Messenger RNAs can be toxic through the activation of Toll-like receptors (TLRs) and therefore modifications of mRNA have been established. These modifications encompass combining 2-thiouridine and 5-methylcytidine to reduce immune stimulation or the inclusion of pseudouridine in the mRNA (Ψ-mRNA) structure to prevent activation of pattern recognition receptors (PRRs).[44]

Chemical modification of siRNAs include replacing the 2'OH ribose group with –O-methyl or 2'fluoro groups, incorporation of locked (extra bridge between the 2' oxygen and 4' carbon of the ribose ring) and unlocked (acyclic analogue of RNA missing the bond between C2′ and C3′ atoms of the ribose ring) nucleic acids and substituting phosphodiester bonds with phosphorothioate or barophosphonate links. These modifications can prevent both endonuclease degradation and recognition by the host innate immune system.[65]

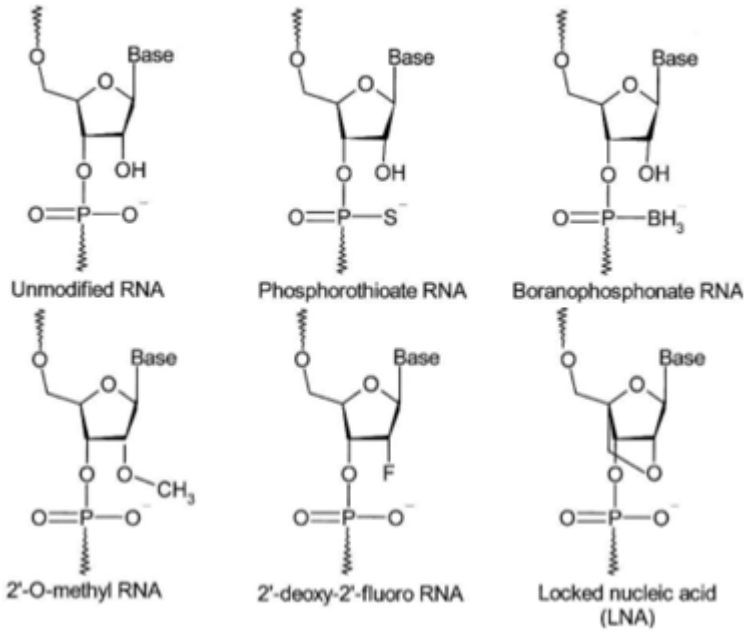


Figure 13: Most common chemical modifications in siRNAs [65]

Polymeric and poly-conjugate nanoparticles

Polyplexes are spherical shaped nanoparticles resulting in the condensation of negatively charged DNA or RNA with cationic polymers and have been shown to

localize intact to the nucleus, thus capable of delivering DNA to its target site successfully.

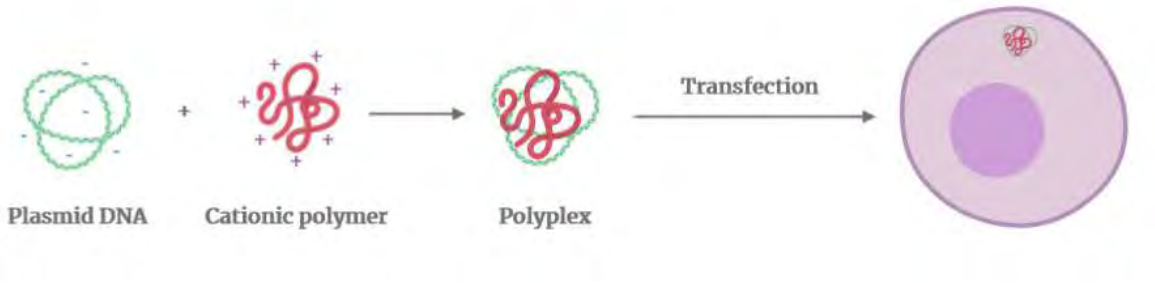


Figure 14: Polyplex formation

Examples of polyplexes include poly(l-lysine) (PLL) and polyethylenimine (PEI).[44] While PLL has shown poor transfection efficacy and significant *in vitro* cytotoxicity, PEI with regularly incorporated nitrogen atoms shows a high charge density at low pH helping with DNA condensation and endosomal escape (following the so-called proton sponge effect). To avoid the trapping of transgenes in lysosomes/endosomes of PLL-based therapies, endosomolytic groups such as chloroquine, histidine or imidazole are commonly used.[66] Hydrophilic poly(ethylene-glycol) (PEG) used as a co-polymer increases the stability and biocompatibility of both PLL and PEI polyplexes, in addition to an increased passive targeting of tumours. Additionally, degradable disulphide crosslinks and alkylation of PEI are used to reduce toxicity and increase potency respectively.

Poly(2-dimethylamino-ethylmethacrylate) (PDMAEMA) and polyamidoamine (PAMAM) are also cationic polymers used in gene vectors, with PMAM being the most used dendrimer-based vector.[66]

The optimal transfection-efficient concentration of polymer differs according to the polymer used, and is known as the polymer amine to DNA phosphate ratio.

The first siRNA delivery system used in clinical trials for cancer was a cyclodextrin polymer (CDP). CDPs are interesting vectors owing to their low toxicity and polycationic charge. A clinical study (**CALAA-01**) is currently underway of a siRNA nanoparticle formulation consisting of CDP, PEG and human transferrin (Tf) as

targeting ligand, directed towards transferrin receptors which are typically upregulated in solid tumours.[67]

Conjugate siRNA systems are siRNAs covalently attached to precisely defined delivery ligands. Examples of liver-targeted conjugate systems include dynamic polyconjugates (DPC) and N-acetylgalactosamine (GalNAc) conjugates.[44]

The system is composed of a membrane-disrupting polymer, shielding polymers and targeting ligands, each providing a particular function in the delivery of the siRNA.

A new generation DPC is the PBAVE polymer which is not covalently attached to the siRNA transgene, but rather co-injected with cholesterol modified siRNA and these two co-localize in endosomes of target cells. Conjugates are well-defined structures of minimal material and broad therapeutic windows.

Lipid-based vectors

Lipoplexes or liposomes are spontaneously formed structures encompassing cationic lipids, neutral lipids and DNA or RNA, where the transgene is captured in lipid bilayers arranged in lamellar or hexagonal formations.[68]

DOTMA is a synthetic cationic lipid which spontaneously forms small liposomes that encapsulates DNA for delivery to a variety of mammalian cell lines. DOTMA is composed of a cationic head and a hydrophobic tail with a linking group in between.[44]

The low efficacy when using cationic lipids comes from their instability and rapid clearance and they have been known to trigger either inflammatory or anti-inflammatory responses. A way of increasing liposomal stability and transfection activity is by incorporating ‘helper lipids’ which are neutral lipids, such as DOPE and cholesterol.[69]

Liposomes delivering miRNAs are being investigated in oncology due to the capacity of certain miRNAs to downregulate various cancer-related genes, miR34a in liver cancer for example.[44] Off-target effects are of a concern,[46] although these can be readily predicted through bioinformatic analyses.

Efficient transfection of mRNA has been shown after complexing the mRNA to reagents such as Stemfect or lipofectamine or to a hydrophobic poly(β-amino ester) that is coated with a positively charged lipid layer.

Other lipid-based nanoparticles include SNALPs and MCNPs.[70] Stable nucleic acid-lipid particles (SNALP) are lipid-based nanoparticles (LNPs) smaller than 200nm in diameter encapsulating genetic material, including siRNAs.[44] Certain SNALP delivery mechanisms involve the apolipoprotein E (apoE). Membrane/core nanoparticles (MCNP) are composed of an inorganic core surrounded by a lipid bilayer. These particles can be very small (~30nm) resulting in higher tissue or cellular uptake efficiency and are more stable than hollow liposomes.

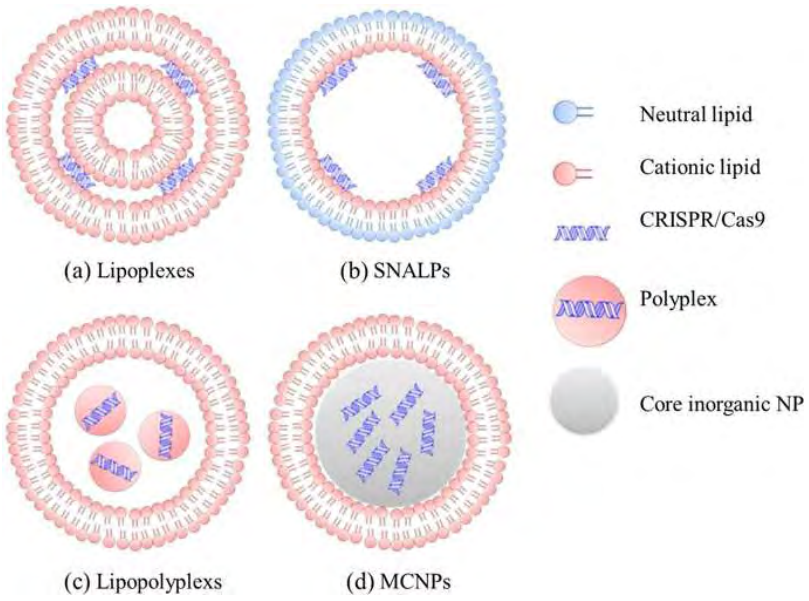


Figure 15: Examples of lipid-based vectors for CRISPR-CAS9 delivery [70]

Exosomes

Exosomes are extracellular vesicles composed of plasma membrane, ranging from 30 to 150nm in size and produced by the endosomal compartment of most eukaryotic cells. They are able to carry a variety of genetic material (DNA, mRNA, iRNA and lncRNA) as well as low molecular weight lipids and proteins. Recently, they have been investigated as gene delivery vectors, with advantages including bio-compatibility,

potentially long expression cycles, and efficient target recognition. *Mendt et al.* have generated clinical-grade exosomes derived from mesenchymal stem/stromal cells (MSCs) for the delivery of siRNA targeting oncogenic Kras in the treatment of pancreas ductal adenocarcinoma (PDAC).[71] These exosomes appear to maintain robust anti-KrasG12D activity and the associated pancreatic cancer cell apoptosis efficacy in mice while avoiding adverse immune reactions.

Engineered exosomes with polymers are therefore getting attention, most recently by a polymer-based precipitation of extracellular vesicles containing miRNAs from patients' serum as potential biomarkers in gastric cancers.[72]

Nanoghosts

Nanoghosts are stem cells turned into a drug delivery system that specifically target cancer cells. These nanoparticles are derived from mesenchymal stem cells (MSC-NG) which have been emptied of their cytoplasm and nucleus. They retain however the surface-associated mechanisms of MSCs which include tropism to inflammation and their capacities in immunomodulation and immune evasiveness.[73][74]

Evaluation of nanoghosts as cancer-targeted vectors is still at its infancy with many unanswered questions that still need to be addressed.

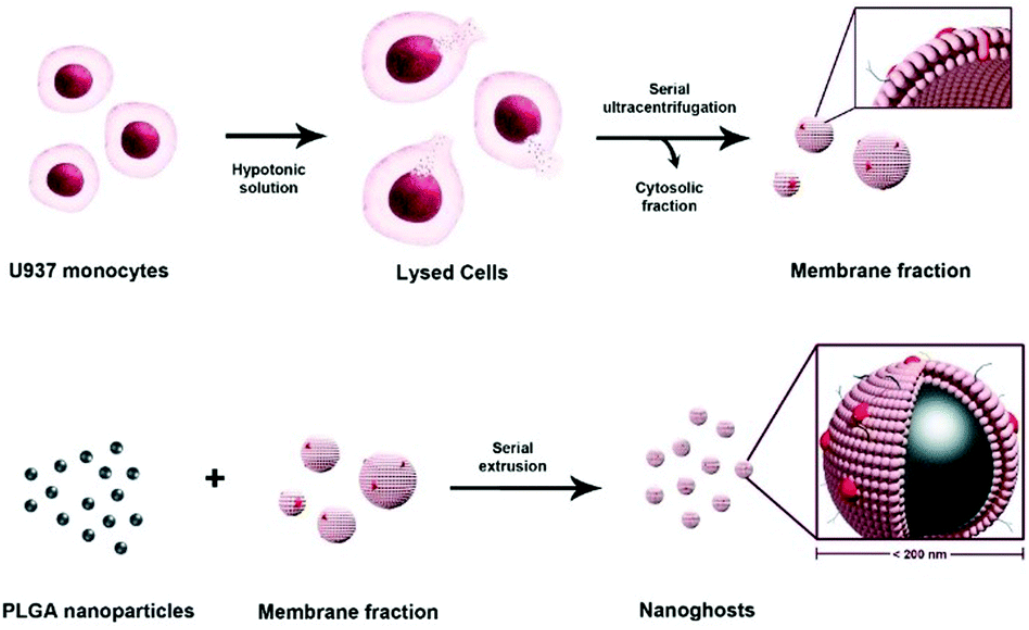


Figure 16: Illustration of nanoghost preparation [73]

Viral gene delivery

Viral vectors represent the majority of vectors used for gene therapy in clinical trials. These virus-based therapeutics can either contain DNA or RNA with their genome being either single-stranded (ss) or double-stranded (ds), with RNA viruses making up around 70% of all viruses. A virus genome is surrounded by a core, which plays a role in host cell attachment and protection from nuclease enzymes.

Viruses can also be categorized as non-enveloped or enveloped depending on the presence or not of a lipid bilayer originating from the host cell's membrane and obtained during virus egress.

A virus is incapable of replicating on its own and needs a host cell, acquiring metabolic and biosynthetic elements required for successful viral replication. Some DNA and single-stranded RNA viruses need the host cell's replication machinery and therefore enter the nucleus of the cell, ssRNA viruses being armed with a reverse transcriptase allowing dsDNA transcription from their genome. Other viruses carry their own replication machinery and replicate in the cytoplasm of the cell, in viral replication centres which are usually peri-nuclear so as to take advantage of other necessary factors.

The idea of systemically delivering viral therapies in order to combat not only the primary tumour but detected/undetected metastases as well is an attractive one. Although, safety is a fundamental issue concerning viral-based therapies, with the most frequent side-effect encountered in gene therapy being a transient fever and flu-like symptoms, with risks of hypersensitivity and leukocytopenia.

Depending on the strain used and on the oncogenic target, viruses can be either developed as vectors carrying specific therapeutically intended transgenes (recombinant vectors), or can themselves act as a drug through their oncolytic activity.

Viral vectors

Virus vectors can be either integrating or non-integrating depending on whether the transgene is incorporated into the genome of the host cell upon delivery. The choice of vector will depend on various factors, such as the virus' tropism (dividing or non-dividing cells), the packaging capacity, the duration of transgene expression and the potential immune response triggered against the virus [75] when delivered *in vivo*. Furthermore, viral vectors can either be replication deficient, replication competent or replication-conditional. The commonly used viruses for vectors are adenoviruses (Ads), adeno-associated viruses (AAVs), retroviruses/lentiviruses, Baculoviruses (BV), Herpes Simplex virus (HSV) and poxviruses.[49][76]

- Adenoviral vectors

Adenoviruses are double-stranded DNA viruses from the *Adenoviridae* family that infect quiescent or slowly dividing cells, and can therefore be lost in quickly dividing target cells. They are non-enveloped with an icosahedral capsid and ~90nm in size. The adenovirus genome is non-integrating since it remains as an episome in the host cell's nucleus. The genome ranges between 26 and 45 kb flanked by two terminal inverted repeats (TIRs).[77]

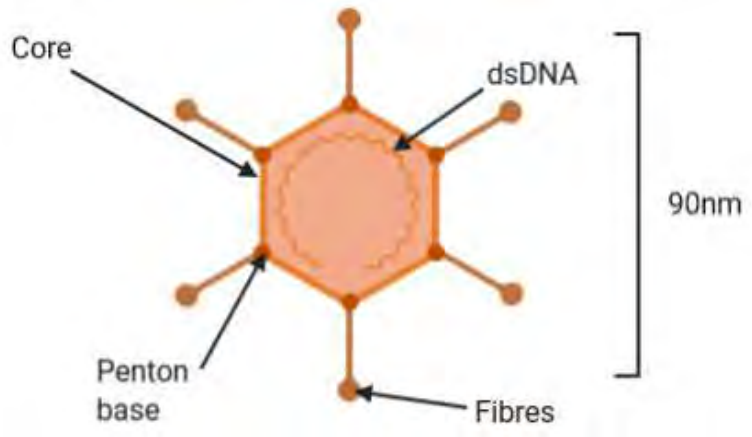


Figure 17: Adenovirus

The first adenovirus vectors were derived from the Ad5 strain, with the E1 and E3 genes deleted, allowing for an insert of 7.5 kb. They are capable to transduce a

plethora of host cells, both quiescent and dividing. However, due to the presence of certain viral genes, the expression was in general transient and accompanied by an immune response, leading to a significant toxicity and loss of the transduced cell.[69] Techniques investigating the diminishing of host immune responses include PEG-shielded Ad vectors and immunosuppression.

Helper-dependant adenoviral-vectors (HDAd) or gutted/gutless vectors are synthetic adenoviruses with all viral genes excised apart from ITRs and packaging signals. Since it does not possess the genes encoding enzymes or structural proteins, it is unable to replicate without the assistance of a helper virus (HV). The helper virus is able to replicate and express all the proteins necessary for replication and assembly of the helper-dependant adenovirus.[78]

However, to obtain a relatively pure transgene-carrying vector population, it is important to inhibit the propagation of the infectious helper virus. An approach is to limit the packaging capacity of the helper virus through gene deletion, and since the HDAd contains the wild-type packaging signal, this will trump viral packaging over that of the HV.

Using helper-dependant adenoviral-vectors increases the insert capacity to 30 kb, allows for long-term transgene expression without chronic toxicity.

Due to their highly efficiency in transduction and immunostimulatory action, adenoviral vectors have been commonly used as vaccine carriers. Furthermore, replication-competent adenoviruses are also studied as oncolytic agents (see below).

- Adeno-associated viral vectors

Adeno-associated viruses are small (20-25nm) non-enveloped icosahedral viruses of the *Parvoviridae* family containing single-stranded DNA of 4.7kb flanked by ITRs. The viral genome codes for three genes: *rep*, *cap* and *AAP* that express non-structural proteins, structural proteins and the assembly-activating protein respectively.[77]

Similarly to the HDAd, AAVs are helper-dependant viruses, in the absence of which the AAV genome remains as an episome in the host's nucleus or integrates, albeit infrequently, the cell's genome on chromosome 19. Helper viruses, such as

Adenovirus or Herpes Simplex virus either enhance AAV protein production to increase replication, or provide directly the proteins necessary for replication.

Recombinant adeno-associated viruses (rAAVs) are engineered AAVs containing a transgene instead of the *rep* and *cap* genes which are henceforth provided for by the helper virus. It is possible to produce different serotypes of rAAVs through pseudotyping which consists in changing the capsid of the helper virus while keeping the same transgene cassette, tweaking the tropisms to find the most adapted vector for the job.

Like Ads, AAVs have a broad tropism and infect both non-dividing and slowly dividing cells, showing no cytotoxicity since its capsid is less immunogenic than that of adenoviruses. A drawback of AAVs is the limited packaging space of less than 5kb, although this can be overcome by trans-splicing, splitting the expression of the transgene over two vectors.

AAV vectors are widely used as a research tool to test novel therapies, assess gene function and knock-down gene expression. They are also used in a variety of clinical trials in oncology and for diverse cancers.

- Retroviral and lentiviral vectors

Retroviruses are large enveloped viruses of 80-120nm with two copies of positive single-stranded RNA. Gamma-retroviruses possess the *gag*, *pol* and *env* genes flanked by long terminal repeats (LTRs). Lentiviruses are complex retroviruses encoding extra genes (*rev*, *tat* …) that aid in viral replication, binding, infection and release.[77][69]

The positive RNA is transcribed into dsDNA by the viral reverse transcriptase and transported to the cell's nucleus, either through nuclear pores (Lentivirus and pre-integration complex) or when the nuclear membrane disassembles (gamma-retrovirus). Lentiviruses are therefore used to transduce dividing and non-dividing cells whereas gamma-retroviruses are generally used to transduce dividing cells only. In both cases, viral dsDNA integrates the host's genome - integration sites varying according to the retrovirus - and remains a permanent part of host cells. Retroviruses

preferentially integrate in promoter regions, while lentiviruses generally integrate in genes that are actively transcribed.

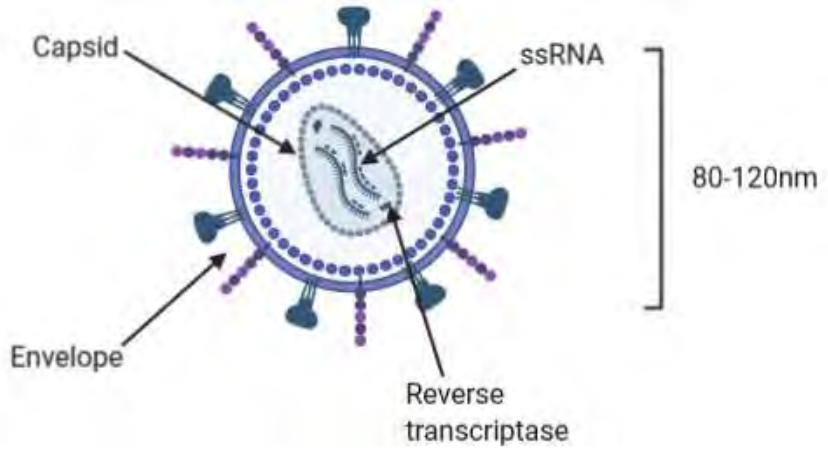


Figure 18: Lentivirus

Most retroviral vectors derive from the Murine Leukaemia virus (MLV), where first generation vectors had a deletion of non-essential genes to allow a transgene of up to 9kb. Second generation retroviral vectors are recombinant particles generated from triple transfection of three separate plasmids, one containing the transgene between the LTRs, another with *gag*/*pol* and a final with *env*. Most recent retroviral vectors are self-inactivating (SIN) where the enhancer/promoter of LTR is deleted, averting the risk of activating nearby genes, notably to prevent from previous disasters during the original X-SCID clinical trial.

Lentiviral vectors are derived from the human immunodeficiency virus (HIV), feline immunodeficiency virus (FIV), or the equine infectious anaemia virus (EIAV). Initial lentiviral vectors were formed from three plasmids, similar to that of retroviral vectors. More recent lentiviral vectors are packaged as four plasmids with the fourth containing the *rev* coding gene, and deletion of the gene encoding *tat*, to further prevent recombination and production of live virus.

Comparably to AAVs, pseudotyping of the retroviral/lentiviral envelope's glycoproteins is an option to engineer the vector's tropism, which is already very broad, for example with the glycoprotein G of the vesicular stomatitis virus (VSV). Furthermore, their integrative genome allows for long-term transgene expression, although with the risk

of insertional mutagenesis by either disrupting or inappropriately activating transcription of a nearby host gene.

Retroviral and lentiviral vectors are commonly used in *ex vivo* gene delivery (e.g. in hematopoietic disorders). Lentiviral vectors are also routinely used in the generation of chimeric CAR-T cells for leukaemia and have recently been investigated in the delivery of the genome editing tool CRISPR-CAS9.[52]

- Baculovirus vectors

Baculoviruses (BV) are rod-shaped 40-50nm by 200-400nm, with a polyhedron coat. Their genome is a complex circular ds DNA. They are insect viruses, non-pathogenic to humans, the most commonly studied strain being the *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV).[79]

Although baculoviruses don't naturally infect mammalian cells, recombinant BVs equipped with a mammalian expression cassette are highly efficient in transient transduction of human cells, including primary cells and embryonic stem cells.

Since BVs are naturally non-pathogenic for humans, there is no detectable pre-existing immunity to them, an advantage over other potentially immunogenic viral vectors. In addition, they have no perceived cytotoxic effect and have limited risk of insertional mutagenesis as their genome is non-integrative to that of the host cell. Finally, BV vectors are genetically easy to manipulate and cultivate and allow for a relatively large insert of 38kb.

Recombinant BV vectors are therefore attractive for short-term and high-level transgene expressions. They are actively being studied in vaccination, tissue engineering/regenerative medicine and cancer therapy.

- Herpes Simplex virus vectors

HSV is a dsDNA (152kb) virus with an icosahedral capsid and envelope, and 150-200nm in size. The genome is made up of long and short segments capped by inverted repeat sequences. The viral genome codes for the three classes of genes α

(transcriptional regulatory proteins), β (transcriptional factors like DNA polymerase), γ (structural proteins).[69][80]

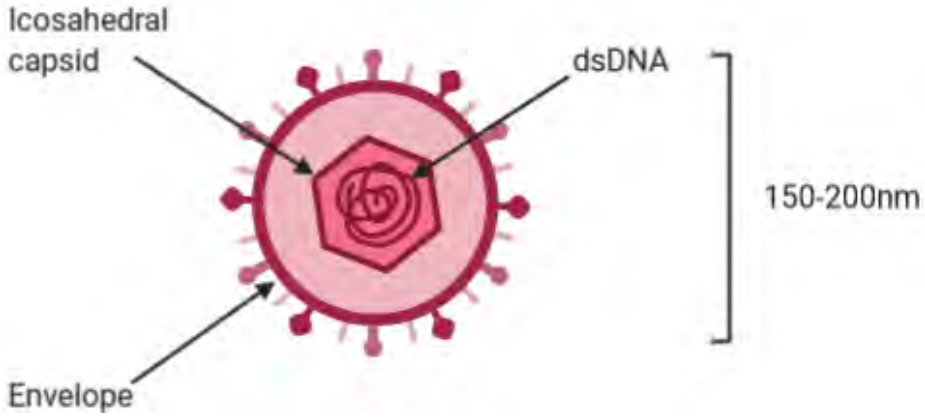


Figure 19: Herpes Simplex virus

A particularity of HSV is the ability to remain latent in the host cells of sensory neurones after initial infection and to reappear spontaneously. Man being the natural host and with the ability of reactivating infection after latency, Herpes Simplex virus has been engineered to produce a safe vector.[81] The most common serotype is HSV-1 which has been designed into two types of vectors:

The first is a replication deficient vector in which case the α genes are deleted allowing an insert of up to 150kb, with a complementing cell line needed to provide the products necessary for viral replication. In this case, the recombinant HSV can infect non-complementing cells without cytotoxicity.

The second is the generation of an amplicon vector where, similarly to HDAd and rAAV vectors, the plasmid with the transgene (amplicon) is transduced with a helper virus. The problem of this technique being low titres of amplicon with remaining cytotoxic helper virus, several methods are being investigated to counteract these issues. The most recent methods include using a cre-lox system to excise the packaging system of helper viruses, a bacterial artificial chromosome containing the packaging system instead of the helper virus or finally by incorporating the plasmid-maintenance functions of Epstein–Barr virus in the amplicon plasmid.

HSV vectors are used in cancer immunotherapy, either by expressing immunomodulatory cytokines, such as granulocyte/macrophage-colony stimulating factor (GM-CSF) in tumour cells, and in cancer vaccines.

- Poxvirus vectors

Poxviruses are large enveloped dsDNA viruses that replicate in the cytoplasm. Poxviruses are one of the first animal viruses to be used as a gene-transfer vector, the most commonly studied being derived from the Vaccinia virus (VACV), in particular the Modified Vaccinia Ankara (MVA, 178kb) and the highly attenuated vaccinia virus strain (NYVAC, 192kb). These strains present an interest due to their natural replication deficiency and their inability to produce infectious particles in human tissue.

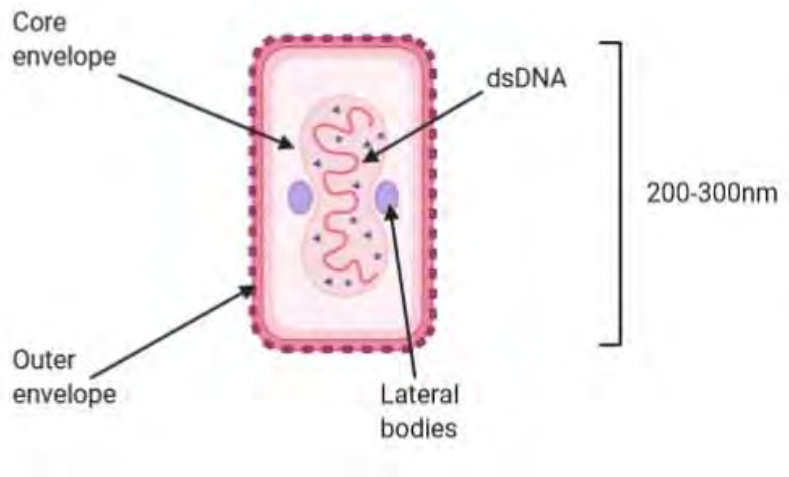


Figure 20: Poxvirus

More recently, poxviruses have been studied for their oncolytic activity. This includes not only the Vaccinia virus but also Myxoma virus (MYXV). On top of their natural oncolytic activity, it is possible to easily engineer these poxviruses to integrate 25 kb of foreign DNA. Since their replication happens in the cytoplasm of target cells, there is no risk of genome integration and insertional mutagenesis.

Given poxvirus' preferential replication in tumour cells, having replication-efficient vectors would in this case be beneficial so as to amplify the resulting transgene's therapeutic effect while leaving noncancerous cells unscathed.[82]

Viral vector	Vector size	Insert size	Possible toxicity	Integrative vs non-integrative	Dividing vs non-dividing cells	Difficulty of production
Adenovirus (Ad) Helper-dependant Ad (HDAd)	90nm	7.5 kb 30kb	Yes No	Non-integrating	Quiescent + slow dividing cells	Difficult
Adeno-associated virus (AAV) Recombinant AAV (rAAV)	20-25nm	<5kb	No	Non-integrating/ Integrating	Quiescent + slow dividing cells	Medium
Retrovirus Lentivirus	80-120nm	9kb	Yes	Integrating	Dividing cells Both	Easy
Baculovirus (BV)	40-50x200-400nm	38kb	No	Non-integrating	Both	Easy
Herpes simplex virus (HSV)	150-200nm	150kb	Yes	Non-integrating	Both	Difficult
Poxvirus (VACV) (MYXV)	200-300nm	25kb	Yes No	Non-integrating	Both	Easy

Table 2: Comparison of viral vectors

Oncolytic virotherapy

The idea of treating cancer patients with replicating viruses comes from observed clinical tumour regression after natural virus infections.[83] To be considered an oncolytic agent, a virus should possess certain qualities:

- tumour-selective infection, replication, and propagation
- reshaping of the tumour microenvironment (TME)
- release of tumour antigens and induction of adaptive antitumor immune responses

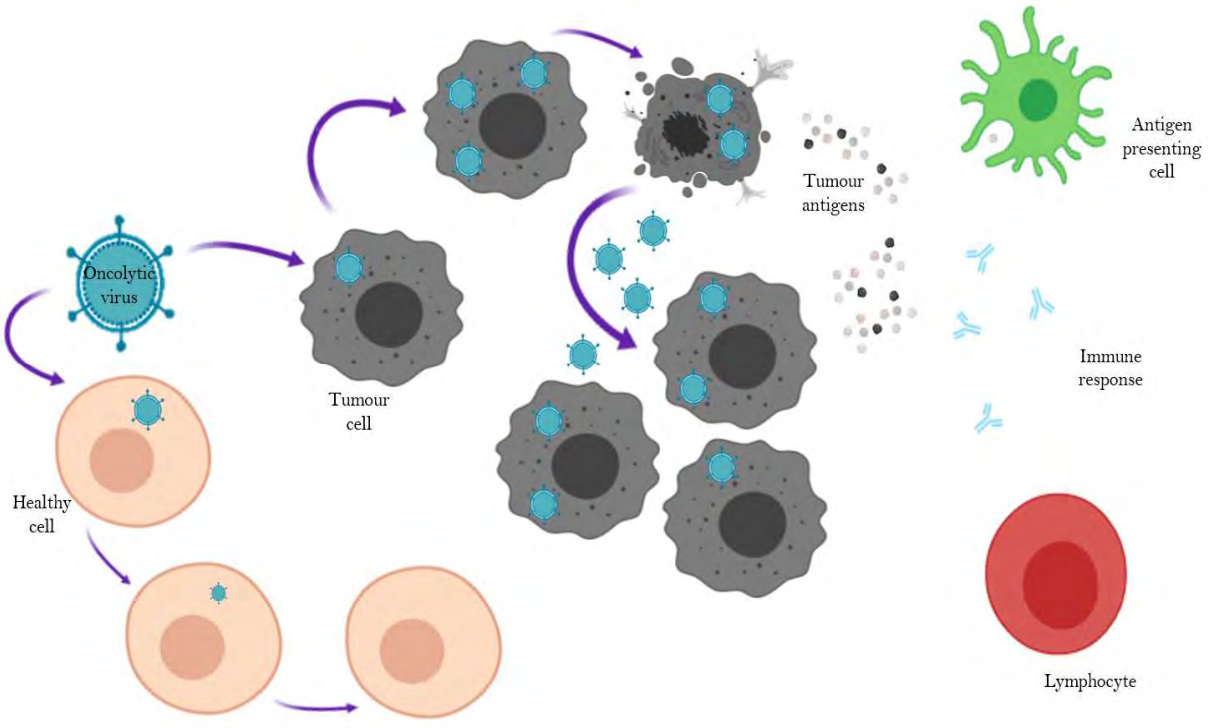


Figure 21: Schematic representation of oncolytic viruses

When examining the "hallmarks of cancer",[1] one can notice properties of the cancer cell that are ultimately beneficial to viral replication. These include sustained proliferation, resistance to apoptosis, and immune evasion. In addition, certain malignant cells readily present or up-regulate membrane receptors necessary in viral entry as well as certain signalling pathways which are appropriated by the oncolytic virus (e.g. wnt/β-catenin pathway) and the downregulation of antiviral (Interferon) and anti-proliferative signalling.

The microenvironment of a tumour (TME) is comprised of stroma, vasculature, immune cells, fibroblasts and signalling molecules. In most neoplasms, the TME is characteristically immunosuppressed and therefore a compelling niche for viral replication. Viral infection of the tumour and lysis of tumoral cells causes local inflammation, innate immune activation, and danger signal (DAMPs and PAMPs) liberation, ultimately turning an immunologically cold tumour to hot. Furthermore, the release of tumour neo-antigens could potentially act as an *in situ* tumour vaccine.

Examples of viruses used for their oncolytic properties are presented in the table below:

	DNA	RNA	
		Positive strand	Negative strand
Single-stranded	- Parvovirus (MVM, LuIII, H-1PV)	- PVSRIPO derived from Polio virus - Coxsackie virus - Toca 511 derived from amphotropic murine leukemia virus - Zika virus	- Measles virus (MeV) - Influenza virus - Vesicular stomatitis virus (VSV) - Newcastle virus (NDV) - Mumps virus - Maraba virus
Double-stranded	- Poxvirus (VACV, MYXV) - Herpes Simplex virus (HSV) - Adenovirus (Ad)	- Reovirus	

Table 3: Oncolytic viruses currently investigated

There has as yet not been any comparisons between these viruses, but those with a tissue-specific tropism may be preferable when combating tumours originating from these tissues. Oncolytic viruses are sought out for their natural ability to replicate preferentially in tumours, but can be engineered to increase their therapeutic potential.[84]

Increasing the anti-tumour potential of OVs can be done by modifying receptor tropism or by including site-specific targets such as through a tumour-specific promoter. Therapeutic potential can also be increased by inserting certain transgenes, whereas the OV acts as a vector on top of its oncolytic manoeuvre. OV-delivery of therapeutic genes include prodrug convertases, toxins, sodium iodide symporter for radiotherapy, and immunomodulators.

It is also possible to act on the TME by encoding factors that degrade the extracellular matrix (matrix metalloproteinase for example) or fusogenic proteins.

Finally it is possible to engineer the deletion of virulence factors (e.g. thymidine kinase) or include tracers allowing the monitoring of viral spread and pharmacokinetics. These include marker proteins or imaging reporters, demonstrating the theranostic (diagnostic and therapeutic) aspect of oncolytic virotherapy.

In parallel with viral vectors, it is important to evaluate the safety of the final drug with regards to the viral strain used and the genetic changes applied to it, in addition to finding the optimal route of delivery.

To adapt oncolytic viruses to clinical situations it is important to know the pharmacokinetics and pharmacodynamics as well as the potential drug combinations, particularly immunomodulators.[85] In order to do so it is primordial to understand the mechanisms of action involved, potential resistances and biomarkers of response.

Finally, in the new-fangled age of personalized medicine, a goal of oncolytic virotherapy is to adapt the virus in question to a specific tumour expression or immunological profile.

The first oncolytic virus used in the US was Talimogene laherparepvec (**Imylgic**), a modified herpes virus encoding the granulocyte-macrophage colony-stimulating factor (GM-CSF), and approved by the FDA in 2015 for the treatment of Melanoma.

Bacterial and yeast gene delivery

The use of bacteria for gene therapy delivery falls into the category of 'non-viral' vectors, explored for their safety and efficient delivery of genetic material to target cells. Similarly to viral vectors, bacterial vectors can either be used for specific intra-tumoral replication or for plasmid transfer into target cells, known as bactofection.[86]

Tumour specific replication

Certain bacterial strains have been shown to have an effect on tumour populations. These strains include *Clostridium perfringens*, Bifidobacterium, Salmonella, *Escherichia coli*, *Vibrio cholera* and *Listeria monocytogenes*. The exact mechanisms for specific tumour localisation and replication of these bacteria are still being investigated and certain theories elucidated.

Initially, this phenomenon was thought to be linked to the hypoxic nature of certain solid tumours. Indeed, low oxygen levels in solid tumours, supporting the development of intra-tumoral necrotic regions, is largely due to the rapid growth of tumours and insufficient blood supply. These features were believed to act as a niche for anaerobic or facultatively anaerobic bacteria, with necrotic regions further providing nutrients such as purines, as well as chemo-attracting agents such as aspartate, ribose and galactose to name a few.

However, other elements of the tumour microenvironment, independent of bacteria or tumour origin, also contribute to tumour colonising bacteria.

The fenestration in neoangiogenic tumour vasculature would promote circulating bacteria to enter and replicate inside the tumour. Similarly, IV administered bacteria have been found to target and reside in cutaneous wound during healing, although contrary to tumours, bacterial presence in healing wounds was transient. This is most likely due to clearance by the immune system which is often lacking in tumours, caused

by a variety of mechanisms employed by cancer cells to avoid detection by the immune system.

With the accumulation of bacteria in tumours, non-invasive strains can be engineered to secrete therapeutic proteins or to act indirectly on the tumour microenvironment such as with anti-angiogenics and immune therapies.

Furthermore, given their tumour-specificity, bacteria vectors can be administered systemically or be engineered to express imaging agents allowing detection of bacteria in tumour sites – a valuable tool in cancer diagnostics and prognostics. Several reporter systems for imaging tumours are being explored: fluorescent and luminescent genes are available for bacteria as well as positron emission topography (PET) scanning combined with thymidine kinase (TK) gene-expressing bacteria.

Bactofection

Bactofection describes the use of bacteria as vectors carrying plasmids of therapeutic intention and to deliver it to target cells. In this case, the bacteria enters host cells, by active invasion of non-phagocytic cells (e.g. most tumour cells) or endocytosis in phagocytic immune cells (as in genetic vaccination), then is lysed either spontaneously or through induction to release the transgene within the target cell.

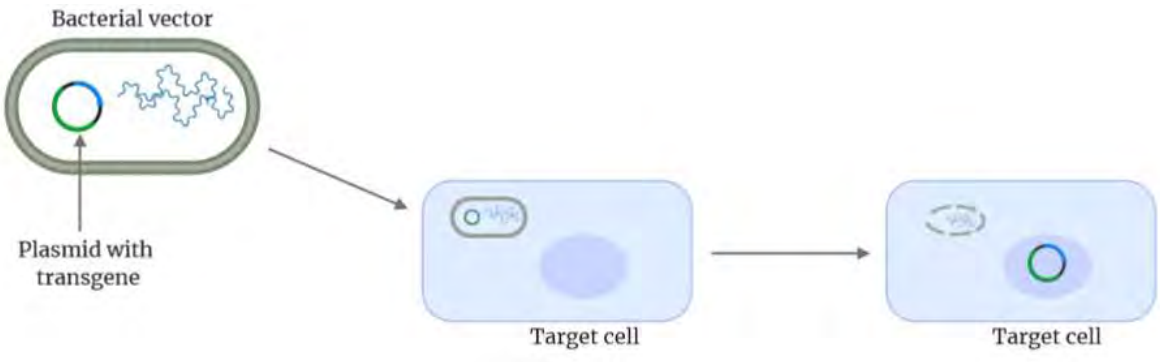


Figure 22: Mechanism of bactofection

Bacteria used in bactofection include Salmonella spp., L. monocytogenes and E. coli. Unlike Salmonella and E.coli which remain in the host cell's phagosome, L. monocytogenes is able to escape the phagolysosome, allowing a more efficient delivery of its contents into the cytosol. Preclinical trials have shown promising results of anti-tumour responses using these bactofection vectors in addition to vaccination strategies when targeting antigen presenting cells (APCs).

Plasmids used in bactofection resembles those used with other non-viral vectors, containing a bacterial origin of replication, an antibiotic resistance cassette and the transgene, usually under the dependence of a constitutive, ubiquitous eukaryotic promoter, such as the early viral promoter of CMV.

There are certain areas of concern using this technique which includes the risk of infecting healthy tissue when using such pathogenic strains. Furthermore, unlike nuclear-replicating viruses that deliver DNA directly to the target organelle, bacteria-based vectors deliver plasmids to the cytoplasm leaving trafficking to the nucleus as a significant rate-limiting step. Although, it is possible to use bacterial vectors to carry other molecules, such as RNA or proteins, thus trouncing this obstacle step.

Transfection efficiency depends on the bacterial vector chosen and the target cell. It is, however, possible to increase intracellular plasmid release by incorporating the phage lysin genes and/or use of antibiotics. Also, non-invasive bacteria can be engineered into bactofection agents through the incorporation of invasive genetic elements, for example the recombinant E. coli expressing the invasin gene from Yersinia pseudotuberculosis.

Yeast

Yeast organisms have contributed to research in cancer through understanding and elucidating the mechanisms of tumorigenesis, the discovery of potential targets (i.e. with small-molecule drug assay), production of anti-cancer drugs and more recently, in therapy with yeast-based vaccinations.[87]

Indeed, they are of particular interest in gene therapy vaccines due to their time- and cost-efficiency and their simplicity of engineering. Despite being non-pathogenic, the

recombinant yeasts explored for vaccines are engineered to present tumour associated or specific antigens (TAAs/TSAs) and have shown to induce immunological responses by being taken up by APCs, such as dendritic cells and macrophages, and surface presentation of these foreign antigens to lymphocytes. Furthermore, certain components of yeasts such as their cell wall, due to their microbial-like composition, are natural adjuvants to vaccines with the ability to stimulate or modulate immune responses.[88]

The most commonly used yeast for vaccines is *Saccharomyces cerevisiae*, having undergone extensive safety assessments for human delivery. Various preclinical and clinical trials are underway of S. cerevisiae vaccines for multiple types of cancer, including pancreatic cancer.

Combination therapy

Combination therapy allows for reduced toxicity, synergistic effects or multiple targeting against a pathology characterised by a high plasticity of cells and by the development of resistance during treatment. With regards to gene therapy, combination treatments can be through the association of a gene-based therapy with conventional treatments (chemotherapy, radiotherapy, surgery); or combination with another gene therapy product.

Combining gene therapy products can be done by co-administering the two different vectors each expressing their gene of interest, although both vectors must deliver their contents to the target site and have their material expressed with these cells to have an efficient combined treatment. To ensure both genes arrive at destination it is possible of incorporating them both into one construct, each containing its promoter, thus limiting the amount of supplementary genetic material to administer.

It is important to take into account the risk of competition between the promoters used for each transgene at the risk of selecting the expression of one over the other. A useful tool in the expression of multiple genes in one construction is the internal ribosome entry site (IRES) element. IRES are RNA elements within the mRNA sequence that allow direct recruitment of ribosomes for gene translation (known as polycistronic mRNA).[64] Therefore by separating the transgenes with IRES sequences it is possible to express both genes of interest from one transcription unit and obliterate any competition between the two. Furthermore IRES are often induced under stress conditions such as hypoxia, found in solid tumours. However, polycistronic systems vary depending on the IRES used, the genes expressed and the relative distance of the genes to the IRES sequence – it is therefore recommended to place the more important gene in front of the lesser.

Another strategy is to use the 2A peptides found in a multiple of viruses.[64] These self-cleaving peptides cause ribosome skipping, instead of true proteolytic cleavage, which results in multiple polypeptides being translated from the same mRNA. Different 2A peptides, named after the viral strain in which they were first discovered, have different efficiencies of self-cleaving and may result in unpredictable outcomes. By combining

2A peptides and IRES sequences, it is possible to generate four separate peptides from one transcript.

As mentioned previously, oncolytic viruses are used for their tumour-targeting abilities but can also be engineered so as to express additional genes to help eradicate cancerous cells. Such as with viral vectors, the possibility of engineering OVs depends on the virus used and the intended target.

An example of an engineered OV is the Cancer Terminator virus (CTV).[89][75] The CTV is a chimeric tropism-modified type 3 and 5 adenovirus that replicates selectively in cancer cells. This chimeric adenovirus encodes the melanoma differentiation associated gene-7/Interleukin-24 (mda-7/IL-24), a multifunctional anti-cancer toxic cytokine which is secreted and stimulates the immune system to attack distant metastatic tumours.[37][90] Combining the CTV with ultrasound targeted microbubble destruction (UTMD, the sonoporation of gene-carrying vectors) allows protective systemic transport of the CTV in microbubbles and focal release of the trapped material by ultrasound.

Finally, it is possible to associate a gene therapy treatment with current treatments. This can either be with chemotherapies such as in studies of triblock copolymer containing siRNA associated with cisplatin in the NF-Kappa B (NF-KB) targeted treatment of metastatic breast cancer[66][91] or adenoviral vector containing p53 gene therapy associated with cabazitaxel in metastatic prostate carcinoma.[92] Although less common, gene-based treatments have also been studied in association with radiotherapy or surgery.

Neoangiogenesis and Enhanced permeation and retention (EPR)

As tumours grow, they develop the formation of new blood vessels, known as neoangiogenesis. These blood vessels, however, differ from normal vessels by their disorganisation with incomplete endothelial linings and blind ends which are also found in lymphatic capillaries and wound healing blood vessels. Furthermore, blood vessels in solid tumours are often found to have the characteristics of high endothelial venules (HEVs) typical of lymph nodes. These tumour-associated HEVs (TA-HEV) may act as important entry points for infiltration of cytotoxic T cells into solid tumours.[93]

Normal inflammatory tissue and tumour tissue both display similar vascular permeability or macromolecular extravasation.[94] Indeed, clearance of a macromolecule such as albumin is observed to be much faster in normal tissue than in solid tumours due to the poor architecture and fenestration of endothelial cells of the rapidly forming neoangiogenic vasculature making it highly permeable. This, as well as a defective lymphatic drainage system habitually found in solid tumours, led to the controversial concept of enhanced permeation and retention (EPR).

EPR is a phenomenon where macromolecules, such as nanoparticles used in gene therapy, accumulate in the tumour microenvironment augmenting the passive targeting of these therapeutics to the site of action therefore improving on pharmacokinetics as opposed to conventional therapies.[95]

Although promising in preclinical models of rodents, this was found not to be the case in humans, with only 0.7% (median) of administered nanoparticles to be delivered to a solid tumour.[96]

The bystander effect

The bystander effect describes the result of a therapeutic agent on cells either nearby or distant from the exposed target.[97] This is mainly due to the transfer of signal or

factors from the targeted cells to those surrounding. These bystander effects were initially described following radiation therapy but can also be observed after treatment of diverse stress-inducing agents.

Studies have shown that the bystander effect can be either damaging or helpful. Indeed, in some cases peripheral cells showed genomic instability, unstable clones and hypersensitivity; while in other cases bystander cells were reported to mitigate the damages of exposed cells, known as the ‘rescue effect’. Some studies have shown no bystander effect, showing us that this reaction is dependent on a variety of factors.

IV. PRODUCT DEVELOPMENT: FROM LAB TO PATIENTS

Once the gene therapy construct has been decided upon and the target defined, there are steps to be carried out before the final treatment is put on the market. This section will describe the course of action of a gene therapy product to a patient-administered drug.

After determining gene, cell function and cell targets, and the best corresponding transgene and vector, there are two major steps to go through before arriving at a final therapeutic product; steps that take years of collaborative work to accomplish. These steps are grossly divided in non-clinical studies and clinical trials.

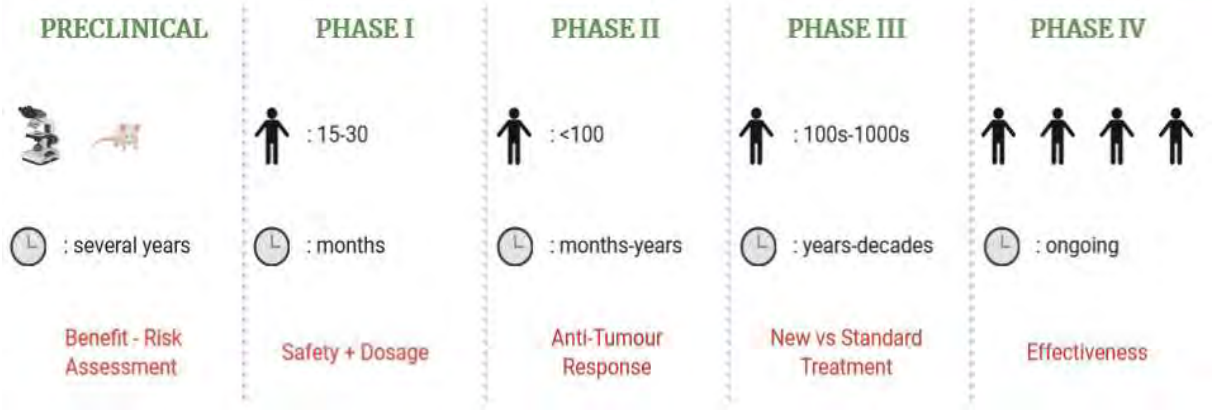


Figure 23: Illustration of preclinical and clinical studies

The EMA has issued guidelines on the 'quality, non-clinical and clinical aspects of gene therapy medicinal products' (EMA/CAT/80183/2014).[98]

This half of the process is further separated in proof of concept (POC) of the product and preclinical studies *in vivo*. These studies are to provide sufficient information related to the efficacy, safety, dose and optimal application of potential treatments before human use.

In vitro studies:

The 'proof of concept' chapter involves primarily *in vitro* experiments with the transgene-vector construct resembling as closely as possible the one intended for human administration. Similarly, the cellular models used to investigate the novel GTMP should take after the malignancy for which the treatment is intended.

The *in vitro* models can be either two-dimensional (2D) or three-dimensional (3D). These models are composed of either established cultures stored in cell banks (the American Type Culture Collection (ATCC)[99] for example) or primary cells isolated directly from donors. Even though primary cells closely mimic the genetic characteristics of tumours, they are in general difficult to isolate and have a short life span, contrary to established cell lines. Cell cultures can either be adherent to a surface, such as glass or plastic culture dishes (e.g. fibroblast cells) or be cultured in suspension (e.g. lymphocytes).[100]

2D cultures are more cost-effective and easier to grow than 3D cultures with more readily available tests and culture media. On the other hand, 2D cultures bring forth a loss of the cell-to-cell and cell-to-environment interactions readily found in tumours. This leads to changes in cell characteristics such as morphology and cell division, intracellular functions and a loss of polarity. These characteristics are more easily preserved in 3D cultures. In addition, 2D cultures have unlimited access to nutrients, oxygen and other compounds which is not the case in 3D cultures and tumours whose access depends on the architecture. Architecture in 3D models resembles that of

tumours, with apoptosis in the central part and cell growth, gene expression, signalling and metabolism depending on topology.[100][101]

	2D culture	3D culture
Time for culture formation	Minutes-hours Long-term cultures easy to maintain	Hours-days Long-term cultures difficult to maintain
Cell characteristics	Different morphology and cell division, loss of phenotype and polarity	Preserved morphology, cell division, phenotype and polarity
Cost of maintenance	Cheap, commercially available tests and media	Expensive, time-consuming and less commercially-available components
Exposure to oxygen, nutrients, metabolites and signal molecules	Unlimited access	Variable access depending on structure architecture
Drug sensitivity	Little resistance – inaccurate portrayal of drug effects	More accurate depiction of drug effects, metabolism and resistance
Gene and protein expression	Different gene and protein expressions to those found *in vivo*	Often similar gene and protein signatures to their *in vivo* tissue origin
Data analysis	Data easily replicable and interpretable	Difficult to replicate and interpret experiments

Table 4: General differences between 2D and 3D cell models

3D culturing techniques include scaffold based techniques[101] such as hydrogel-based support, polymeric hard material-based support, hydrophilic glass fibre, and organoids; and scaffold-free methods such as hanging drop microplates, magnetic levitation, and spheroid microplates with ultra-low attachment coating (*Annexe 3*).[102]

Multicellular tumour spheroid (MCTS) is an example of cancer cell aggregates grown in 3D to mimic the *in vivo* TME. Different microenvironments found in various tumours can be replicated using different culture methods of these MCTS. These methods include static suspension, hanging drop methods, magnetic levitation, spinner bioreactor, rotational bioreactor, microfluidic system, and gel embedding.[102]

Another 3D cancer model gaining popularity is the tumour-on-a-chip technique which has proven useful in drug testing studies even though limited by a lack of vascular network.

Another recent and interesting use of three-dimensional models is the 3D biomimetic microtissue used to culture cancer cells, effectively modelled to mimic cancer metastases.[101][102]

Spheroid structures are simple clusters of cells generated through a variety of techniques and are able to provide intensive cell-to-cell contacts with excellent regenerative properties. Spheroids can mimic complex tissue morphologies through co-culture and can include the incorporation of biomaterials to improve function and shaping of the spheroid models.[103] Organoids are more advanced structures that resemble a near-physiological (or pathological) tissue organisation mirroring to a certain degree organ functionality.[104] Organoids are useful in understanding tumour modelling and gene and cell function in cancer.

Both 2D and 3D models can be used in co-cultures.[100] Co-cultures involve the growth of different types of cells in a same culture so as to explore the interactions between them. Co-cultures are either direct, mixing the cells, or indirect where different cells are separated by a physical barrier.

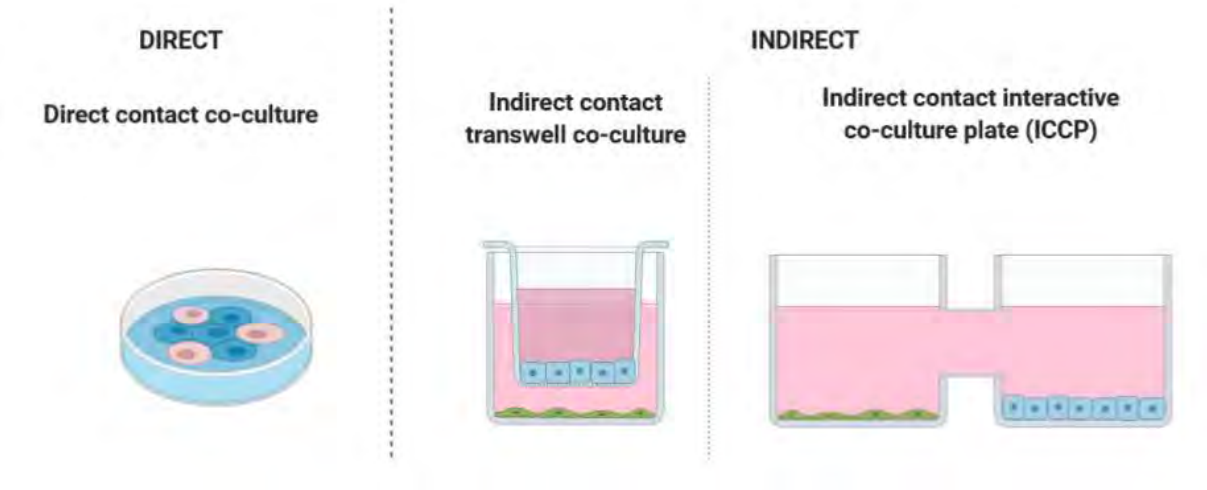


Figure 24: Cell co-culture techniques

In vivo studies:

Murine cancer models offer the most advanced preclinical opportunity to investigate the complexities of human cancers, with continuous development to optimise

preclinical efficacy to guide clinical trial designs. Multiple murine models are currently used:[105][106]

- Cell line-derived xenograft (CDX) model: tumour cell lines are transplanted or injected in immunocompromised mice. This is the most time and cost-consuming model. Although CDX models represent the genetic aspects of the tumour cell lines, they fail to predict human efficacy for most cancer protein-targeted therapies.

- Patient-derived xenograft (PDX) model: surgically-obtained tumour samples from patients are subcutaneously or orthotopically implanted in immunocompromised mice (nude, SCID, and NOD/SCID strains). The tumour is fully-formed after around 2-4 months. PDX preserve tumour architecture and the histologic and molecular heterogeneity characteristics of those in patients. Transplant take rates and tumour growths vary according to the samples and quantity used as well as tumour origin and recipient strain. Similarly to primary cells, therapeutic studies are the most representative in low-passage models.

- Genetically engineered mouse (GEM) cancer model: mouse genome is altered through genetic engineering techniques so cancers are developed intrinsically with stroma in immunocompetent mice. Although being the most complete representation of cancer development, GEM models are the most challenging to work with.

- GEM-derived allograft (GDA) model: tissue fragments from GEM tumours or metastatic lesions are engrafted subcutaneously or orthotopically in an immunocompetent syngeneic mouse. GDA models combine the genetic similarities of GEM mice to the ease of PDX transplantation technology and is of particular interest when investigating metastases. Indeed, a GDA model takes less time to develop (1-2 months) than GEM models and is less challenging than PDX models. Stem-cell derived chimeric mice are engineered through implantation of GEM-derived or genetically manipulated embryonic stem cells (ESCs) into pre-implantation embryos thus generating mice chimeric for mutant and wild type cells.

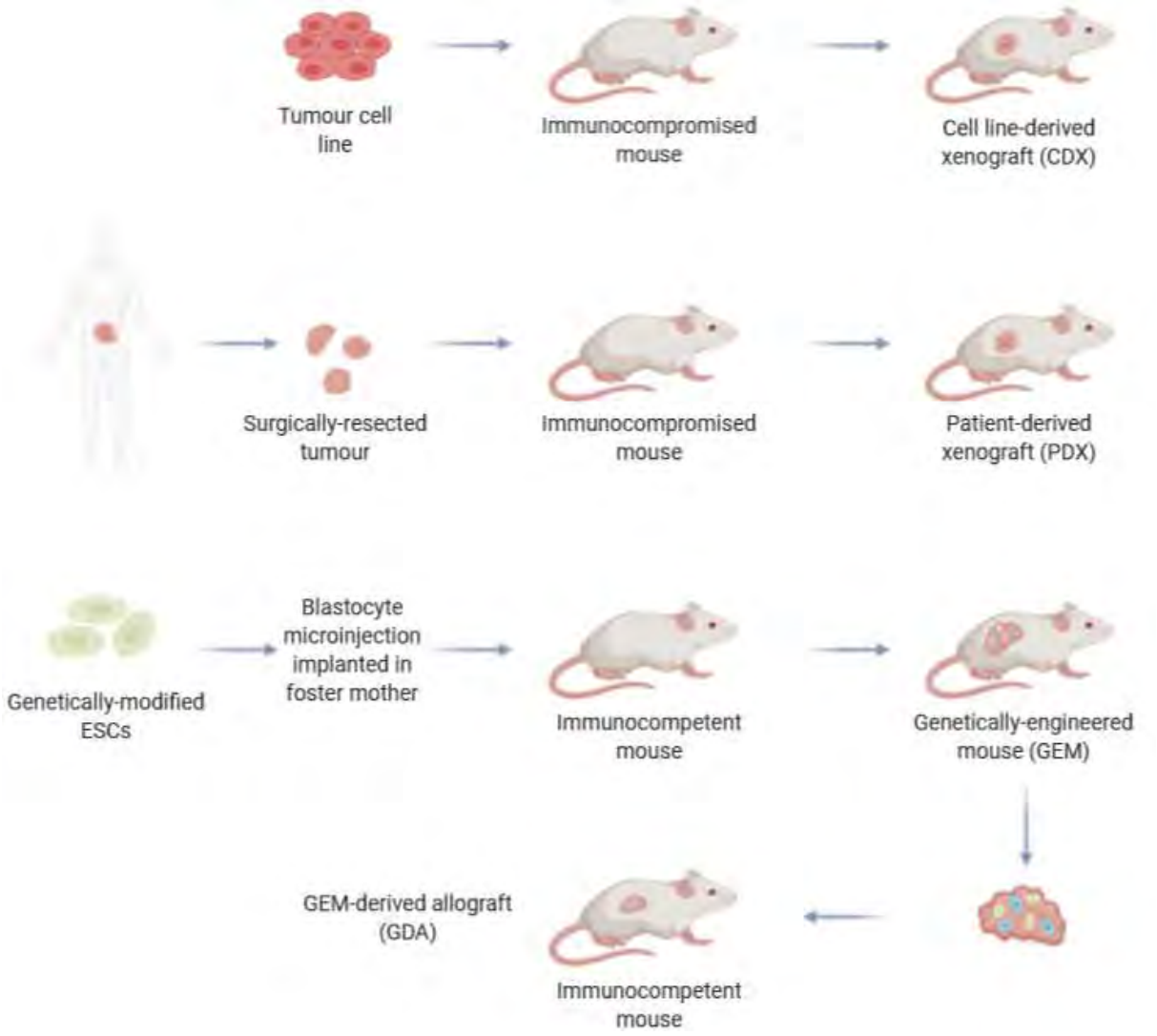


Figure 25: Preclinical mice models

There are various requirements of preclinical experiments before moving on to clinical trials and human use. Similar GTMPs may be used as scientific guidance but because of the singularities of each gene-based product, the non-clinical study program should be done on a case-by-case basis. These studies are carried out according to the good laboratory practice (GLP) measures to ensure the uniformity, consistency, reliability, reproducibility, quality, and integrity of pharmaceutical non-clinical safety tests.

Pharmacodynamics include the demonstration of expression and production of the correct transgene in the appropriate target organ (specificity) supporting the related

biological effect to molecular mechanism of action. In addition, any consequences of aberrant gene product formation has to be elucidated.

Biodistribution studies have to include information on all organs, investigating GTMP persistence, mobilisation and shedding with fitting observation time periods.

The first **dose** and schedule of administration used in clinical trials is based on the rationale justifying that of which the gene transfer is assumed to modify the disease pathway.

These dose recommendations need to take into account **toxicity** studies. The toxicity studies are to be carried out using the dose, route and number of administrations intended in clinical stages. The toxic potential of GTMP products is influenced by the number of vector particles and their composition, the expression and/or integration of the delivered gene and drug substance purity.

Other preclinical studies include: Immunogenicity/toxicity, repro- and genotoxicity, carcinogenicity and tumorigenicity as well as approval of delivery devices and excipients.

Certain preclinical studies are in accordance to the type of gene therapy or vector. For example, integration studies, germline transmission and immune-toxicological safety when dealing with plasmids and nucleic acid vaccines. Viral and bacterial vectors are accompanied by risks of replication-competence and genomic integration, latency/reactivation, shedding and immunogenicity that need to be explored.

Selectivity of tumour cells over normal cells of oncolytic viruses needs to be established in cell models before *in vivo* explorations. Furthermore, with OVs, selection of the animal model, pharmacology studies and toxicity should take into account the viral strain used and the intended indication.[107]

Clinical trials

Clinical trials are put in place to determine the safety and efficacy of medicine in humans. In the European Union this will be under regulation (EU) No 536/2014, building on the existing EU Clinical Trial Directive (EC) No. 2001/20/EC, in order to harmonise the safety and transparency of trial information through a clinical trials information system (CTIS). The CTIS is to be set in vigour in December 2020 and although it outlines the centralised functional specifications, the clinical trials authorisations and oversight will be dependent on the member states (*Annexe 2*).

Ethical principles for medical research involving human subjects follow the Declaration of Helsinki, a policy drafted up by the World Medical Association (WMA). Physicians are further bound by the Declaration of Geneva and the International Code of Medical Ethics ensuring the patient's central role and safety in treatment strategies. The good clinical practice (GCP) is an international standard regarding ethical and scientific quality for creating, recording and reporting trials.

Patients are recruited or revoked from a clinical trial according to the inclusion and exclusion criteria respectively. Outside of exceptions, patient enrolment is only possible after having obtained informed consent from each subject or the subject's legally authorised representative. Trial protocols are divided into different phases:[108]

- Phase I:

This first phase is to determine a safe dose of treatment through escalated increase until reaching the maximum tolerated dose (MTD) as well as establishing the potential dose-limiting toxicity (DLT) and the highest dose with acceptable toxicity (RP2D). This phase is either a first-in-man study, evaluating a new drug, or can be used to explore different administration routes and duration of administration. The general effect of the treatment on the body is also recorded, an example being the side effects graded by the CTCAE (Common Terminology Criteria for Adverse Effects).[109] Around 15 to 30 people take part in a phase I trial and, contrarily to other drugs (phases Ia in healthy volunteers and Ib in patients), is uniquely performed on patients after failure of their

standard treatment. The patients enrolled are, apart from the targeted malignancy, as healthy as possible to avoid variability between subjects.

- Phase II:

Usually less than a hundred patients are enrolled in this phase of trials which determines the anti-tumour effect of the treatment on the targeted cancer(s) or cellular/molecular defect. A method of measuring the response to an anti-cancer drug is by using the RECIST (response evaluation criteria in solid tumours) guidelines.[110] Through the use of imagery such as X-rays, CT scan or MRI scan, it is possible to evaluate whether a solid tumour has shrunk, gotten bigger or stayed the same. RECIST criteria then divide the patients' response into several categories:

Complete response (CR)	Disappearance of all target lesions. Any pathological lymph nodes (whether target or nontarget) must have reduction in short axis to <10 mm.
Partial response (PR)	At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters
Progressive disease (PD)	At least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm (Note: The appearance of 1 or more new lesions is also considered progression).
Stable disease (SD)	Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study

Table 5: RECIST criteria [110]

The success or failure of this phase depends on the tumoral context and the intended effect of the studied therapy.

- Phase III:

The purpose of a phase III is to compare the new treatment (or new use of treatment) to the current standard therapy. The treatment objectives are well defined by this stage – target tumour localisation, progression stage etc. This step includes hundreds to thousands of patients to confirm its efficacy and is most often randomised and multicentric. The efficacy criteria studied here are much lengthier and so this phase is

generally more time-consuming than the previous. These include progression-free survival (PFS), overall survival (OS) and survival without relapse. Other objectives are to evaluate the effectiveness, monitor minor side effects and collect information that will allow the safe use of the medication.

- Phase IV/pharmacovigilance:-

This phase is undertook after licensed approval of the treatment. These postmarketing studies allow for perpetual evaluation of benefits and risks during the entirety of the drug's active medical use. Other studies include pharmacokinetics and bioequivalence in specific populations (paediatrics, geriatrics…).

Some protocols have trials that combine two phases, phase I/II or phase II/III, allowing for questions to be answered quicker or inclusion of fewer patients, but also tend to have additional inclusion criteria. This is especially the case of phase I/II trials in onco-haematology.

Clinical trials for GTMPs generally follow the same regulations as clinical trials for other medication in oncology, although given the biological complexities of these treatments, certain considerations are to be reviewed.

Firstly, the balance between benefits versus risks of using a GTMP product in comparison to conventional therapies needs to be accounted for while considering the indication at hand. When using a biological vector (viral or bacterial) the choice has to be justified regarding the tropism of the microorganism used. Furthermore, the ICH (international conference of harmonisation) provides guidelines on the possible choices of control groups and alternatives if necessary as well as guidelines for trials in small populations when the conditions targeted for treatment are extremely rare.

The usual **pharmacokinetic** studies evaluating absorption, distribution, metabolism and excretion (ADME) are usually not needed for gene-based treatments. However, depending on the GTMP studied, other studies are to be carried out which are decided upon on a case by case basis.

An example of these studies includes viral and vector shedding and the potential of transmission or lack thereof (which has to be justified) to a third party. The ICH provides recommendations on the design of these studies. On top of shedding analyses, dissemination, clearance and possible germline transmissions of the GTMP/vector need to be evaluated. These will contribute to the planning of long-term follow-up studies.

Biodistribution analyses need to take into account the intended target, indication and administration as well as vector kinetics. Different techniques can be used for these studies which can be invasive, such as biopsies and fluid collection, or non-invasive techniques like imaging techniques when invasive ones are unfeasible.

In addition, pharmacokinetics of the expressed transgene (e.g. product protein) are also to be carried out including drug concentration and half-life. The correlation between the level and duration of expression and clinical efficacy/safety have to be determined. Other undesired aspects such as expressed vector genes, genetic polymorphism and the potential interference of residual endogenous proteins also need to be investigated.

Pharmacodynamics are performed to evaluate either the function/expression of the nucleic acid, of the expressed drug or sometimes of the vector itself (for example with oncolytic viruses).

An important aspect of GTMP clinical trials is the **immune** aspect. This includes the potential pre-existing immunity to the vector or the immune response triggered by the transgene or the vector, especially when considering multiple administrations. An evaluation of the cellular and humoral immune responses to the genetic or vectoral component is to be provided in relation to treatment timing and safety/efficacy.

Efficacy studies are to be carried out in the target population supporting the proposed posology and evaluate the duration of therapeutic effect. Clinical evaluation is done according to the intended timing of treatment efficacy.

The **safety** of GTMPs is very important and has to be considered throughout all the steps of the treatment procedure. Indeed, not only are the side effects linked to the transgene product or vector logged into a database, but the safety of administration

route (invasive procedures, the need of local/general anaesthesia) and the use of immunosuppressants or chemotherapeutics should also be addressed. Other safety considerations include the potential off-target effects (unintended transduction of tissues, for example) or malignancies induced.

With replication-competent viral vectors and in particular **oncolytic viruses** there are specific technical challenges, reviewed in the ICH recommendations for OVs.[107] These reviews include pharmacokinetics, pharmacodynamics, and biological activity monitored primarily through polymerase chain reaction (PCR) and infectivity assays. Other criteria include biosafety, immunity and immune responses with extra consideration to minimise exposure of third parties, especially people with suppressed or compromised immune systems. When determining the appropriate route of administration, a step-by-step approach has often been used. This involves starting with intratumoral injection, moving on to regional or local administration and then to systemic administration.

A useful tool in controlling unwanted or excessive viral replication is by having an antiviral therapy at hand, a sort of antidote so to speak. For example, an out of control HSV oncolytic virus could be controlled with ganciclovir.

Exceptions

In some cases, subjects of research studies are considered particularly vulnerable or not in a position to give their consent. In these cases and depending on the context, their participation will depend on the benefits/risks of the proposed treatment, consideration of persons close to the patient and the research ethics committee or fellow physicians not involved in the research.

This is the case, for example, in compassionate (the 'hospital exemption' clause)[111] or emergency research trials of treatments which could potentially provide life-saving therapies to patients in life-threatening situations who cannot enter in clinical trials, improve emergency medical therapies that currently have poor clinical outcomes, or to advance knowledge through collection of information about effectiveness and safety. The ethical standpoint in these situations is primordial, with the patient's well-being in the centre of all decisions.

Although the EMA provides recommendations through the CHMP (Regulation (EC) No 726/2004), the regulation and implementation of these uses are ultimately up to the member state in question. For example, the UK issued the Mental Capacity Act in 2005 to guide health professionals in these situations.[112] The EMA underlines however, that compassionate use of treatment can only be applied to medicines undergoing clinical trials or that have entered the MAA process.

V. PANCREATIC CANCER AND THE DRUGS OF TOMORROW

A summary of pancreatic cancer

Pancreatic cancer (PC) is the fourth leading malignancy in developed countries, twelfth worldwide, with an occurrence of over 450 thousand diagnosed cases worldwide in 2018 and accounting for over 430 thousand deaths that same year.[113] It is estimated to rise to second place on the prevalence podium in the Western world by the year 2030, with worldwide incidence and mortality rates predicted to nearly double by 2040 (*fig. 26*).[114] Pancreatic cancer is mostly diagnosed in patients over seventy years of age but is increasing among the younger population, and slightly more common in males than females although this gap is getting narrower.

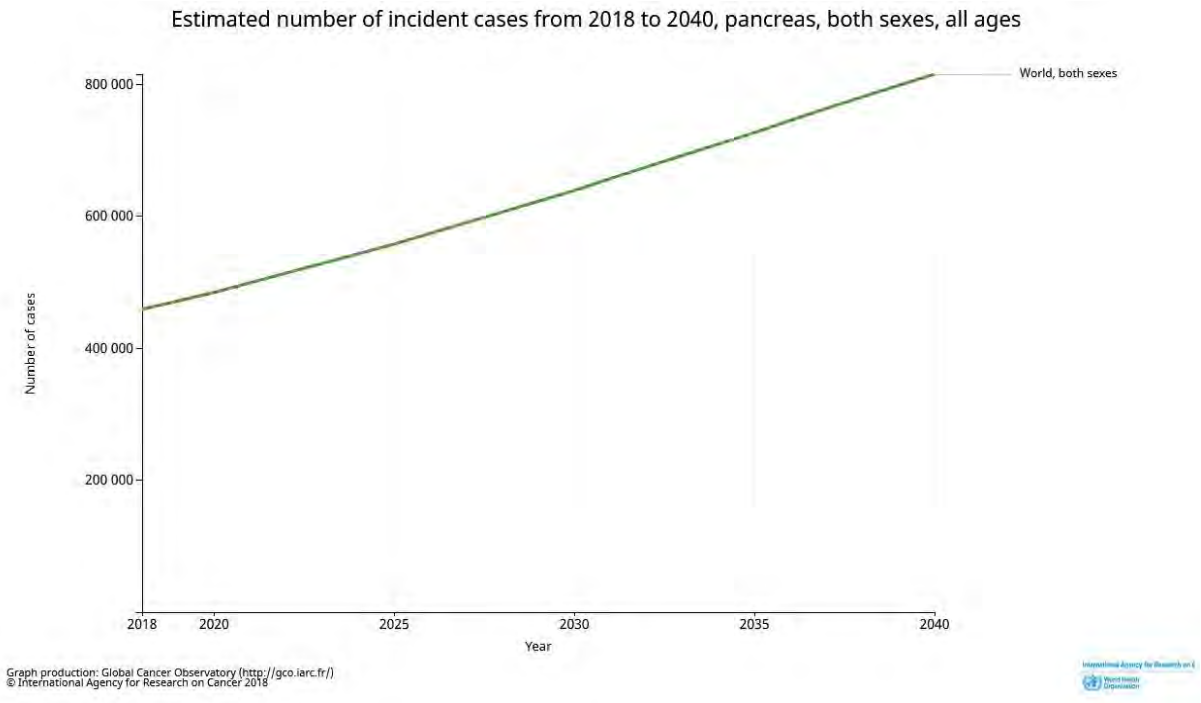


Figure 26: Estimated evolution of worldwide pancreatic incidence rate [114]

The risk factors associated to developing pancreatic cancer are multiple with many still remaining unasserted. These factors include chronic pancreatitis, excess tobacco and alcohol consummation, obesity, diabetes mellitus and genetic predispositions.

The most common and dire form of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), progressing from exocrine cells and accounting for nine out of ten cases of PC. Symptoms of PDAC are non-specific although tumours located in the head of the pancreas, which are more frequent (70-80%) than those in the body or tail (20-30%), tend to be symptomatic - and therefore diagnosed - at an earlier stage. These symptoms include an intense abdominal pain, jaundice and diabetes in half of the cases. Other less common symptoms include acute pancreatitis, thromboembolism, intestinal occlusion and other digestive problems.[115]

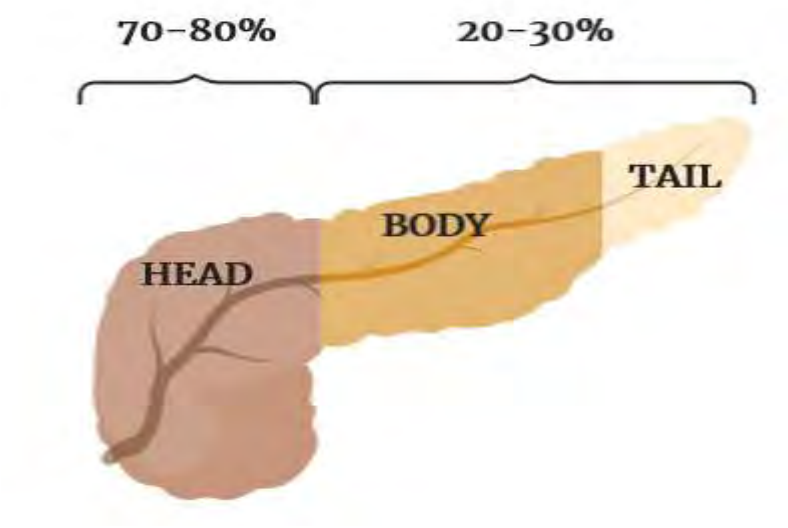


Figure 27: Tumour occurrence depending on localisation in the pancreas

Diagnostic of pancreatic cancer is often late since clinical signs generally appear at a later stage of disease progression. Indeed, a mere 15-20% of patients are diagnosed at a time point where the tumour is operable. Additionally, there are no early biomarkers of PC, further hindering early diagnostic. The carbohydrate antigen 19-9 (CA 19-9) found in serum has a high sensitivity and specificity but an insufficient predictive positive value for diagnostic purposes and is only used for prognostic information, as patients may lack the gene encoding for the chemical modification, and because Ca 19-9 levels can be elevated in patients with pancreatitis. Other biomarkers are under investigation, such as thrombospondin A2, circulating tumour DNA (ctDNA),

circulating tumour cells (CTC) and circulating extracellular vesicles, but are not yet validated for clinical use.[116]

PC is the digestive cancer with the worst prognosis, having a five year overall survival rate of 7-8%, all stages considered. The fact that the mortality rate is close to the incidence rate can be at least partly explained by the lack of an early diagnosis, an ineffective treatment plan and the high relapse rate.

The best therapeutic option remains surgery which, *in fine*, is only possible in less than a fifth of diagnosed patients accompanied by a high morbidity risk and a probability of relapse of around 80%. Tumours are divided in operable, borderline, locally advanced or metastatic.

There are several drugs available for the treatment of PC depending on disease progression, the results of tumour biopsy and patients' characteristics (age, well-being, co-morbidities etc.) but to this day pancreatic cancer remains an incurable pathology. **Gemcitabine** (Gemzar) is available, either alone or associated to **5-Fluorouracil** (5-FU) or **Nab-Paclitaxel** (Abraxane). 5-FU can also be used with **folic acid** (LV5-FU2) alone or in an association protocol of **FOLFOX** (Oxaliplatin + LV5-FU2), **FOLFIRI** (Irinotecan + LV5-FU2) or **FOLFIRINOX** (Oxaliplatin + LV5-FU2 + Irinotecan). In some cases, radiotherapy is integrated into the treatment plan as a neoadjuvant (before surgery) or for locally advanced tumours.[115]

These chemotherapy regimens are accompanied by numerous side effects and the risk of innate or acquired resistance. In addition, these treatment protocols are inadequate when faced with the cellular and molecular complexity of pancreatic cancers and their tumour microenvironment (TME).[117] Due to these shortcomings, gene therapy has a promising future where current treatments are lacking.

Many sources and databases exist that register clinical trials, either nationally or worldwide. The clinical trials studied henceforth were obtained from multiple references so as to have an utmost complete index. These sources include: the national institute of health (NIH) U.S. national library of medicine,[118] the Wiley 'Gene Therapy Clinical Trials Worldwide' online library,[119] the UMIN clinical trials registry (UMIN-CTR)[120] and the WHO international clinical trials registry platform (WHO-ICTRP).[121]

According to the Wiley 'Gene Therapy Clinical Trials Worldwide' online library, last updated in September 2019, there were 2333 ongoing or recently completed clinical trials of gene therapy, 1606 of them (68.8%) in relation to cancer diseases.

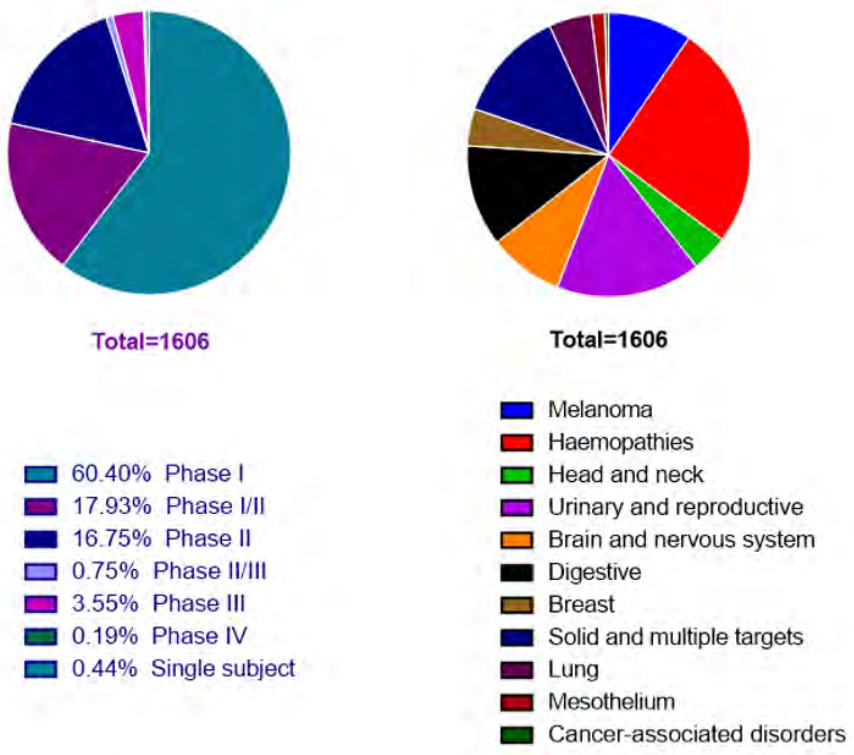


Figure 28: Distribution of gene therapy clinical trials in oncology 2019 according to phase of trial (left) and arbitrary category of tumour (right)

The great majority of ongoing cancer-related trials are in phases I to II (*fig. 28*). This could be explained by the fact that gene therapy is still an innovative field and so many

therapeutics are only just arriving at the clinical trial stage or failed in early phases. Another reason could be the fact that certain aspects of gene-based products are unforeseeable, especially when transitioning to *in vivo* administrations. This is especially the case when considering viral or bacterial vectors. To this regard, it would be interesting to know how many clinical trials do not make it to clinical trials or to the next phase.

When dividing these trials according to indication category instead of trial phase (*fig.28*), we can see that the majority are for haemopathies, followed by urinary and reproductive tumours. For clarification: i) retinoblastomas were included to the 'brain and nervous system' category, ii) solid and multiple targets include all unspecific targets (advanced carcinomas, for example) as well as studies aiming multiple organs or tumour types, iii) head and neck category also consists of cancers of the ear, nose and throat.

Pancreatic cancer makes up a significant portion of clinical trial indications of the 'Digestive' category as well as several 'solid and multiple targets' trials. After compilation of the current gene therapy trials enrolling patients with all forms of pancreatic cancer from the sources cited above, 51 trials are in an active or recruiting status, 58 trials have been completed and 23 have been prematurely terminated or withdrawn (*fig. 29*).

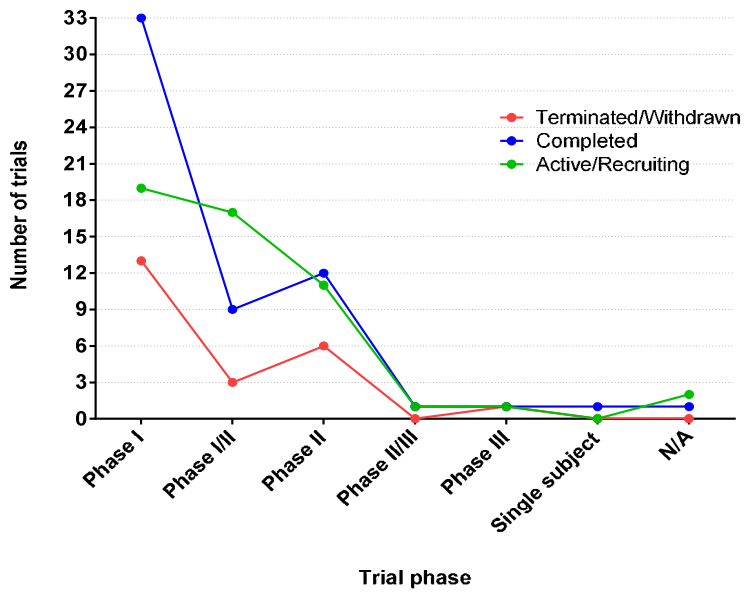


Figure 29: GTMP clinical trials for pancreatic cancer depending on phase and trial status

According to the Wiley online library a single patient compassionate study was carried out in 2011 in the USA for recurrent PDAC. This involves the intravenous delivery of bi-lamellar invaginated vesicle (BIV)-liposome enveloping a bi-functional shRNA-based composition (**bi-shRNAPDX-1**). This gene silencing iRNA targets the pancreatic and duodenal homeobox 1 (PDX-1), an oncogene that regulates the initiation and maintenance of pancreatic malignancy.[122] However, no other details of this study have been recorded despite hopeful results in preclinical models of mice and pigs.

It would be interesting to investigate the reasons behind the prematurely terminated and withdrawn trials and the status of those completed but which have not moved on to the next phase. An example is the phase III trial concluded in 2016 in the USA for a **HyperAcute** vaccine.[123] The Hyperacute vaccine consists in intradermally (ID)-administered irradiated allogeneic pancreatic cancer cells transfected to express the murine gene encoding Alpha-(1,3) galactosyltransferase. Murine alpha-gal epitopes induce a hyperacute rejection of the cancer cell allograft resulting in the rapid activation of antibody-dependent cell-mediated cytotoxicity (ADCC) towards allograft cells and, in consequence, endogenous pancreatic cancer cells. The murine alpha-gal gene is transfected in allogeneic pancreatic cancer cells by means of a retroviral vector. The results of the phase III have not yet been published and so whether this drug will be market-approved will have to wait and see.

Also, it is important to note that certain clinical trials are the succession of others found in the 'completed' section. For example, when looking at clinical trials in France, one can see a phase I trial completed and an ongoing phase II trial (*Annexe 4 (a)*), both on the study TherGAP[124] which, after positive results from the phase I completed in 2013, a phase II was launched in 2017.

So in order to decrypt the potential future drugs for pancreatic cancer and to avoid counting multiple times the same trial in different phases, the following analyses will be done on ongoing (active and recruiting) clinical trials and those completed in the last two years (2018≤), totalling up to 66 trials.

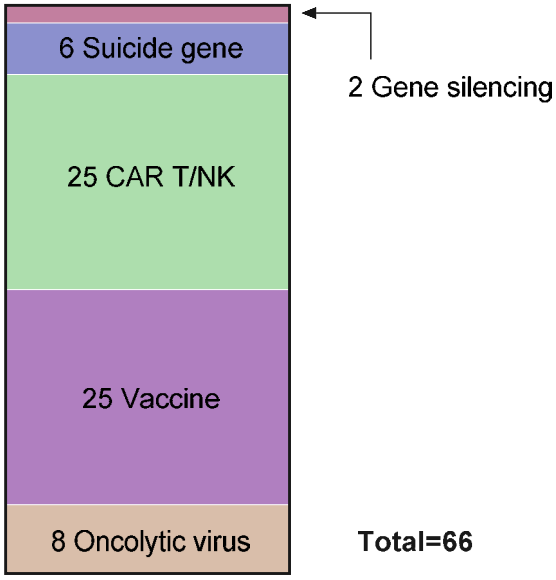


Figure 30: Ongoing or recently completed pancreatic cancer GTMP trials depending on gene-therapy technique

In these 66 trials, there are a variety of gene therapy techniques studied (*fig. 30*), the majority being vaccines and CAR-T/NK cells. There are also trials involving oncolytic viruses and suicide genes, and gene silencing being the least studied.

These GTMP compounds are either delivered in monotherapy or associated to chemotherapies, targeted therapies, immunotherapies and other treatments such as Stereotactic Body Radiation Therapy (SBRT), the benefits of these associations being a goal of the trials.

As a whole, biological (viral, bacterial and yeast) vectors are the more popular choice (*Annexe 4 (b)*) even though when separated, the naked/plasmid DNA category is the most popular (*Annexe 4 (c)*) especially for vaccines, followed by retrovirus and lentivirus vectors mainly in CAR-T cell engineering.

The most common route of administration studied in clinical trials is intravenous (IV) popular for CAR-T cell delivery. Other commonly studied routes include subcutaneous and intradermal exclusively to vaccines, and intratumoral, a predilection for OV delivery (*Annexe 4 (d)*).

Vaccines

As described in chapter III, vaccines are composed of genetically engineered cells that present tumour-associated antigens or epitopes to the immune system and prompting an immune response towards the tumour cells presenting these TAAs or oncoproteins.

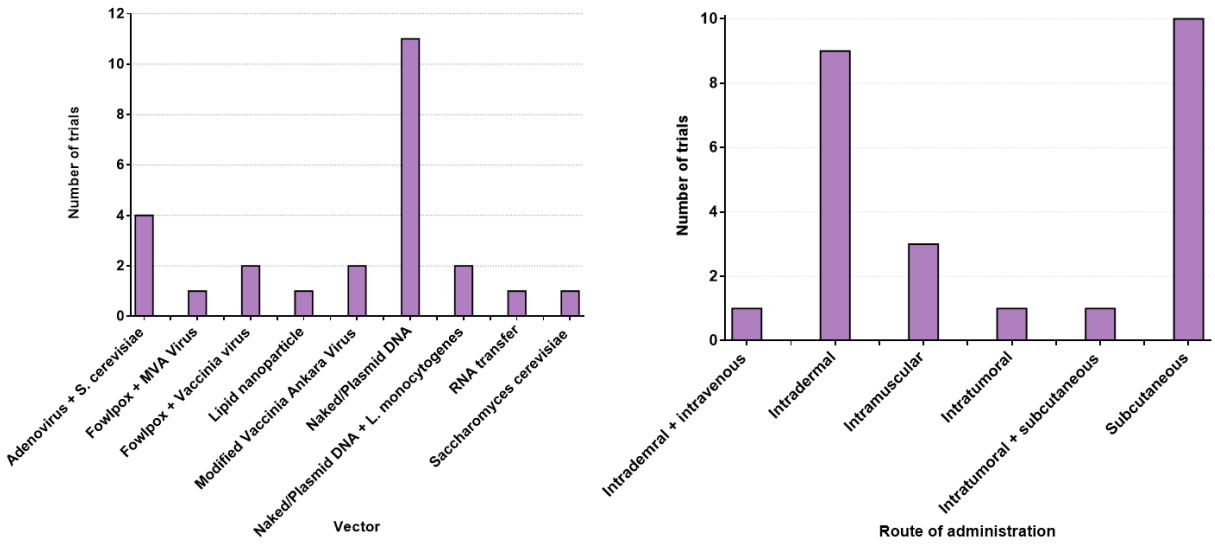


Figure 31: Vaccine trials repartition according to vector used (left) or route of administration (right)

The majority of vaccines are composed of naked or plasmid DNA (*fig. 31*). An example of a DNA vaccine is composed of autologous pancreatic cancer cells genetically modified to secrete the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) and then irradiated to prevent further cell division (**GVAX**).[123][125] GM-CSF stimulates the immune system by promoting the activation of DCs and by increasing antigen presentation to B and T cells. Additionally, GM-CSF enhances ADCC and interleukin-2 (IL-2)-mediated lymphokine-activated killer cell function. Seven trials of phases between I to II in the USA are currently underway studying intradermal administrations of GVAX. Three other phase II trials involving GVAX vaccine are active, and accompanied by a second vaccine composed of live, attenuated *Listeria monocytogenes* expressing mesothelin (**CRS-207**) delivered intravenously (Intravenous + intradermal). Mesothelin is a tumour-associated antigen (TAA) overproduced by certain tumours, including pancreatic.[126]

Other DNA vaccines encode cytokines such as GM-CSF (phase II, ID), antigens such as mesothelin (phase I, IM) or the human telomerase reverse transcriptase

(hTERT/**INO-1400**, phase I, IM).[123][127] The enzyme hTERT prolongs cells' lifespan by maintaining the lengths of telomeres, playing an important role in tumour cell immortality.

There are two trials of tumour vaccines which use RNA transgenes. The first is a lipid nanoparticle (LNP)-formulated mRNA-derived vaccine targeting the four most commonly occurring KRAS oncogenic mutations: G12D, G12V, G13D and G12C (**V941**).[123][128] After intramuscular vaccination, the mRNA is taken up by APCs and the epitopes presented at the surface by the major histocompatibility complex (MHC). This occasions the induction of cytotoxic and memory T lymphocytes directed toward tumour cells harbouring these mutations. The second is a recently started (2020) phase I trial involving a perinodal delivery of a DC vaccine loaded with pancreatic adenocarcinoma lysate plus mRNA as adjuvant therapy following completion of standard chemotherapy (**DECIST**).[129]

A popular route of administration of these vaccines is by subcutaneous (SC) injection (*fig. 31*), this is especially the case for vaccines involving a biological vector. Four phase I/II USA trials involve a cocktail of drugs administered by SC, comprising two gene-based therapies (QUILT-3.070, -3.039, -3.080, -3.060 NANT vaccines).[130] The first is an adenoviral serotype 5 vector with deleted E1 and E2b genes encoding the human carcinoembryonic antigen (CEA), a TAA overexpressed in various tumours (Ad5-CEA(6D), **ETBX-011**).[131] The deletion of E1 and E2B early genes potentially bypasses an anti-adenovirus immunity. The second is a heat-killed recombinant Saccharomyces cerevisiae yeast transfected with genes encoding mutated forms of RAS oncoproteins (**GI-4000**).[123][132] A Saccharomyces cerevisiae transfected with YE-NEO-001 neoepitope (QUILT-2.025 NANT vaccine)[133] was approved by the FDA for a phase I trial in 2018.

Other subcutaneously-administered vaccines involve viral vectors such as the replication-deficient modified vaccinia Ankara (MVA) virus – an attenuated strain of vaccinia virus. MVA encoding the p53 gene (**p53MVA**)[134] is studied in mutant p53 over-expressing cancers in a phase I trial. The p53 gene is a tumour suppressor gene with a key role in cell division and cell death and commonly mutated in cancer cells. Another phase I MVA trial (**MVA Brachyury-TRICOM**)[135] encodes the brachyury gene, a member of the T-box family of transcription factors that is overexpressed in

numerous cancer cell types and is correlated with increased epithelial-mesenchymal transition (EMT), cancer resistance and cancer progression. This same vaccine also associates a triad of T-cell co-stimulatory molecules, B7.1, ICAM-1 and LFA-3 (TRICOM).[136] The TRICOM vaccine is also investigated in a phase I/II trial combination with a fowlpox encoding CEA and mucin-1 (MUC-1) TAAs as booster (**CV-301**).[136][137] The final two trials, one in phase I and one in phase III, combine the fowlpox virus encoding for CEA and MUC-1 (**Falimarev**/PANVAC-F) and a vaccinia virus encoding the same TAAs in addition to TRICOM (**Inalimarev**/PANVAC-V).[136][138]

CAR-T/NK cells

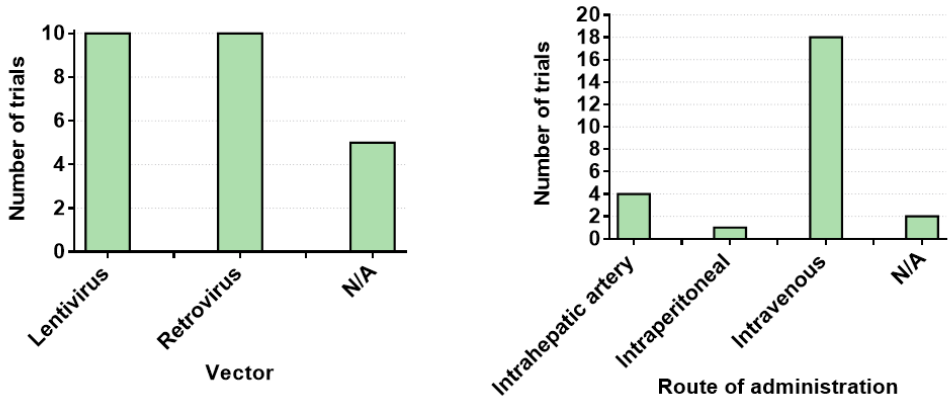


Figure 32: CAR-T/NK cell trials repartition according to vector used (left) or route of administration (right)

CAR-T/NK cells are *ex vivo* engineered T lymphocytes or natural killer cells reinjected into the patient in order to direct these immune cells towards the lysis of tumour cells.

Although vector information is incomplete on a portion of these trials, the only two vectors used to transduce these CAR-T cells are retrovirus and lentivirus vectors (*fig. 32*). Four trials in the USA – two phase I active, one phase II/III recruiting and one phase I complete – involve intrahepatic artery delivery of retroviral (one N/A)-transduced **CEA-CAR T**.[129] All four trials are aimed to combat liver metastases of PC. Similarly, a phase I trial targeting peritoneal metastases or malignant ascites (IPC) use the intraperitoneal route to deliver anti-CEA CAR-T cells.

The rest of the trials involving CAR-T cells are administered by IV and transduced to express a variety of TAAs. A popular choice is mesothelin (**meso-CAR T**)[129]

investigated in five phase I trials (of which three recently completed) and one phase I/II in China, and one phase I trial in the USA. Other antigens studied in phase I or I/II trials in the USA or China include: **CEA**, **MUC-1**, **KRASG12V**, **KRASG12D**, **CLD18** (Claudin-18), **CD133**, **ROR2** (receptor tyrosine kinase-like orphan receptor 2), **EpCAM** (epithelial cell adhesion molecule), **CD70** and **PSCA** (prostate stem cell antigen).[139]

A single clinical trial involves CAR-NK cells engineered with a lentivirus vector to express the **ROBO-1** (Roundabout homolog 1) receptor which has been found to be overexpressed in pancreatic tumours.[140][141] ROBO-1 is a member of the axon guidance receptor family with a reported role in T cells chemotaxis modulation and tumour angiogenesis. This BiCAR-NK trial is a recruiting phase I/II trial designed in China.

Oncolytic viruses

Oncolytic viruses are replication-competent viruses that target and lyse tumour cells while sparing healthy cells. In these ongoing or recently completed clinical trials for PC, eight are related to OVs, in addition to the two oncolytic adenoviral vectors carrying suicide genes. These eight trials involve four virus strains: adenovirus, herpes simplex virus, reovirus and parvovirus H1 (H-1PV).

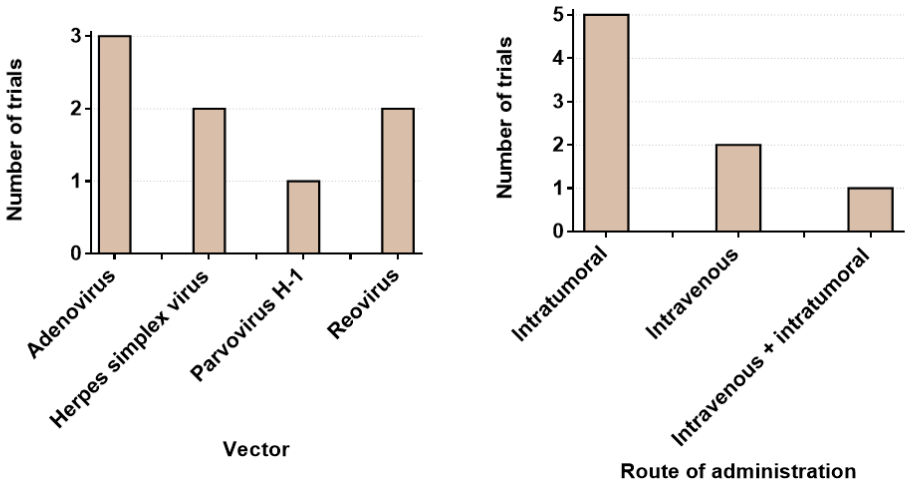


Figure 33: OVs trials repartition according to vector used (left) or route of administration (right)

In these OV studies, the adenovirus and HSV are administered intratumorally whereas the reovirus is delivered intravenously and the H-1PV uses both routes of

administration (*fig. 33*). A completed phase I and an ongoing phase I trial, both in Spain, investigate a PH20 hyaluronidase-expressing adenovirus (**VCN-01**).[142] Hyaluronic acid is a glycosaminoglycan found in the TME frequently overproduced by tumour cells, contributing to tumour cell growth, metastatic capacities and resistance to chemotherapeutics. The hyaluronidase expressed by the OV degrades hyaluronic acid which decreases the interstitial space viscosity and the tumour interstitial fluid pressure (IFP) resulting in increased viral spread and facilitating the access of other drugs. The third adenoviral-centred research is an ongoing phase I/II USA and Sweden trial of a modified immunostimulatory adenovirus encoding TMZ-CD40L and 4-1BBL (**delolimogene mupadenorepvec**, LOAd7).[143]

A spontaneously attenuated replication-competent strain of HSV-1 (**TBI-1401(HF10)**) [143] is currently being investigated in a phase I trial in Japan in combination with standard chemotherapy. Talimogene laherparepvec (OncoVEXGM-CSF/**T-vec**), a genetically-modified HSV-1 was approved by the FDA in 2015 for melanoma (**Imlygic**) and is now in a phase I study in the USA for pancreatic cancer.

Wild-type serotype 3 Dearing strain reovirus (**pelareorep**)[143] is being tested as an OV against PC in a recently completed phase I/II trial in China (REOLYSIN) and an active phase II trial in the USA.[144]

Finally, a phase I/II German study has recently finished exploring parvovirus H1 (**ParvOryx**),[143] administered both in IV and IT in liver metastases, as an OV treatment in patients with metastatic inoperable PC.[145]

Suicide genes

To recapitulate, suicide or tumour suppressor genes once incorporated into a tumour cell help induce cell death, for example by increasing the metabolism of chemotherapies into their active form.

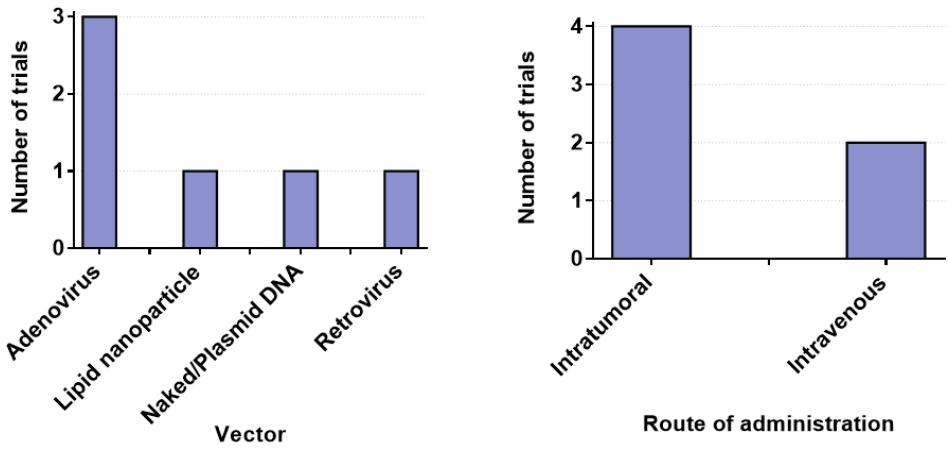


Figure 34: Suicide gene trials repartition according to vector used (left) or route of administration (right)

As mentioned previously, there is currently a phase II trial in France regarding the combination of **CYL-02** DNA plasmid and gemcitabine (TherGAP) by IT injection.[146] The CYL-02 plasmid encodes the mouse somatostatin receptor subtype 2 (sst2) and the fusion protein of human deoxycytidine kinase (DCK) and uridine monophosphate kinase (UMK) complexed to a synthetic polyethylenimine carrier. Expression of the DCK::UMK fusion protein converts gemcitabine into its toxic metabolite. Expression of the sst2 protein - whose gene expression is often lost in pancreatic and colorectal cancers and which negatively regulates multiple processes such as epithelial cell proliferation – is believed to induce both anti-oncogenic and local antitumor bystander effects. Combining the two allows for a lower dose of gemcitabine to cause tumour cell lysis.

Another DNA-based suicide gene (*fig. 34*) is a recruiting phase II trial in the USA and Taiwan using a liposome to carry a plasmid containing the wild-type p53 gene (**SGT-53**)[147] and injected intravenously.

Other suicide gene-based clinical trials involve a viral vector such as IT injection of adenovirus or IV delivery of retrovirus (*fig. 34*). A recruiting phase II study in USA and Mexico uses an adenoviral vector engineered to bear the HSV thymidine kinase (HSV-TK) gene (**aglatimagene besadenovec**).[148] The phase I investigations involving adenoviral vectors involve double suicide fusion genes: a yeast cytosine deaminase (yCD) and a mutant form of HSV-1 thymidine kinase (HSV-1 TKSR39). The first, which also incorporates the adenovirus death protein (ADP) gene with potential oncolytic

activity (**Ad5-yCD/mutTK(SR39)rep-ADP**)[149] is recently completed and located in South Korea while the second which additionally includes the natural killer (NK) cells promoting IL-12 gene (**Ad5-yCD/mutTK(SR39)rep-IL12**)[150] is still ongoing and based in the USA. The final suicide gene clinical trial is a phase I/II research in the USA (BLESSED) based on a retrovector with a cytocidal cyclin G1 construct (**DeltaRex-G**).[151] Cyclin G1 is one of the target genes of p53 and induces a cell cycle arrest between the G2 and M phases.

Gene silencing

There are only two clinical trials involving gene silencing mechanism (*fig.30*) both delivered directly into the tumour. One a completed phase I trial in Japan involving a siRNA directed against NIMA Related Kinase 2 (NEK2) expression. NEK2 is a serine/threonine kinase involved in cell division and mitosis and which is abnormally expressed in PDAC.[152] The other is a biodegradable polymeric matrix containing a siRNA for the mutated KRASG12D (**siG12D-LODER**)[153] currently undergoing phase II in the United States and Israel.

VI. Discussion - Conclusion

Solid cancers remain a major health challenge in terms of research, not only due to their structure and organisation but also in the molecular and genetic variations present between tumours as well as within the same tumour. When adding on the tumour microenvironment with cancer-associated cells, vasculature and the body's immune response (or lack of), the weapons used to tackle this disease must also be diverse and intricate. Developing gene-based therapies against tumours contributes to the diverse lines of attack already established for cancers and can potentially overcome certain obstacles encountered with these strategies, the lack of tumour selectivity with chemotherapies for example.

The techniques for gene therapy medicinal products (GTMPs) development have evolved immensely since the first discovery of DNA in 1944. This involves development of both the therapeutic transgene in itself, which can take various genetic forms, and of its transport and delivery, either by the procedure of administration or the progress in vector design. These GTMPs can have both a direct effect on tumour cells through induction of cell lysis and potentialising the tumoral efficacy of chemotherapies, or indirectly through immuno-stimulating/modulating signalling. Due to the complexities and increased ethical and safety considerations involved with gene-based products, regulations and guidelines have been set up by health administrations to ensure the best efficacy and safety during the development, testing and use of these medicines. The European Medicines Agency (EMA) ensures a coordination between member states of the European Union in order to facilitate access to new therapies and optimise patient care.

GTMPs having been defined by the EMA as possessing recombinant nucleic acid, one can debate on whether oncolytic viruses can be considered as gene therapy products. However, considering the safety implications when using viruses in therapies, specific control and monitoring of viral genes activities is essential, similarly to other gene-based compounds. OVs have therefore been included in the accounts of gene therapy

techniques and applications. In addition, recombinant oncolytic viruses are being conceptualised to express transgenes of tumour suppressor genes or immune-stimulation on top of their oncolytic capacities.

Given the high mortality and relapse rate associated with pancreatic cancer (PC), novel treatments including gene therapy are actively being investigated. Even though there are no gene therapies for PC currently on the market, a significant amount of clinical trials are underway especially in active and recruiting or recently completed phases (*fig. 29*). The majority of these trials involve immune-stimulating techniques such as vaccines and CAR-T/NK cells. Pancreatic cancer is considered a 'cold' tumour in that the tumoral environment is lacking and suppressed of immune activity. Vaccines and CAR-T/NK cells could help activate a specific immune response towards tumour cells, thereby turning them 'hot'. These immune strategies have functioned for other types of cancer and although there are some promising results in patients with PC, no significant improvement in patient survival has yet been observed.[154]

Other techniques investigated include gene silencing and suicide gene approaches which also display potential in PC. In addition to suppressing oncogenes or inducing tumour suppressor genes, these strategies have the potential of triggering bystander ramifications through the diffusion of therapeutic effect, i.e. pro-apoptotic signalling, on environing tumour cells. Problems with these techniques have arisen however, such as poor tumour cell uptake efficiency and degradation and clearance, limits which are being tackled by optimising delivery vectors. Such vectors include viral, lipid and polymer-based nanoparticles.[155]

Vectors and routes of administration are adapted according to the intended target and transgene approach. This is seen in clinical trials for PC by favouring the intradermal, subcutaneous and intramuscular route for vaccine administrations (*fig. 31*), optimising access to antigen presenting cells such as dendritic cells, an essential step in the induction of an immune response.[156] Another example is the intrahepatic arterial delivery of CAR-T cells when targeting liver metastasis and the intraperitoneal administration when aiming at metastases and malignant ascites (*fig. 32*). Clinical trials involving adenoviruses alone (excluding vaccines combining S. cerevisiae) exclusively use an intratumoral approach of delivery (*figs. 33, 34*). Adenoviruses (especially helper-associated adenoviruses) are popular in gene therapy constructs due to their

large transgene holding capacity and their low risk of inducing mutagenesis. Nevertheless, they are also characterised by a large tropism, pre-existing immunity and liver sequestration by coagulation factors when delivered systemically.[157] Intratumoral delivery of adenoviral vectors, whether for delivering suicide genes or for their oncolytic activity, seems to be the optimal choice to ensure tumour specificity and efficiency.

Pancreatic cancers are known for their molecular heterogeneity, contributing to the difficulty in pinpointing targets and finding a fitting treatment.[158] This can explain the large spread of vectors and transgenes that are being researched in clinical trials. Although no ground breaking treatment for PC has emerged, some promising results in these gene therapy clinical trials gives hope to uncovering more effective and safe treatment protocols than those in play today. Breaking the code of pancreatic tumours rather than using brutal force will definitely help gene therapy in defeating this currently incurable disease.

Bibliography

[1] D. Hanahan and R. A. Weinberg, 'Hallmarks of Cancer: The Next Generation', *Cell*, vol. 144, no. 5, pp. 646–674, Mar. 2011, doi: 10.1016/j.cell.2011.02.013.

[2] 'Cancer today'. http://gco.iarc.fr/today/home (accessed May 05, 2020).

[3] R. L. Siegel, K. D. Miller, and A. Jemal, 'Cancer statistics, 2019', *CA A Cancer J Clin*, vol. 69, no. 1, pp. 7–34, Jan. 2019, doi: 10.3322/caac.21551.

[4] 'HPV and Cancer - National Cancer Institute', Jan. 03, 2019. https://www.cancer.gov/about-cancer/causes-prevention/risk/infectious-agents/hpv-and-cancer (accessed May 05, 2020).

[5] Y. A. Fouad and C. Aanei, 'Revisiting the hallmarks of cancer', p. 21.

[6] 'What is Cancer Surgery?', *Cancer.Net*, Mar. 31, 2011. https://www.cancer.net/navigating-cancer-care/how-cancer-treated/surgery/what-cancer-surgery.

[7] S. Tohme, R. L. Simmons, and A. Tsung, 'Surgery for Cancer: A Trigger for Metastases', *Cancer Res*, vol. 77, no. 7, pp. 1548–1552, Apr. 2017, doi: 10.1158/0008-5472.CAN-16-1536.

[8] 'Brachytherapy'. Gustave Roussy, 2017, [Online]. Available: https://www.gustaveroussy.fr/en/brachytherapy.

[9] M. Drăgănescu and C. Carmocan, 'Hormone Therapy in Breast Cancer', *chr*, vol. 112, no. 4, p. 413, 2017, doi: 10.21614/chirurgia.112.4.413.

[10] 'Hormone Therapy for Prostate Cancer'. https://www.cancer.org/cancer/prostate-cancer/treating/hormone-therapy.html (accessed May 09, 2020).

[11] A. Hoos, 'Development of immuno-oncology drugs — from CTLA4 to PD1 to the next generations', *Nat Rev Drug Discov*, vol. 15, no. 4, pp. 235–247, Apr. 2016, doi: 10.1038/nrd.2015.35.

[12] K. A. Schafer, 'The Cell Cycle: A Review', *Vet Pathol*, vol. 35, no. 6, pp. 461–478, Nov. 1998, doi: 10.1177/030098589803500601.

[13] 'Sécuriser la dispensation des anticancéreux', *Le Moniteur des Pharmacies*, no. 3293, Nov. 02, 2019.

[14] S. El Anbassi and V. Bianchi, *Médicaments*. De Boeck, 2015.

[15] M. J. Lind, 'Principles of cytotoxic chemotherapy', p. 5, 2007.

[16] V. Malhotra and M. C. Perry, 'Mechanisms, Toxicities and the Therapeutic Window', vol. 2, no. 4, p. 3.

[17] PubChem, 'Mitotane'. https://pubchem.ncbi.nlm.nih.gov/compound/4211.

[18] ‘VIDAL - Asparaginase’. https://www.vidal.fr/substances/3877/asparaginase/.

[19] X.-H. Tang and L. J. Gudas, ‘Retinoids, Retinoic Acid Receptors, and Cancer’, *Annu. Rev. Pathol. Mech. Dis.*, vol. 6, no. 1, pp. 345–364, Feb. 2011, doi: 10.1146/annurev-pathol-011110-130303.

[20] R. Kannaiyan and D. Mahadevan, ‘A comprehensive review of protein kinase inhibitors for cancer therapy’, *Expert Review of Anticancer Therapy*, vol. 18, no. 12, pp. 1249–1270, Dec. 2018, doi: 10.1080/14737140.2018.1527688.

[21] U. S. FDA, ‘Herceptin Final Labeling’, p. 33, 2010.

[22] ‘Regulation (EC) No 1394/2007 of the European Parliament and of the Council’. .

[23] ‘Directive 2001/83/EC of the European Parliament and of the Council’. .

[24] X. M. Anguela and K. A. High, ‘Entering the Modern Era of Gene Therapy’, *Annu. Rev. Med.*, vol. 70, no. 1, pp. 273–288, Jan. 2019, doi: 10.1146/annurev-med-012017-043332.

[25] ‘Regulation (EC) No 726/2004 of the European Parliament and of the Council’. .

[26] T. T. L. Yu, P. Gupta, V. Ronfard, A. A. Vertès, and Y. Bayon, ‘Recent Progress in European Advanced Therapy Medicinal Products and Beyond’, *Front. Bioeng. Biotechnol.*, vol. 6, p. 130, Sep. 2018, doi: 10.3389/fbioe.2018.00130.

[27] ‘Cancer Gene Therapy Market Growth Statistics Forecasts 2025’, *Global Market Insights, Inc.* https://www.gminsights.com/industry-analysis/cancer-gene-therapy-market.

[28] S. I. Hajdu, ‘A note from history: Landmarks in history of cancer, part 1’, *Cancer*, vol. 117, no. 5, pp. 1097–1102, Mar. 2011, doi: 10.1002/cncr.25553.

[29] V. T. DeVita and S. A. Rosenberg, ‘Two Hundred Years of Cancer Research’, *N Engl J Med*, vol. 366, no. 23, pp. 2207–2214, Jun. 2012, doi: 10.1056/NEJMra1204479.

[30] J. Burley and J. Harris, *A Companion to Genetics*. Wiley-Blackwell, 2002.

[31] D. Dickson, ‘NIH censure for Dr Martin Cline’, *Nature*, vol. 291, no. 5814, pp. 369–369, Jun. 1981, doi: 10.1038/291369a0.

[32] J. Trojan *et al.*, ‘Methodology for Anti-Gene Anti-IGF-I Therapy of Malignant Tumours’, *Chemotherapy Research and Practice*, vol. 2012, pp. 1–12, 2012, doi: 10.1155/2012/721873.

[33] D. W. Andrews *et al.*, ‘Results of a Pilot Study Involving the Use of an Antisense Oligodeoxynucleotide Directed Against the Insulin-Like Growth Factor Type I Receptor in Malignant Astrocytomas’, *JCO*, vol. 19, no. 8, pp. 2189–2200, Apr. 2001, doi: 10.1200/JCO.2001.19.8.2189.

[34] S. E. Raper *et al.*, ‘Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer’,

Molecular Genetics and Metabolism, vol. 80, no. 1–2, pp. 148–158, Sep. 2003, doi: 10.1016/j.ymgme.2003.08.016.

[35] P. P. Denèfle, 'Introduction to Gene Therapy: A Clinical Aftermath', in *Viral Vectors for Gene Therapy*, vol. 737, O.-W. Merten and M. Al-Rubeai, Eds. Totowa, NJ: Humana Press, 2011, pp. 27–44.

[36] M. Cavazzana-Calvo, 'Gene Therapy of Human Severe Combined Immunodeficiency (SCID)-X1 Disease', *Science*, vol. 288, no. 5466, pp. 669–672, Apr. 2000, doi: 10.1126/science.288.5466.669.

[37] D. Cross and J. K. Burmester, 'Gene Therapy for Cancer Treatment: Past, Present and Future', *Clinical Medicine & Research*, vol. 4, no. 3, pp. 218–227, Sep. 2006, doi: 10.3121/cmr.4.3.218.

[38] Administrator, 'Oncorine'. http://www.genetherapynet.com/oncorine.html.

[39] Eurolab, 'Neovasculogen listing in Eurolab'. https://www.eurolab.ua/medicine/drugs/5411/.

[40] A. Shahryari *et al.*, 'Development and Clinical Translation of Approved Gene Therapy Products for Genetic Disorders', *Front. Genet.*, vol. 10, p. 868, Sep. 2019, doi: 10.3389/fgene.2019.00868.

[41] Anonymous, 'Zalmoxis', *European Medicines Agency*, Sep. 17, 2018. https://www.ema.europa.eu/en/medicines/human/EPAR/zalmoxis.

[42] 'Rigvir cancer treatment at center of fresh controversy'. https://eng.lsm.lv/article/society/health/rigvir-cancer-treatment-at-center-of-fresh-controversy.a313826/.

[43] European Commission and Directorate-General for Research and Innovation, *100 radical innovation breakthroughs for the future.* 2019.

[44] H. Yin, R. L. Kanasty, A. A. Eltoukhy, A. J. Vegas, J. R. Dorkin, and D. G. Anderson, 'Non-viral vectors for gene-based therapy', *Nat Rev Genet*, vol. 15, no. 8, pp. 541–555, Aug. 2014, doi: 10.1038/nrg3763.

[45] M. S. Ebert and P. A. Sharp, 'MicroRNA sponges: Progress and possibilities', *RNA*, vol. 16, no. 11, pp. 2043–2050, Nov. 2010, doi: 10.1261/rna.2414110.

[46] D. S. Hong *et al.*, 'Phase 1 study of MRX34, a liposomal miR-34a mimic, in patients with advanced solid tumours', *Br J Cancer*, Apr. 2020, doi: 10.1038/s41416-020-0802-1.

[47] Z. P, 'Suicide Gene Therapy for Cancer; Old Dog New Tricks', *J Genet Syndr Gene Ther*, vol. 04, no. 04, 2013, doi: 10.4172/2157-7412.1000139.

[48] Y. Zhang, 'Human Telomerase Reverse Transcriptase (hTERT) Is a Novel Target of the Wnt-β-Catenin Pathway in Human Cancer', *J Bio Chem*, p. 18, Sep. 2012, doi: 10.1074/jbc.M112.368282.

[49] M. H. Amer, 'Gene therapy for cancer: present status and future perspective', *Mol and Cell Ther*, vol. 2, no. 1, p. 27, 2014, doi: 10.1186/2052-8426-2-27.

[50] D. Holoch and D. Moazed, ‘RNA-mediated epigenetic regulation of gene expression’, *Nat Rev Genet*, vol. 16, no. 2, pp. 71–84, Feb. 2015, doi: 10.1038/nrg3863.

[51] K. E. Ormond *et al.*, ‘The clinical application of gene editing: ethical and social issues’, *Personalized Medicine*, vol. 16, no. 4, pp. 337–350, Jul. 2019, doi: 10.2217/pme-2018-0155.

[52] T. Zhan, N. Rindtorff, J. Betge, M. P. Ebert, and M. Boutros, ‘CRISPR/Cas9 for cancer research and therapy’, *Seminars in Cancer Biology*, vol. 55, pp. 106–119, Apr. 2019, doi: 10.1016/j.semcancer.2018.04.001.

[53] V. Corless, ‘First human trial finds CRISPR-edited cells for cancer treatment are safe and durable’, *Advanced Science News*, Feb. 07, 2020. https://www.advancedsciencenews.com/crispr-edited-cells-for-cancer-treatment-are-safe-and-durable/.

[54] S. Shankar, A. Sreekumar, D. Prasad, A. V. Das, and M. R. Pillai, ‘Genome editing of oncogenes with ZFNs and TALENs: caveats in nuclease design’, *Cancer Cell Int*, vol. 18, no. 1, p. 169, Dec. 2018, doi: 10.1186/s12935-018-0666-0.

[55] P. E. Mangeot *et al.*, ‘Genome editing in primary cells and in vivo using viral-derived Nanoblades loaded with Cas9-sgRNA ribonucleoproteins’, *Nat Commun*, vol. 10, no. 1, p. 45, Dec. 2019, doi: 10.1038/s41467-018-07845-z.

[56] S. Chira, D. Gulei, A. Hajitou, A.-A. Zimta, P. Cordelier, and I. Berindan-Neagoe, ‘CRISPR/Cas9: Transcending the Reality of Genome Editing’, *Molecular Therapy - Nucleic Acids*, vol. 7, pp. 211–222, Jun. 2017, doi: 10.1016/j.omtn.2017.04.001.

[57] J. Dörrie, N. Schaft, G. Schuler, and B. Schuler-Thurner, ‘Therapeutic Cancer Vaccination with Ex Vivo RNA-Transfected Dendritic Cells—An Update’, *Pharmaceutics*, vol. 12, no. 2, p. 92, Jan. 2020, doi: 10.3390/pharmaceutics12020092.

[58] S. Patel *et al.*, ‘Beyond CAR T Cells: Other Cell-Based Immunotherapeutic Strategies Against Cancer’, *Front. Oncol.*, vol. 9, p. 196, Apr. 2019, doi: 10.3389/fonc.2019.00196.

[59] M. Felices, T. R. Lenvik, Z. B. Davis, J. S. Miller, and D. A. Vallera, ‘Generation of BiKEs and TriKEs to Improve NK Cell-Mediated Targeting of Tumor Cells’, in *Natural Killer Cells*, vol. 1441, S. S. Somanchi, Ed. New York, NY: Springer New York, 2016, pp. 333–346.

[60] L. Cao *et al.*, ‘Cytokine gene transfer in cancer therapy’, *STEM CELLS*, vol. 16, no. S2, pp. 251–260, Jun. 2009, doi: 10.1002/stem.5530160831.

[61] S. Mehierhumbert and R. Guy, ‘Physical methods for gene transfer: Improving the kinetics of gene delivery into cells’, *Advanced Drug Delivery Reviews*, vol. 57, no. 5, pp. 733–753, Apr. 2005, doi: 10.1016/j.addr.2004.12.007.

[62] ‘Biolistic®PDS-1000/He Particle Delivery System’. Bio-Rad Laboratories.

[63] T. Suda and D. Liu, ‘Hydrodynamic Gene Delivery: Its Principles and Applications’, *Molecular Therapy*, vol. 15, no. 12, pp. 2063–2069, Dec. 2007, doi: 10.1038/sj.mt.6300314.

[64] C. Liu, ‘Strategies for Designing Transgenic DNA Constructs’, in *Lipoproteins and Cardiovascular Disease*, vol. 1027, L. A. Freeman, Ed. Totowa, NJ: Humana Press, 2013, pp. 183–201.

[65] D. De Paula, M. V. L. B. Bentley, and R. I. Mahato, ‘Hydrophobization and bioconjugation for enhanced siRNA delivery and targeting’, *RNA*, vol. 13, no. 4, pp. 431–456, Feb. 2007, doi: 10.1261/rna.459807.

[66] Q. Zheng, D. Lin, L. Lei, X. Li, and S. Shi, ‘Engineered Non-Viral Gene Vectors for Combination Cancer Therapy: A Review’, *j biomed nanotechnol*, vol. 13, no. 12, pp. 1565–1580, Dec. 2017, doi: 10.1166/jbn.2017.2489.

[67] M. E. Davis, ‘The First Targeted Delivery of siRNA in Humans via a Self-Assembling, Cyclodextrin Polymer-Based Nanoparticle: From Concept to Clinic’, *Mol. Pharmaceutics*, vol. 6, no. 3, pp. 659–668, Jun. 2009, doi: 10.1021/mp900015y.

[68] C. Roma-Rodrigues, L. Rivas-García, P. V. Baptista, and A. R. Fernandes, ‘Gene Therapy in Cancer Treatment: Why Go Nano?’, *Pharmaceutics*, vol. 12, no. 3, p. 233, Mar. 2020, doi: 10.3390/pharmaceutics12030233.

[69] W. Walther and U. S. Stein, Eds., *Gene Therapy of Cancer*, vol. 542. Totowa, NJ: Humana Press, 2009.

[70] S. Zhen and X. Li, ‘Liposomal delivery of CRISPR/Cas9’, *Cancer Gene Ther*, Nov. 2019, doi: 10.1038/s41417-019-0141-7.

[71] M. Mendt *et al.*, ‘Generation and testing of clinical-grade exosomes for pancreatic cancer’, *JCI Insight*, vol. 3, no. 8, p. e99263, Apr. 2018, doi: 10.1172/jci.insight.99263.

[72] K. Y. Chung, J. M. Quek, S. H. Neo, and H. P. Too, ‘Polymer-Based Precipitation of Extracellular Vesicular miRNAs from Serum Improve Gastric Cancer miRNA Biomarker Performance’, *The Journal of Molecular Diagnostics*, p. S1525157820300441, Mar. 2020, doi: 10.1016/j.jmoldx.2020.01.016.

[73] S. Krishnamurthy, M. K. Gnanasammandhan, C. Xie, K. Huang, M. Y. Cui, and J. M. Chan, ‘Monocyte cell membrane-derived nanoghosts for targeted cancer therapy’, *Nanoscale*, vol. 8, no. 13, pp. 6981–6985, 2016, doi: 10.1039/C5NR07588B.

[74] L. Kaneti *et al.*, ‘Nanoghosts as a Novel Natural Nonviral Gene Delivery Platform Safely Targeting Multiple Cancers’, *Nano Lett.*, vol. 16, no. 3, pp. 1574–1582, Mar. 2016, doi: 10.1021/acs.nanolett.5b04237.

[75] S. K. Das *et al.*, ‘Gene Therapies for Cancer: Strategies, Challenges and Successes’, *J. Cell. Physiol.*, vol. 230, no. 2, pp. 259–271, Feb. 2015, doi: 10.1002/jcp.24791.

[76] O.-W. Merten and M. Al-Rubeai, Eds., *Viral Vectors for Gene Therapy: Methods and Protocols*, vol. 737. Totowa, NJ: Humana Press, 2011.

[77] Y. H. Chen, M. S. Keiser, and B. L. Davidson, 'Viral Vectors for Gene Transfer', *Current Protocols in Mouse Biology*, vol. 8, no. 4, p. e58, Dec. 2018, doi: 10.1002/cpmo.58.

[78] A. Rosewell, F. Vetrini, and P. Ng, 'Helper-Dependent Adenoviral Vectors', p. 34, 2014.

[79] T. W. Kwang, X. Zeng, and S. Wang, 'Manufacturing of AcMNPV baculovirus vectors to enable gene therapy trials', *Molecular Therapy - Methods & Clinical Development*, vol. 3, p. 15050, 2016, doi: 10.1038/mtm.2015.50.

[80] S. El Anbassi, V. Bianchi, and N. Duployez, *Bacteriologie - Virologie*. De Boeck, 2013.

[81] R. H. Lachmann, 'Herpes simplex virus-based vectors', *International Journal of Experimental Pathology*, p. 14, 2004.

[82] S. J. Conrad and J. Liu, 'Poxviruses as Gene Therapy Vectors: Generating Poxviral Vectors Expressing Therapeutic Transgenes', *Viral Vectors for Gene Therapy*, vol. 1937, pp. 189–209, 2019, doi: 10.1007/978-1-4939-9065-8_11.

[83] M. E. Davola and K. L. Mossman, 'Oncolytic viruses: how "lytic" must they be for therapeutic efficacy?', *OncoImmunology*, vol. 8, no. 6, p. e1581528, Jun. 2019, doi: 10.1080/2162402X.2019.1596006.

[84] C. E. Engeland, Ed., *Oncolytic Viruses*, vol. 2058. New York, NY: Springer New York, 2020.

[85] J. Raja, J. M. Ludwig, S. N. Gettinger, K. A. Schalper, and H. S. Kim, 'Oncolytic virus immunotherapy: future prospects for oncology', *j. immunotherapy cancer*, vol. 6, no. 1, p. 140, Dec. 2018, doi: 10.1186/s40425-018-0458-z.

[86] C. K. Baban, M. Cronin, D. O'Hanlon, G. C. O'Sullivan, and M. Tangney, 'Bacteria as vectors for gene therapy of cancer', *Bioengineered Bugs*, vol. 1, no. 6, pp. 385–394, Nov. 2010, doi: 10.4161/bbug.1.6.13146.

[87] R. Ferreira, A. Limeta, and J. Nielsen, 'Tackling Cancer with Yeast-Based Technologies', *Trends in Biotechnology*, vol. 37, no. 6, pp. 592–603, Jun. 2019, doi: 10.1016/j.tibtech.2018.11.013.

[88] R. Kumar and P. Kumar, 'Yeast-based vaccines: New perspective in vaccine development and application', *FEMS Yeast Research*, vol. 19, no. 2, Mar. 2019, doi: 10.1093/femsyr/foz007.

[89] L. Emdad, S. K. Das, X.-Y. Wang, D. Sarkar, and P. B. Fisher, 'Cancer terminator viruses (*CTV*): A better solution for viral-based therapy of cancer', *J Cell Physiol*, vol. 233, no. 8, pp. 5684–5695, Aug. 2018, doi: 10.1002/jcp.26421.

[90] C. C. Cunningham *et al.*, 'Clinical and local biological effects of an intratumoral injection of mda-7 (IL24; INGN 241) in patients with advanced carcinoma: a phase I study', *Molecular Therapy*, vol. 11, no. 1, pp. 149–159, Jan. 2005, doi: 10.1016/j.ymthe.2004.09.019.

[91] H. Yu *et al.*, 'Triple-Layered pH-Responsive Micelleplexes Loaded with siRNA and Cisplatin Prodrug for NF-Kappa B Targeted Treatment of Metastatic Breast Cancer', *Theranostics*, vol. 6, no. 1, pp. 14–27, 2016, doi: 10.7150/thno.13515.

[92] R. E. Tamura, M. G. Lana, E. Costanzi-Strauss, and B. E. Strauss, 'Combination of cabazitaxel and p53 gene therapy abolishes prostate carcinoma tumor growth', *Gene Ther*, vol. 27, no. 1–2, pp. 15–26, Feb. 2020, doi: 10.1038/s41434-019-0071-x.

[93] L. Martinet *et al.*, 'High endothelial venules (HEVs) in human melanoma lesions: Major gateways for tumor-infiltrating lymphocytes', *OncoImmunology*, vol. 1, no. 6, pp. 829–839, Sep. 2012, doi: 10.4161/onci.20492.

[94] H. Maeda, 'Tumor-Selective Delivery of Macromolecular Drugs via the EPR Effect: Background and Future Prospects', *Bioconjugate Chem.*, vol. 21, no. 5, pp. 797–802, May 2010, doi: 10.1021/bc100070g.

[95] S. Wilhelm *et al.*, 'Analysis of nanoparticle delivery to tumours', *Nat Rev Mater*, vol. 1, no. 5, p. 16014, May 2016, doi: 10.1038/natrevmats.2016.14.

[96] F. Danhier, 'To exploit the tumor microenvironment: Since the EPR effect fails in the clinic, what is the future of nanomedicine?', *Journal of Controlled Release*, vol. 244, pp. 108–121, Dec. 2016, doi: 10.1016/j.jconrel.2016.11.015.

[97] N. Verma and A. B. Tiku, 'Significance and nature of bystander responses induced by various agents', *Mutation Research/Reviews in Mutation Research*, vol. 773, pp. 104–121, Jul. 2017, doi: 10.1016/j.mrrev.2017.05.003.

[98] 'Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products', p. 46.

[99] 'ATCC Cell Lines'. https://www.lgcstandards-atcc.org/en/Products/Cells_and_Microorganisms/Cell_Lines.aspx.

[100] M. Kapałczyńska *et al.*, '2D and 3D cell cultures – a comparison of different types of cancer cell cultures', *aoms*, 2016, doi: 10.5114/aoms.2016.63743.

[101] K. Duval *et al.*, 'Modeling Physiological Events in 2D vs. 3D Cell Culture', *Physiology*, vol. 32, no. 4, pp. 266–277, Jul. 2017, doi: 10.1152/physiol.00036.2016.

[102] C. Jensen and Y. Teng, 'Is It Time to Start Transitioning From 2D to 3D Cell Culture?', *Front. Mol. Biosci.*, vol. 7, p. 33, Mar. 2020, doi: 10.3389/fmolb.2020.00033.

[103] M. W. Laschke and M. D. Menger, 'Life is 3D: Boosting Spheroid Function for Tissue Engineering', *Trends in Biotechnology*, vol. 35, no. 2, pp. 133–144, Feb. 2017, doi: 10.1016/j.tibtech.2016.08.004.

[104] B. Artegiani and H. Clevers, 'Use and application of 3D-organoid technology', *Human Molecular Genetics*, vol. 27, no. R2, pp. R99–R107, Aug. 2018, doi: 10.1093/hmg/ddy187.

[105] C.-P. Day, G. Merlino, and T. Van Dyke, ‘Preclinical Mouse Cancer Models: A Maze of Opportunities and Challenges’, *Cell*, vol. 163, no. 1, pp. 39–53, Sep. 2015, doi: 10.1016/j.cell.2015.08.068.

[106] A. Azmi and R. M. Mohammad, *Animal Models in Cancer Drug Discovery*. Academic Press, 2019.

[107] ‘ICH Considerations: Oncolytic Viruses’, p. 6, 2009.

[108] ‘Phases of clinical trials’, *Cancer Research UK*, Oct. 21, 2014. https://www.cancerresearchuk.org/about-cancer/find-a-clinical-trial/what-clinical-trials-are/phases-of-clinical-trials.

[109] ‘Common Terminology Criteria for Adverse Events (CTCAE)’, p. 196.

[110] ‘Rationale Grows for the Immune-Related Response Criteria’, *targetedonc*. https://www.targetedonc.com/view/rationale-grows-for-the-immunerelated-response-criteria.

[111] ‘Advanced therapy medicines: exploring solutions to foster development and expand patient access in Europe’.

[112] ‘Mental Capacity Act 2005’, p. 185, 2005.

[113] ‘Pancreas fact sheet’. Globocan 2018 - WHO.

[114] ‘Cancer tomorrow’. http://gco.iarc.fr/tomorrow/home.

[115] ‘Cancer du pancréas’. Thésarus National de Cancérologie Digestive, Nov. 25, 2019, [Online]. Available: www.tncd.org/www.snfge.org.

[116] X. Zhang, S. Shi, B. Zhang, Q. Ni, X. Yu, and J. Xu, ‘Circulating biomarkers for early diagnosis of pancreatic cancer: facts and hopes’, p. 22.

[117] K.-Y. Li *et al.*, ‘Pancreatic ductal adenocarcinoma immune microenvironment and immunotherapy prospects’, *Chronic Diseases and Translational Medicine*, vol. 6, no. 1, pp. 6–17, Mar. 2020, doi: 10.1016/j.cdtm.2020.01.002.

[118] ‘Home - ClinicalTrials.gov’. https://clinicaltrials.gov/.

[119] ‘Gene Therapy Clinical Trials Worldwide’. http://abedia.com/wiley/index.html.

[120] ‘UMIN Clinical Trials Registry (UMIN-CTR)’. https://www.umin.ac.jp/ctr/.

[121] ‘WHO | Welcome to the WHO ICTRP’, *WHO*. http://www.who.int/ictrp/en/.

[122] N. Roy *et al.*, ‘PDX1 dynamically regulates pancreatic ductal adenocarcinoma initiation and maintenance’, *Genes Dev.*, vol. 30, no. 24, pp. 2669–2683, Dec. 2016, doi: 10.1101/gad.291021.116.

[123] D. Schizas *et al.*, ‘Immunotherapy for pancreatic cancer: A 2020 update’, *Cancer Treatment Reviews*, vol. 86, p. 102016, Jun. 2020, doi: 10.1016/j.ctrv.2020.102016.

[124] L. Buscail, *La Thérapie Génique*, Grancher. 2017.

[125] J. Nemunaitis *et al.*, '630. A Phase 2 Randomized Study of GM-CSF Gene-Modified Autologous Tumor Cell Immunotherapy (CG8123) with and without Low-Dose Cyclophosphamide in Advanced Stage Non- Small Cell Lung Cancer (NSCLC)', *Molecular Therapy*, vol. 13, p. S243, 2006, doi: 10.1016/j.ymthe.2006.08.705.

[126] D. T. Le *et al.*, 'Results from a Phase IIb, Randomized, Multicenter Study of GVAX Pancreas and CRS-207 Compared with Chemotherapy in Adults with Previously Treated Metastatic Pancreatic Adenocarcinoma (ECLIPSE Study)', *Clin Cancer Res*, vol. 25, no. 18, pp. 5493–5502, Sep. 2019, doi: 10.1158/1078-0432.CCR-18-2992.

[127] R. Benedetti, C. Dell'Aversana, C. Giorgio, R. Astorri, and L. Altucci, 'Breast Cancer Vaccines: New Insights', *Front. Endocrinol.*, vol. 8, p. 270, Oct. 2017, doi: 10.3389/fendo.2017.00270.

[128] L. Talukdar, 'KRAS vaccine (mRNA-5671)', p. 7.

[129] J. Bian and K. Almhanna, 'Pancreatic cancer and immune checkpoint inhibitors—still a long way to go', *Translational Gastroenterology and Hepatology*, p. 5, 2020.

[130] A. Melão, 'NANT Cancer Vaccine Clinical Trial Plans Expanding to Include...', *Immuno-Oncology News*, Jun. 12, 2017. https://immuno-oncologynews.com/2017/06/12/nant-cancer-vaccine-program-is-going-to-target-multiple-cancers/.

[131] M. E. Gatti-Mays *et al.*, 'A Phase I Trial Using a Multitargeted Recombinant Adenovirus 5 (CEA/MUC1/Brachyury)-Based Immunotherapy Vaccine Regimen in Patients with Advanced Cancer', *The Oncol*, p. theoncologist.2019-0608, Oct. 2019, doi: 10.1634/theoncologist.2019-0608.

[132] M. L. Hartley, N. A. Bade, P. A. Prins, L. Ampie, and J. L. Marshall, 'Pancreatic cancer, treatment options, and GI-4000', *Human Vaccines & Immunotherapeutics*, vol. 11, no. 4, pp. 931–937, Apr. 2015, doi: 10.1080/21645515.2015.1011017.

[133] I. NantBioScience, 'QUILT-2.025 NANT Neoepitope Yeast Vaccine (YE-NEO-001): Adjuvant Immunotherapy Using a Personalized Neoepitope Yeast-Based Vaccine To Induce T-Cell Responses In Subjects W/ Previously Treated Cancers', *TrialBulletin.com*. https://trialbulletin.com/lib/entry/ct-03552718.

[134] V. Chung *et al.*, 'Evaluation of safety and efficacy of p53MVA vaccine combined with pembrolizumab in patients with advanced solid cancers', *Clin Transl Oncol*, vol. 21, no. 3, pp. 363–372, Mar. 2019, doi: 10.1007/s12094-018-1932-2.

[135] C. R. Heery *et al.*, 'Phase I Study of a Poxviral TRICOM-Based Vaccine Directed Against the Transcription Factor Brachyury', *Clin Cancer Res*, vol. 23, no. 22, pp. 6833–6845, Nov. 2017, doi: 10.1158/1078-0432.CCR-17-1087.

[136] D. Repáraz, D. Llopiz, and P. Sarobe, 'When Cancer Vaccines Go Viral', *Clin Cancer Res*, vol. 25, no. 16, pp. 4871–4873, Aug. 2019, doi: 10.1158/1078-0432.CCR-19-1652.

[137] M. E. Gatti-Mays *et al.*, 'A Phase I Dose-Escalation Trial of BN-CV301, a Recombinant Poxviral Vaccine Targeting MUC1 and CEA with Costimulatory Molecules', *Clin Cancer Res*, vol. 25, no. 16, pp. 4933–4944, Aug. 2019, doi: 10.1158/1078-0432.CCR-19-0183.

[138] H. L. Kaufman *et al.*, 'Poxvirus-based vaccine therapy for patients with advanced pancreatic cancer', *J Transl Med*, vol. 5, no. 1, p. 60, Dec. 2007, doi: 10.1186/1479-5876-5-60.

[139] A. I. Ali, A. J. Oliver, T. Samiei, J. D. Chan, M. H. Kershaw, and C. Y. Slaney, 'Genetic Redirection of T Cells for the Treatment of Pancreatic Cancer', *Front. Oncol.*, vol. 9, p. 56, Feb. 2019, doi: 10.3389/fonc.2019.00056.

[140] N. Xia *et al.*, 'Robo1-specific CAR-NK Immunotherapy Enhances Efficacy of ^{125}I Seed Brachytherapy in an Orthotopic Mouse Model of Human Pancreatic Carcinoma', *Anticancer Res*, vol. 39, no. 11, pp. 5919–5925, Nov. 2019, doi: 10.21873/anticanres.13796.

[141] Q. Yue and B. Jianzhong, 'Killing effect of Robo1 targeted Chimeric Antigen Receptor modified NK92 cells against glioma and neuroblastoma cells', *National Medical Journal of China*, vol. 98, no. 11, pp. 860–866, Mar. 2018, doi: 10.3760/cma.j.issn.0376-2491.2018.11.014.

[142] A. Rodriguez-Garcia *et al.*, 'Safety and Efficacy of VCN-01, an Oncolytic Adenovirus Combining Fiber HSG-Binding Domain Replacement with RGD and Hyaluronidase Expression', *Clinical Cancer Research*, vol. 21, no. 6, pp. 1406–1418, Mar. 2015, doi: 10.1158/1078-0432.CCR-14-2213.

[143] I. Eissa *et al.*, 'The Current Status and Future Prospects of Oncolytic Viruses in Clinical Trials against Melanoma, Glioma, Pancreatic, and Breast Cancers', *Cancers*, vol. 10, no. 10, p. 356, Sep. 2018, doi: 10.3390/cancers10100356.

[144] L. Vidal *et al.*, 'A Phase I Study of Intravenous Oncolytic Reovirus Type 3 Dearing in Patients with Advanced Cancer', *Clinical Cancer Research*, vol. 14, no. 21, pp. 7127–7137, Nov. 2008, doi: 10.1158/1078-0432.CCR-08-0524.

[145] A. L. Angelova, M. Witzens-Harig, A. S. Galabov, and J. Rommelaere, 'The Oncolytic Virotherapy Era in Cancer Management: Prospects of Applying H-1 Parvovirus to Treat Blood and Solid Cancers', *Front. Oncol.*, vol. 7, p. 93, May 2017, doi: 10.3389/fonc.2017.00093.

[146] L. Buscail *et al.*, 'First-in-man Phase 1 Clinical Trial of Gene Therapy for Advanced Pancreatic Cancer: Safety, Biodistribution, and Preliminary Clinical Findings', *Molecular Therapy*, vol. 23, no. 4, pp. 779–789, Apr. 2015, doi: 10.1038/mt.2015.1.

[147] S.-S. Kim, J. B. Harford, M. Moghe, A. Rait, and E. H. Chang, 'Combination with SGT-53 overcomes tumor resistance to a checkpoint inhibitor', *OncoImmunology*, vol. 7, no. 10, p. e1484982, Oct. 2018, doi: 10.1080/2162402X.2018.1484982.

[148] L. Aguilar, 'Gene-mediated cytotoxic immunotherapy as adjuvant to surgery or chemoradiation for pancreatic adenocarcinoma', *Cancer Immunol Immunother.*, pp. 727–736, Nov. 2014, doi: DOI 10.1007/s00262-015-1679-3.

[149] J. Lee *et al.*, 'Tolerability and safety of EUS-injected adenovirus-mediated double-suicide gene therapy with chemotherapy in locally advanced pancreatic cancer: a phase 1 trial', *Gastrointestinal Endoscopy*, p. S0016510720301383, Feb. 2020, doi: 10.1016/j.gie.2020.02.012.

[150] S. O. Freytag, Y. Zhang, and F. Siddiqui, 'Preclinical toxicology of oncolytic adenovirus-mediated cytotoxic and interleukin-12 gene therapy for prostate cancer', *Molecular Therapy - Oncolytics*, vol. 2, p. 15006, 2015, doi: 10.1038/mto.2015.6.

[151] S. P. Chawla, H. Bruckner, M. A. Morse, N. Assudani, F. L. Hall, and E. M. Gordon, 'A Phase I-II Study Using Rexin-G Tumor-Targeted Retrovector Encoding a Dominant-Negative Cyclin G1 Inhibitor for Advanced Pancreatic Cancer', *Molecular Therapy - Oncolytics*, vol. 12, pp. 56–67, Mar. 2019, doi: 10.1016/j.omto.2018.12.005.

[152] Z. Ning *et al.*, 'Abnormal expression of Nek2 in pancreatic ductal adenocarcinoma: a novel marker for prognosis', p. 8.

[153] T. Golan *et al.*, 'RNAi therapy targeting KRAS in combination with chemotherapy for locally advanced pancreatic cancer patients', *Oncotarget*, vol. 6, no. 27, Sep. 2015, doi: 10.18632/oncotarget.4183.

[154] A. Amedei, E. Niccolai, and D. Prisco, 'Pancreatic cancer: Role of the immune system in cancer progression and vaccine-based immunotherapy', *Human Vaccines & Immunotherapeutics*, vol. 10, no. 11, pp. 3354–3368, Nov. 2014, doi: 10.4161/hv.34392.

[155] J. Kokkinos *et al.*, 'Targeting the undruggable in pancreatic cancer using nano-based gene silencing drugs', *Biomaterials*, vol. 240, p. 119742, May 2020, doi: 10.1016/j.biomaterials.2019.119742.

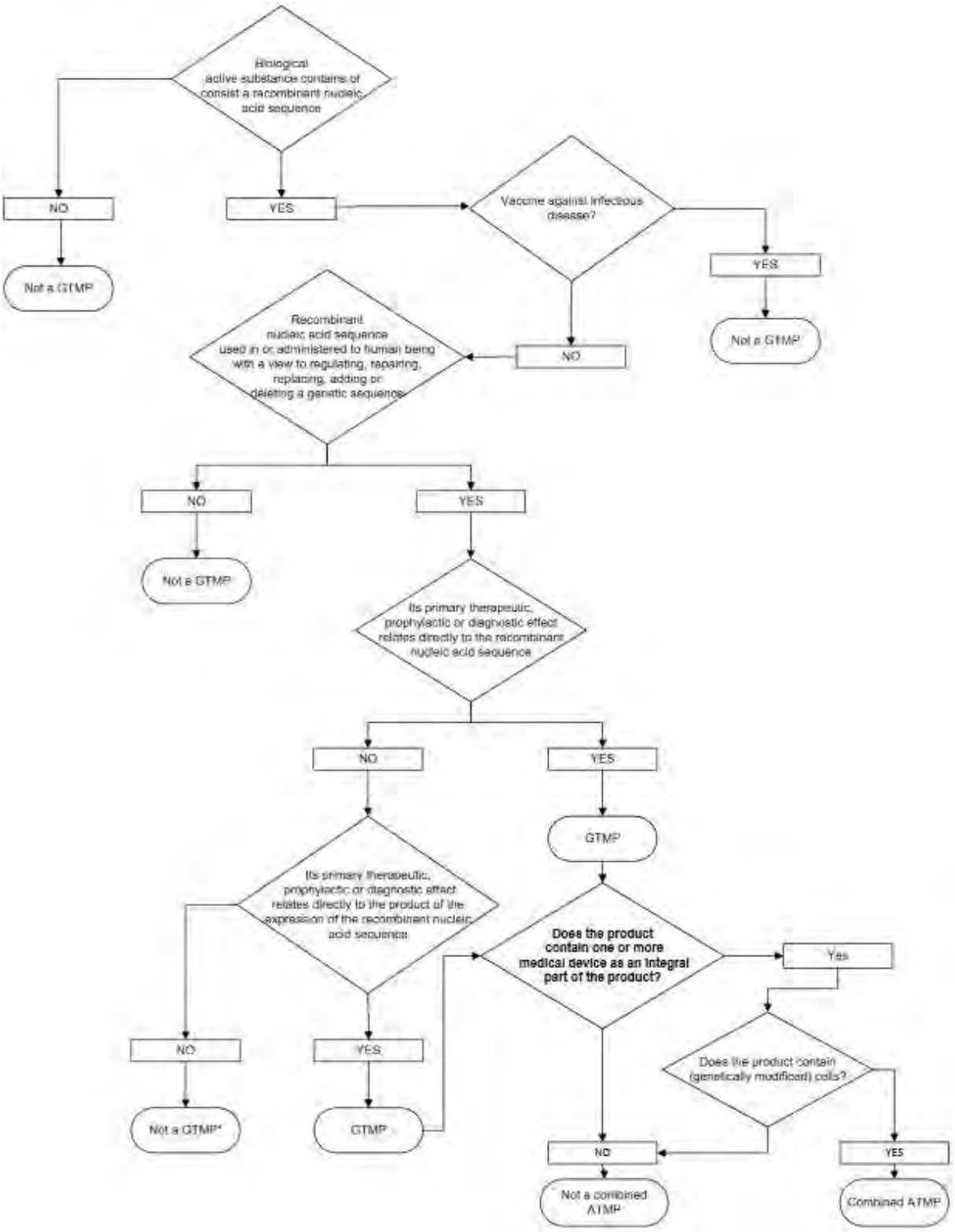
[156] R. F. Donnelly, 'Vaccine delivery systems', *Human Vaccines & Immunotherapeutics*, vol. 13, no. 1, pp. 17–18, Jan. 2017, doi: 10.1080/21645515.2016.1259043.

[157] H. Uusi-Kerttula, S. Hulin-Curtis, J. Davies, and A. Parker, 'Oncolytic Adenovirus: Strategies and Insights for Vector Design and Immuno-Oncolytic Applications', *Viruses*, vol. 7, no. 11, pp. 6009–6042, Nov. 2015, doi: 10.3390/v7112923.

[158] M. Rouanet, 'Gene Therapy for Pancreatic Cancer: Specificity, Issues and Hopes', *Int J Mol Sci.*, p. 25, Jun. 2017, doi: 10.3390/ijms18061231.

Annexe 1

EMA decision tree for GTMPs



*The product can contain genetically modified cells for which specific requirements should be followed (see 'Guideline on human cell-based medicinal products' (EMEA/CHMP/410869/2006)

Annexe 2

List of national competent authorities in the European Economic Area (EEA)

Country	National competent authority
Austria	Austrian Agency for Health and Food Safety
Belgium	Federal Agency for Medicines and Health Products
Bulgaria	Bulgarian Drug Agency
Croatia	Agency for medicinal products and medical devices of Croatia
Cyprus	Ministry of Health - Pharmaceutical Services
Czech Republic	State Institute for Drug Control
Denmark	Danish Medicines Agency
Estonia	State Agency of Medicines
Finland	Finnish Medicines Agency
France	National Agency for the Safety of Medicine and Health Products
Germany	Federal Institute for Drugs and Medical Devices
Germany	Paul Ehrlich Institute
Greece	National Organization for Medicines
Hungary	National Institute of Pharmacy and Nutrition
Iceland	Icelandic Medicines Agency
Ireland	Health Products Regulatory Authority (HPRA)
Italy	Italian Medicines Agency
Latvia	State Agency of Medicines
Liechtenstein	Office of Health / Department of Pharmaceuticals
Lithuania	State Medicines Control Agency
Luxembourg	Ministry of Health
Malta	Medicines Authority
Netherlands	Medicines Evaluation Board
Netherlands	Healthcare Inspectorate
Norway	Norwegian Medicines Agency
Poland	Office for Registration of Medicinal Products, Medical Devices and Biocidal Products
Poland	Chief Pharmaceutical Inspectorate
Portugal	National Authority of Medicines and Health Products
Romania	National Authority of Medicines and Medical Devices
Slovakia	State Institute for Drug Control
Slovenia	Agency for Medicinal Products and Medical Devices of the Republic of Slovenia
Spain	Spanish Agency for Medicines and Health Products
Sweden	Medical Products Agency

Annexe 3

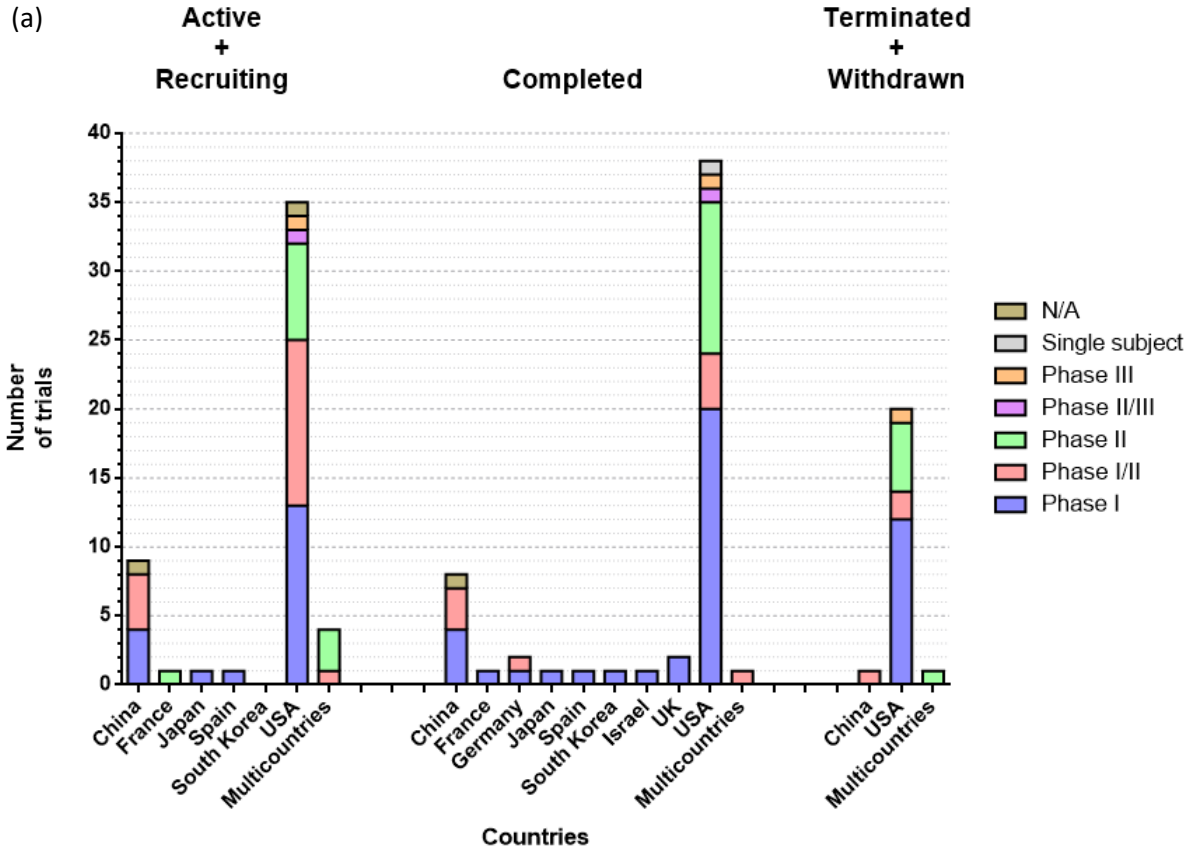
Advanced 3D cell culturing technique comparison

	Function	Preparation	Advantages and Applications
Hydrogel-based support	1. ECM can be replicated (Antoni et al., 2015) 2. Can be loaded with biological fluids and water (Antoni et al., 2015) 3. Can osmoregulate (Godugu et al., 2013)	1. Chemical crosslinking, free radical polymerization, irradiation crosslinking, and physical crosslinking via polyelectric complexation, hydrogen bonding, and hydrophobic association (Godugu et al., 2013)	1. Smart hydrogels can respond to environmental stimuli such as changes in temperature, pH, ionic strength, radiation, metal, electric field and more (Godugu et al., 2013) 2. Intestinal flow and diffusive transport (Langhans, 2018) 3. Act as drug storehouses, tissue barriers, and a bioactive moieties delivery system that stimulates the natural reparative process (Jordan et al., 2004; El-Sherbiny and Yacoub, 2013)
Polymeric hard material based support	1. The scaffold is used to replicate the *in vivo* ECM since cells can attach and form 3D cultures (Dhandayuthapani et al., 2011)	1. The cells are matured on the scaffold to model tumors or tissue (Shantha and Harding, 2003) 2. The cells are then cut to a diameter that fits inside a given test vessel (Hoffman, 2001)	1. The cell treatment procedures are very similar to 2D cell culture (Hoffman, 2001) 2. Very reproducible (Costa et al., 2016) 3. Tumoroids grown using patient samples show promising signs for drug screening and drug development (Peppas et al., 2000) 4. Tissue regeneration in bone, ligaments, cartilage, skeletal and vascular muscle, and central nervous system tissue (Haycock, 2011)
Hydrophilic glass fiber	1. Model the ECM (Cushing and Anseth, 2007) 2. Can be used in migration, invasion, chemo-invasion, and angiogenesis assays (Cushing and Anseth, 2007)	1. Commonly performed using the SeedEZTM lab device by Lena Biosciences 2. 3D cell cultures will be more consistent in shape, spread, thickness, and cell distribution in the X, Y, and Z dimensions (Cushing and Anseth, 2007)	1. Can perform spot culture experiments, mixed cell cultures, sol-state gel suspension experiments, non-contact and contact co-culture methods via the three-dimensional feeder layer technique, stack and culture experiments, and side-by-side cultures (Cushing and Anseth, 2007) 2. Cells may be primary cells, secondary cells, and cell lines of various origins and sources (Cushing and Anseth, 2007) 3. Can culture advanced 3D tumor models for long durations of time *in vitro* (Cushing and Anseth, 2007)
Magnetic levitation	1. The magnetic forces allow cell aggregation while inducing ECM synthesis (Godugu et al., 2013) 2. Promotes cell-cell interaction (Godugu et al., 2013)	1. Created by loading the cells with magnetic nanoparticles and then are exposed to an external magnetic field that causes cells to aggregate into a spheroid (Souza et al., 2010; Talukdar and Kundu, 2012)	1. Does not require a specific medium (Talukdar and Kundu, 2012) 2. Works with normal 2D cell culture techniques (Talukdar and Kundu, 2012) 3. Works with a wide range of cell types (Souza et al., 2010) 4. Not just limited to 96 well-plates (Adine et al., 2018) 5. Takes about 16 h for spheroids to form (Talukdar and Kundu, 2012) 6. Can form a 3D culture without the use of an artificial protein substrate (Talukdar and Kundu, 2012) 7. Can synthesize ECM while forming (Talukdar and Kundu, 2012)
Spheroid microplates with ultra-low attachment coating	1. The ultra-low attachment coating reduces cell adherence to promote spheroid formation (Dhandayuthapani et al., 2011)	1. Typically made out of polystyrene and treated with hydrophilic or hydrophobic coatings or made with natural polymers such as agarose (Haisler et al., 2015) 2. The v-shaped bottomed wells promote consistent spheroid formation in all the wells (Dhandayuthapani et al., 2011)	1. Transfer of spheroids to a new plate is often unnecessary due to the large volume 96- or 384 well plates (Haisler et al., 2015; Imamura et al., 2015) 2. The spheroids of human breast cancer cells mimicked characteristics *in vivo* such as hypoxia, dormancy, anti-apoptotic features, and drug resistance in one study (Coleman et al., 2007) 3. 3D neurospheres have proven useful in studying growth kinetics and drug toxicity (Imamura et al., 2015)

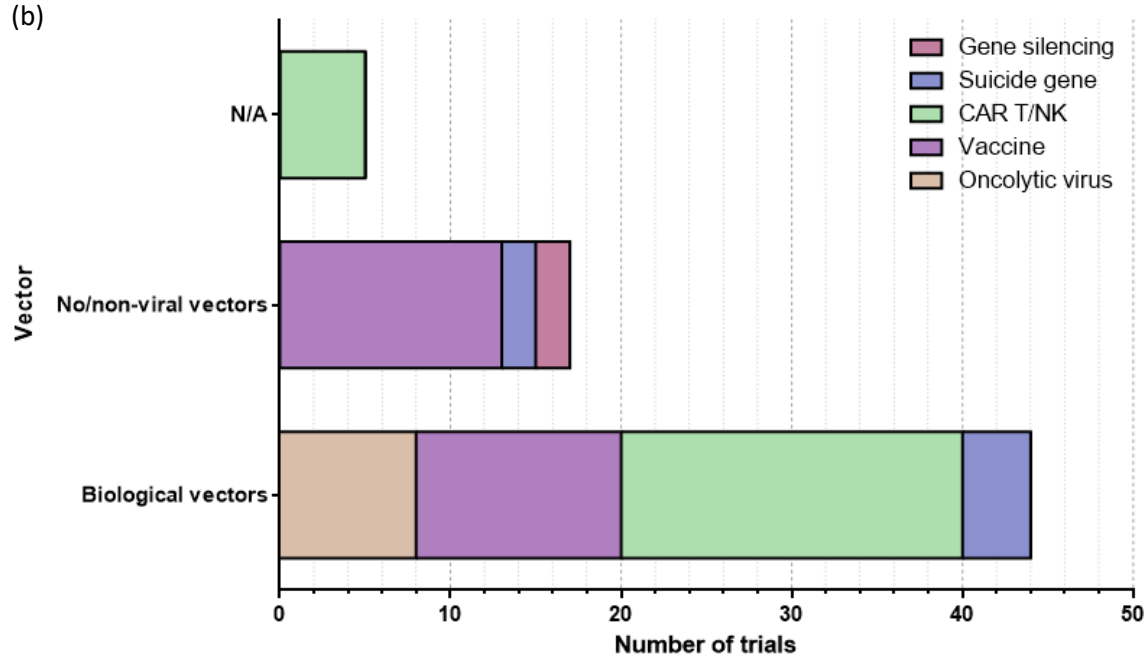
Annexe 4

Supplementary graphs of clinical trial repartitions related to pancreatic cancer

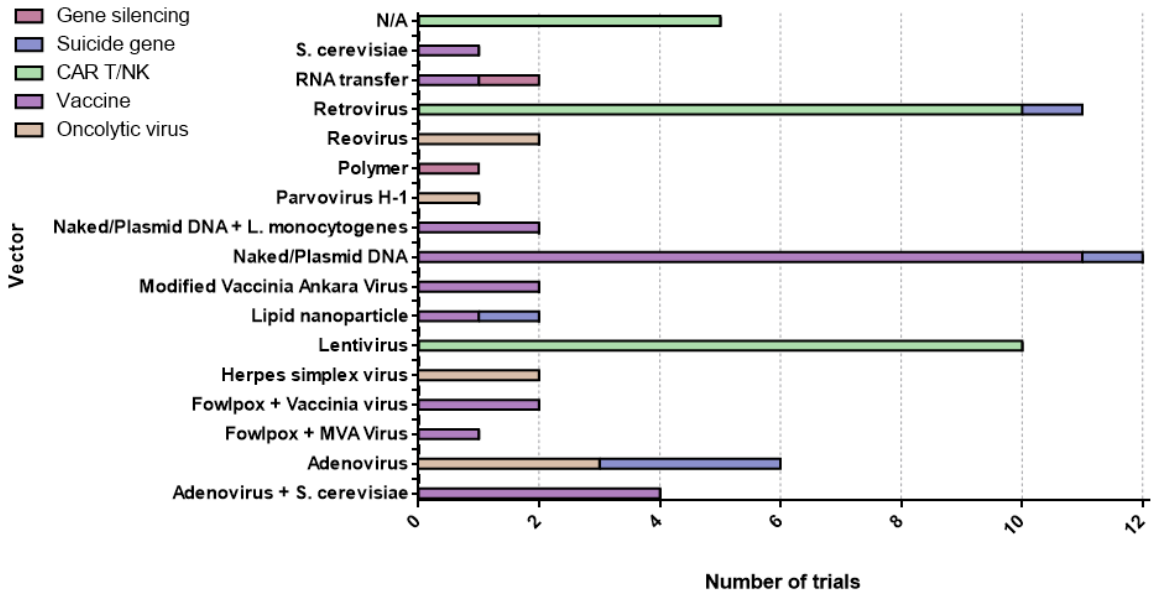
(a)



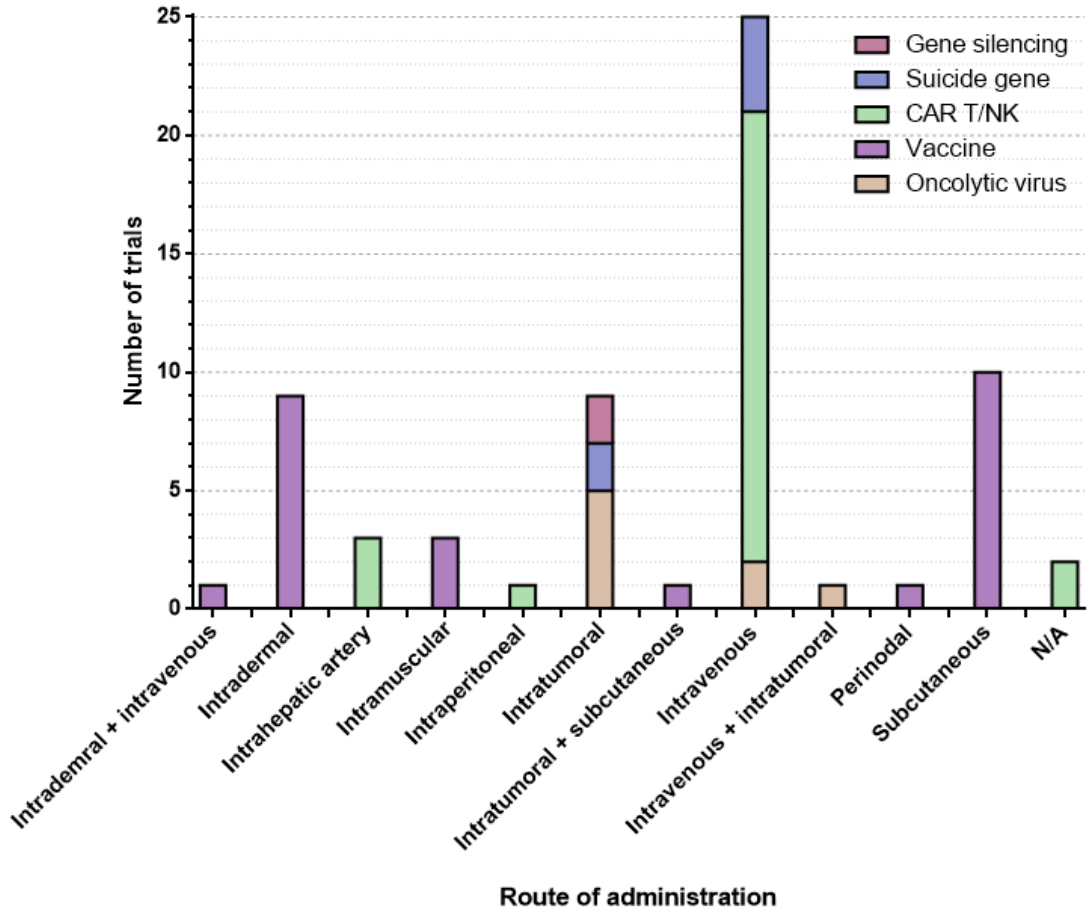
(b)



(c)


Gene silencing
Suicide gene
CAR T/NK
Vaccine
Oncolytic virus
Vector
N/A
S. cerevisiae
RNA transfer
Retrovirus
Reovirus
Polymer
Parvovirus H-1
Naked/Plasmid DNA + L. monocytogenes
Naked/Plasmid DNA
Modified Vaccinia Ankara Virus
Lipid nanoparticle
Lentivirus
Herpes simplex virus
Fowlpox + Vaccinia virus
Fowlpox + MVA Virus
Adenovirus
Adenovirus + S. cerevisiae
0
2
4
6
8
10
12
Number of trials

(d)


Number of trials
0
5
10
15
20
25
Gene silencing
Suicide gene
CAR T/NK
Vaccine
Oncolytic virus
Intrademral + intravenous
Intradermal
Intrahepatic artery
Intramuscular
Intraperitoneal
Intratumoral
Intratumoral + subcutaneous
Intravenous
Intravenous + intratumoral
Perinodal
Subcutaneous
N/A
Route of administration

GENE THERAPY IN ONCOLOGY:
FROM BENCH TO BEDSIDE

ABSTRACT:

Anticancer drugs have continuously evolved in synergy with our understanding of the biological and molecular cogs in the cancer machine. This is also the case for advanced medicinal products, particularly gene therapy treatments, which are directed by regulations and guidelines so as to ensure the best efficacy and safety in human use. Gene therapy product conception varies with regards to the genetic material in play (transgene), the utility of a vector, and its nature – the majority being of biological origin. Complex tumours that are unscathed by current treatments, such as pancreatic cancer, have the most to gain from gene therapy. This work assembles the latest techniques developed with regards to gene therapy, as well as an analysis of the ongoing or recently completed clinical trials in relations to pancreatic cancer, untangling the tools used in this pathology.

KEYWORDS: Gene therapy medicine, European regulation, Pancreatic cancer, Clinical trials

LA THERAPIE GENIQUE EN ONCOLOGIE : DE LA PAILLASSE AU PATIENT

RESUME :

Les traitements anticancéreux ont continuellement évolué en parallèle avec notre compréhension biologique et moléculaire de cette pathologie. Ceci en va du même pour les thérapies innovantes, en particulier les thérapies géniques qui nécessitent des régulations et lignes directrices afin de permettre la meilleure efficacité et sécurité lors de l'usage chez l'Homme. La conception d'un produit de thérapie génique varie selon le choix de matériel génétique mis en jeu (transgène), la présence ou non de vecteur ainsi que la nature de ce vecteur – souvent d'origine biologique. Les tumeurs complexes réfringentes aux traitements d'aujourd'hui, tel que le cancer du pancréas, sont les plus à même de profiter de la thérapie génique. Ce travail recueille les dernières techniques développées dans la thérapie génique ainsi qu'une analyse des essais cliniques en cours ou récemment complétées pour le cancer du pancréas, permettant d'élucider les outils mis en jeu pour cette pathologie.

Titre et résumé en Anglais : voir au recto de la dernière page de la thèse

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DISCIPLINE administrative : DES Innovation Pharmaceutique et Recherche

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MOTS-CLES : Médicament de thérapie génique, Règlementation Européenne, Cancer du pancréas, Essais cliniques

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