UNIVERSITAT ROVIRA i VIRGILI

CAR-T THERAPY AS TREATMENT FOR PANCREATIC DUCTAL ADENOCARCINOMA

- FINAL DEGREE PROJECT -

- BIOCHEMISTRY AND MOLECULAR BIOLOGY –

AUTHOR: RAQUEL DELGADO GIL

MENTOR: CARME GRAU BOVÉ

TARRAGONA, 2022

TABLE OF CONTENTS

1. ACKNOWLEDGEMENTS

First, I would like to express my deep gratitude to my tutor Carme Grau. She has been supporting me and encouraging me from the very first moment. Additionally, she has helped me and given me all the necessary guidelines to be able to carry out this work successfully, always taking into account my ideas at the same time.

Moreover, I would like to thank Rovira i Virgili University for all these years in which I have grown as a person. I am honestly grateful to all my professors for their lessons and wisdom that have helped me get here.

Lastly, but not less important, I am extremely grateful to my family for always believing in me. They have been a fundamental and constant pillar throughout my life and have encouraged, supported, and helped me in every aspect.

2. ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a strongly fatal disease since early diagnosis is usually not possible. Since it is commonly an incurable disease, new treatments are required. This project reviews the current state of knowledge on chimeric antigen receptor (CAR) T-cell immunotherapy to treat PDAC. Several trials are discussed, establishing the importance of rightfully choosing the target antigen, the engineering of the cells, and the application of preconditioning or combinatorial therapies, among other aspects. Efficacy and toxicity depend on these factors, but the promising results published so far indicate that this therapy could be used to treat PDAC shortly.

KEYWORDS: PDAC, pancreas, cancer, immunotherapy, CAR

3. INDEX OF ABBREVIATIONS

5-FU	5-fluorouracil
ALL	Acute lymphoblastic leukemia
ACT	Adoptive cell therapy
ACTH	Adrenocorticotropic hormone
BRM	Biological response modifier
BMSC	Bone marrow stromal cell
CSC	Cancer stem cell
CAF	Cancer-associated fibroblast
CRES	CAR T-cell-related encephalopathy syndrome
CA	Carbohydrate antigen
CEA	Carcinoembryonic antigen
CAR	Chimeric antigen receptor
CLD18.2	Claudin 18.2
CT	Computed tomography
CP	Cyclophosphamide
CRS	Cytokine release syndrome
EUS	Endoscopic ultrasound
EMT	Epithelial-to-mesenchymal transition
ECM	Extracellular matrix
FasL	Fas ligand
FAP	Fibroblast activation protein-α
HSV-TK	Herpes simplex virus thymidine kinase
HER2	Human epidermal growth factor receptor type 2
HLA	Human leukocyte antigen
ICANS	Immune effector cell-associated neurotoxicity syndrome
iCasp9	Inducible caspase 9
iCAR	Inhibitory CAR
IPMN	Intraductal papillary mucinous neoplasm
MRI	Magnetic resonance imaging
MHC	Major histocompatibility complex
MSLN	Mesothelin
MLL	Mixed-lineage leukemia
MUC1	Mucin 1
mcCAR	Multi-chain CAR
MDSC	Myeloid-derived suppressor cell
NCR	Natural cytotoxicity receptors
NK	Natural killer
NET	Neuroendocrine tumor
NKG2D	NK group 2
NHL	Non-Hodgkin lymphoma
PDAC	Pancreatic ductal adenocarcinoma
PanIN	Pancreatic intraepithelial neoplasia
PSC	Pancreatic stellate cell
PET	Positron emission tomography
PSCA	Prostate stem cell antigen
QM	Quasimesenchymal

SAE	Serious adverse event
Treg	Regulatory T cells
scFv	Single-chain variable antibody fragment
SMRP	Soluble MSLN-related peptide
sCAR	Switchable CAR
TCR	T-cell receptor
TME	Tumor microenvironment
TAM	Tumor-associated macrophage
TIL	Tumor-infiltrating lymphocyte
VIP	Vasoactive intestinal peptide

4. INTRODUCTION

4.1. Pancreatic cancer

The pancreas is an organ of the digestive and endocrine system located in the abdominal region, spanning from the duodenum to the spleen (1,2). As a result of its location, it is in contact with a large number of blood and lymphatic vessels, as well as nerve structures (2). It presents both exocrine and endocrine activities. As an exocrine gland, it contributes to the digestive and absorptive processes of food by secreting pancreatic juice into the duodenum, containing bicarbonate and digestive enzymes. Bicarbonate acts by neutralizing acid from the stomach, while the enzymes break down nutrients (1–3). Pancreas also regulates blood sugar levels, as part of its endocrine function, due to the secretion of a variety of hormones such as glucagon, insulin, amylin, somatostatin, ghrelin, gastrin, and pancreatic polypeptide (1,2,4). This endocrine function is carried out by 5% of the pancreatic cells (2,5), which are called "islets of Langerhans" and comprehend α-, β-, δ-, ε- and γ-pancreatic cells (4).

Pancreatic cancer's overall incidence has been gradually rising, representing 2.57% of all tumors in 2020 (6). Frequently, the age at which the cancer is diagnosed is 60-80 years old (7). In 2020 there were 495.773 new cases worldwide, being the twelfth most common type of cancer with a 2.56% of prevalence. Nevertheless, this rank position differs when it comes to mortality in the same year, as it appears to be in the seventh position with 466.003 deaths (4.68% of all cancers) (6). The incidences of new cases and number of deaths are predicted to keep on rising. It has been estimated that in 2040 there will be 801.634 new cases worldwide (2.77% of all cancers), showing an increase of 61.7% compared to the current data. As for the mortality numbers, there will be 765.261 deaths (4.73% of all cancers), increasing 64.2% compared to the current data (6).

Hence, pancreatic cancer is a strongly fatal disease and is progressively becoming a common cause of cancer mortality, presenting an average 5-year survival rate of 10% (3,8). However, this percentage may vary depending on the stage of the tumor at the time of diagnosis. A localized tumor, which has not yet spread outside the pancreas, has a 39% survival rate; a regional one, in which nearby lymph nodes, organs, or tissues have been reached by the tumor, has a 13% survival rate; and a distant one has only a 3% survival rate, when distant organs, tissues or lymph nodes are affected (3,5). Therefore, it is of high importance to detect the tumor as soon as possible.

4.1.1. Risk factors, common symptoms, and diagnosis

Most pancreatic cancers appear sporadically, and there have been described a variety of risk factors that may increase the probability of developing this malignant disease. Risk factors that stand out among others are the following: advanced age, male sex, black race, smoking, chronic pancreatitis, exposure to certain chemicals, obesity, and diabetes mellitus (2,3,5,7,8). Additionally, 10% of pancreatic cancers are due to hereditary causes, so family clinical history is also considered an important risk factor (3,7–9).

Generally, signs of pancreatic cancer usually appear late when the tumor has grown excessively or has spread to other organs (3,7,9,10). Duct obstruction can occur at an early stage, resulting in bilirubin build-up, so that jaundice shows. Hence, yellowing of the eyes, dark urine, light-colored or greasy stools, and itchy and yellow skin are likely to appear. Weight loss, poor appetite, nausea, vomiting, and belly or back pain are some of the common signs that might show, as well as gallbladder and liver enlargement, blood clots, and diabetes (2,3,5,8,9). Nevertheless, these symptoms are quite nonspecific and can also be caused by many other pathologies.

Detecting pancreatic cancer in its early stages is difficult because tumors are not noticeable during a routine physical exam and generally symptoms do not appear soon (3,5,8). Therefore, most patients (80%) already present an advanced or metastatic disease when being diagnosed (1,11). Moreover, as no screening test has proven to reduce mortality risk, they are not routinely performed except in high-risk individuals, including someone with a strong family history of pancreatic cancer or with a genetic syndrome that has been proven to increase the risk (3,7,9). These detection tests are also done if pancreatic cancer-related symptoms appear, to detect the problem and make a diagnosis. The standard detection test is computed tomography (CT) scan (7–9). In addition, other tests may be performed, like magnetic resonance imaging (MRI), endoscopic ultrasound (EUS), cholangiopancreatography, positron emission tomography (PET) scan, angiography, and blood analysis, looking for biomarkers such as carbohydrate antigen (CA) 19-9 or non-specific ones as carcinoembryonic antigen (CEA) or CA125. However, usually, it is necessary to perform a biopsy to be certain about the tumor's nature (3,5,7,8).

4.1.2. Pancreatic cancer classification

Pancreatic cancers can be sorted out in function on where it starts developing, either on the endocrine or the exocrine gland (Table 1). 95% of all pancreatic cancers are exocrine ones, and like all cancers, they can be either malignant or benign. The malignant exocrine pancreatic cancers are extremely aggressive and can also be divided into several types (1,3).

Table 1. Types and subtypes of pancreatic cancers and their prevalence.

Exocrine pancreatic cancer (95%) (1)		**Endocrine pancreatic cancer (<5%)** (3)		
Adenocarcinoma (PDAC)	>90% (1)	Non-functional		50% (3)
Acinar cell carcinoma	1-2% (1)	Functional (50%) (3)	Insulinoma	70% (3)
Squamous cell carcinoma	1-2% (12)		Gastrinoma	n.d.
Adenosquamous carcinoma	1-4% (1)		Glucagonoma	n.d.
Colloid carcinoma	1-3% (1)		VIPoma	n.d.
Signet ring cell carcinoma	n.d.		Somatostatinoma	n.d.
Undifferentiated carcinoma	0,1-6% (13)		ACTH-secreting tumors	n.d.

n.d.: not defined

Adenocarcinoma, or ductal carcinoma (PDAC), is the most common type of pancreatic cancer (>90%). It usually begins to develop in the endothelial cells that line the exocrine ducts. This project will be mainly dedicated to PDAC due to its significant prevalence compared to the other pancreatic cancer types (1,3).

A less common type of PDAC starts developing in the cells responsible for producing pancreatic enzymes. It presents mostly the same symptoms as the typical PDAC and is called acinar cell carcinoma. The other types of exocrine pancreatic cancers, which are uncommon, are squamous cell carcinoma, adenosquamous carcinoma, colloid carcinoma, signet ring cell carcinoma, and undifferentiated carcinoma. Among these, adenosquamous carcinoma is the most aggressive one, whereas colloid carcinoma is the least aggressive one because it does not spread easily (1,3).

On the other hand, only <5% of pancreatic cancers are endocrine ones, which can also be called neuroendocrine tumors (NETs) or islet cell tumors, and have a better prognosis than exocrine cancers. NETs can either be functioning when they affect cells of the “islets of Langerhans” that produce hormones, or non-functioning if the affected cells do not

produce any hormone. The latter ones do not make many symptoms until their size is really big, so they are not usually diagnosed at the early stages (3).

About 50% of NETs are functioning ones and can be classified in function of which hormone's production gets altered. The most common one (70%) is insulinoma (insulin), but they can also be gastrinoma (gastrin), glucagonoma (glucagon), VIPoma (vasoactive intestinal peptide (VIP)), somatostatinoma (somatostatin), or ACTH-secreting tumors (adrenocorticotropic hormone (ACTH)) (1,3).

Among the different subtypes of pancreatic cancer, PDAC will be the focus of this current work. In the following sections, the current knowledge about the pathophysiology of PDAC and the treatment option for this pathology will be reviewed.

4.2. PDAC: classification and pathophysiology

A wide variety of molecular features that can be affected in PDAC has been reported. These genetic expression differences have allowed the identification and classification of these pancreatic cancer cases. Their clinical progression rates differ between them, and so does the prognosis and each treatment's effectiveness. This classification is extremely useful to achieve a more individualized therapy for each one, thereby enhancing the chances of being successful when treating these patients (11,14).

Two PDAC molecular subtypes have been described: classical and quasimesenchymal (QM-PDAC). The former is characterized by overexpression of adhesion and epithelial genes, meanwhile, the latter has overexpressed mesenchyme-associated genes. The classical PDCA has been related to higher expression levels of *GATA6* and *KRAS* (14), and to a better prognosis after resection of the tumor (8,14). Consequently, KRAS- or GATA6-directed therapy appears as a promising treatment for this subtype of PDAC patients (14).

Another two different subtypes associated with low cellularity have also been proposed: exocrine-like and immunogenic. However, their true existence is controversial, since some studies state that their presence is only due to contamination of tumor samples with neighboring tissues (11).

4.2.1. PDAC initiation and development

PDAC mostly arises from pancreatic intraepithelial neoplasia (PanIN) that acquires some alterations at the genome level (8,11), like point mutations in the *KRAS* oncogene, such as aspartic acid, valine, and cysteine mutations. These lead to the GTP-bound KRAS form being permanently active (11), enhancing the RAS and PI3K-AKT signaling pathways, thus leading to higher cell survival and motility (8). These genetic modifications take place in 90% of PDAC and relate to poor prognosis. Nevertheless, some mutations only activate slightly ERK and may relate to a better prognosis (11).

Grade 1 PanIN is characterized by chromosomal instability due to telomere shortening (8). Grade 2 PanIN presents cyclin-dependent kinase inhibitors in its inactive state, like CDKN2A in 46-60% of PDAC, thus promoting uncontrolled cell growth and the cell cycle progression. Late stages (grade 3 and 4 PanIN) of carcinogenesis correlate with inactivating mutations in the tumor suppressor genes *TP53* and *SMAD4*. *TP53* alterations are present in 50-70% of PDAC and enhance cell proliferation and survival and reduce apoptosis and DNA damage recognition. *SMAD4* (60-90% or 31-38% of PDAC) mutations induce tumorigenesis by reducing canonical TFG-β signaling and promoting non-canonical ones (8,11).

On the other hand, cystic neoplasms like intraductal papillary mucinous neoplasm (IPMN), even though originally they are not aggressive since they do not spread easily (1), they can also develop in PDAC (8,11). 41-75% of IPMN and around 4% of PDAC cases have the GNAS complex locus (*GNAS*) mutated, which is fundamental in GPCRs signaling pathways (11). Mutations inactivating SMAD4 are not that common in IPMN cancers (9). Furthermore, germline mutations in DNA repair associated genes, such as *BRCA2*, are also present in 4-7% of PDAC (11).

The epigenome usually also suffers some modifications, as in the genes encoding *SWI/SNF* complexes, which act by remodeling the chromatin, and are mutated in more than 10% of PDAC. Moreover, histone modification enzymes like mixed-lineage leukemia (MLL) histone methylases or histone methyltransferases may also be altered, thus promoting cell proliferation and cell cycle progression, as well as regulating the chromatin. Histone deacetylases like HDAC1 and HDAC2 are significantly expressed in PDAC and enhance epithelial-to-mesenchymal transition (EMT) and metastasis (11).

Several signaling pathways get activated in PDAC cases, regulating, and promoting the development of the malignancy, such as the JAK/STAT and the NF-κB pathways. The JAK/STAT is important in some inflammatory processes and correlates with a poorer prognosis. The NF-κB pathway is also involved in inflammation, but is related as well to tumor growth and progression, metastasis, and therapeutic resistance (11).

However, it has been observed that each signaling pathway is more or less modified depending on the PDAC subtype that the patient suffers from. For example, in the classical one, with higher expression levels of KRAS, the RAS, PIRK/AKT, and WNT pathways are usually more altered, although they are also slightly altered in other subtypes. The RAS pathway gets activated in 90% of PDAC and is involved in the tumor initiation and development, as well as in inflammation, cell transformation, proliferation, and metastasis. The PI3K/AKT pathway gets modified in more than 40% of PDAC being also involved in proliferation and metastasis, just as in cell plasticity, carcinogenesis, and short survival rate. The alteration of the WNT pathway leads to carcinogenesis, tumor progression, therapeutic resistance, invasion, and migration. In the QM-PDAC subtype is highly significant the alteration of the Hippo/YAP pathway, which is involved in tumor progression, therapeutic response, and metabolic homeostasis, and correlates with a poorer prognosis (11).

4.2.2. PDAC tumor microenvironment (TME)

The highly aggressivity and mortality associated with PDAC are mainly due to its hostile microenvironment since it is extremely immunosuppressive. This means that is capable of escaping from being killed by cytotoxic immune cells in a wide variety of ways. It is a highly acidic environment as a consequence of increased lactate secretion (15). Moreover, it is hypoxic because of a lack of blood vessels, low oxygen perfusion, and a high metabolic rate (15,16). These features are also extremely responsible for the general therapeutic resistance of PDAC (10).

Both primary and metastatic PDAC tumors are surrounded by a dense and extensive stroma. It corresponds to 80-90% of the total tumor. It is considered a desmoplastic stroma since it is mainly composed by connective tissue. It contains a big heterogeneous mass of cells including myofibroblasts, pancreatic stellate cells (PSCs), fibroblasts, blood vessels, extracellular matrix (ECM), immune cells, cytokines, and growth factors. Therefore, the stroma functions as a physical barrier that protects PDAC cells and

promotes tumor proliferation, invasion, and metastasis (10,11). Inflammatory cells are present in this desmoplastic stroma, including PSCs, cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs). This inflammation contributes to desmoplasia, tumor progression, and immunosuppression (8,10,11). CAFs are the principal component of the PDAC stroma. They express the fibroblast activation protein-α (FAP) that worse the prognosis. They also stimulate aerobic glycolysis and secrete metabolites of high energy. Therefore, they promote tumor growth even when there are limited nutrients by supplying amino acids, intermediates of the Krebs cycle, and lipids (11).

Metastasis and vascularization are common in PDAC because TAMs elevate glycolytic metabolism. It was also observed that TAMs levels directly correlate with the tumor size, prognosis, and survival of the patient (11). Moreover, the numbers of PSCs are also related to the tumor size, metastasis, and desmoplasia (15). High levels of MDSCs also aggravate the disease, being associated with inflammation and tumor progression (11). The lower survival rate is also linked to Tregs cells as they make it difficult for T cells to recognize cancer cells. Components of the ECM promote water retainment by increasing osmotic pressure and rigidity. They do mechanical forces around the tumor that also act as protection, reducing perfusion, as well as drug sensitivity (15).

In the TME there is a huge infiltration of immunosuppressive leukocytes, and antitumor T cells levels are considerably small (11). Some cells like macrophages and Tregs under hypoxic conditions secrete cytokines such as IL-10 and TGF-β that have immunosuppressive effects by inhibiting cytotoxic T cells (10,11). They are responsible as well for recruiting cells involved in immune evasion, thus regulating the formation of the TME. Neutrophils secrete cytokines as well like TNF-α that enhance metastasis (11).

The expression of major histocompatibility complex I (MHC I) is downregulated, and the antigen recognition and insertion associated with this molecule are inhibited, leading to a lower cell clearance. Fas ligand (FasL) induces apoptosis when it binds to its receptor. PDAC cells express FasL and non-functional Fas receptor, inhibiting its own apoptosis, but promoting immune cells The apoptosis process is also induced by the immune checkpoints PD-L1, which are overexpressed in PDAC cells, also enhancing angiogenesis (10,11,15). The IDO enzyme is also secreted, which decreases the lymphocytes' lifetime and increases the antitumor T cells' response (10). This enzyme is involved as well in the recruitment and differentiation of Tregs (15).

4.3. Treatment options

Surgery, ablation, embolization, radiation, chemotherapy, and immunotherapy are some of the therapies that may be used as treatment options. Sometimes, it is recommended to carry out more than one type of treatment because of the synergistic effect that may increase the response (3,8,9,16,17).

Treatment options for PDAC are different depending on the type and stage of cancer and other factors, such as the overall health (3,7). Commonly, PDAC progression is described following the TNM staging system, which takes into account the primary tumor, lymph nodes, and metastasis. Firstly, the T represents the size of the primary tumor and its growth outside the pancreas. Secondly, the N indicates the level of spread to nearby lymph nodes. Finally, the M corresponds to the distant metastasis (1,7). Afterwards, these values get together and correspond to a numerical stage. The lowest value (S0) corresponds to the early stages when malignant cells are only found in the lining of the pancreas. On the other hand, the highest value (S4) indicates that it has spread to distant organs and may have also affected nearby lymph nodes or organs/tissues (1,5,7).

As has already been said, the treatment, or combination of them, used depends on the stage of the PDAC. They are classified as well according to the probability that it will be possible to remove the tumor or not. Resectable cancers refer to tumors that can be fully removed because it has not spread very much. This corresponds mostly to stages IA, IB, and IIA, where both N and M are 0. On the other hand, if a tumor cannot be totally removed, it is called unresectable. Here it is possible to differentiate between locally advanced, which englobes the rest of the tumor's stages without metastasis (M0), and metastatic cancers, which are stage IV tumors having M1 (3).

Therefore, resectable pancreatic cancers are usually treated by combining surgical resection and chemotherapy using adjuvants such as FOLFIRINOX, gemcitabine, or capecitabine. Moreover, treating also with chemoradiation increase resection rate and disease-free survival (8). A small percentage of locally advanced PDAC patients may benefit from surgical resection. However, the wide majority of these patients and metastatic ones are unsuitable for surgical resection, being an incurable disease. These patients undergo systemic chemotherapy with the aim of palliating symptoms and prolonging life (7,8,10). Therefore, surgical resection remains the only treatment with curative potential due to low survival rates when using the other treatments (8,15–17).

It is worth noticing that the efficacy of treatments is variable depending on the PDAC subtype. For example, the targeted therapy with gemcitabine showed better income in the quasimesenchymal subtype, meanwhile, the erlotinib worked better for the classical one. Therefore, combining both agents would only increase toxicity, not efficacy (14).

Formerly, the main therapies used were surgery, chemotherapy, and radiation. Nonetheless, they only seem to improve slightly the 5-year survival rate, and with considerable toxicities (11). Targeted therapies, like Imatinib (tyrosine-kinase inhibitor) or Erlotinib (EGFR inhibitor), have gained importance since their discovery in the late 1990s (3,15), although they do not imply a significant improvement (11). Therefore, it is necessary to look for a kind of therapy that does act efficaciously when treating this lethal disease, and nowadays immunotherapy has gained attention as a promising tool that may finally achieve this goal (10,17,18).

4.7.1. Immunotherapy

The immune system is a network of cells and proteins that can recognize and fight any unknown substance as part of its normal function, as abnormal cells (3). Immunotherapy is a biological therapy, or biological response modifier (BRM), as substances made from living organisms are used to act against tumor cells (5). It involves either stimulating the patient's immune system or using designed substances to improve the immune system efficiency, therefore fighting cancer cells in a more effective way (3).

Nevertheless, detecting these cancer cells as foreign can be tough as they were initially normal ones and, in addition to that, they can evade the immune system's mechanisms and modify it so that proliferation, invasion, and metastasis of cancer cells are enhanced (3), as it has already been discussed. Immunotherapy involves reversing these methods of immune escape, improving the immune system's ability to attack cancer cells.

Despite being approved to treat many types of cancer, immunotherapy works better for some types of cancer on which it can be used by itself, like metastatic colorectal cancer. However, for other kinds of cancers, it is more beneficial when it is used alongside other therapies, such as melanoma (5,17). Hence, it is not as commonly used as other techniques such as the already mentioned surgery, chemotherapy, or radiation. There is current research on improving immunotherapy by different methods, such as reducing its side effects or looking for solutions to overcome the presented resistance to immunotherapy.

There have been described several types of immunotherapies that can be used to treat cancer (3,5,17):

- Immune checkpoint inhibitors act preventing the immune system to be turned off, thereby allowing molecules like T cells to attack cancer cells.
- Monoclonal antibodies are created to interact with specific targets so that it helps the immune system to act against cancer cells.
- Treatment vaccines help the immune system to recognize and react to specific antigens on cancer cells, destroying them.
- Immunomodulators, such as cytokines or immunomodulatory drugs, enhance the immune response against cancer in a variety of ways.
- Oncolytic viruses are designed to target tumor cells killing them.
- T-cell transfer therapy.

4.7.2. T-cell transfer therapy

The T-cell transfer therapy, which is also called adoptive cell therapy (ACT), adoptive immunotherapy, or immune cell therapy, starts with the leukapheresis procedure. Leukapheresis consists of the collection of the patient's white blood cells, including T cells, which are, afterward, harvested and grown in large numbers in the laboratory. Finally, they are infused back into the patient, enhancing the immune system's ability to attack cancer (3,5,10,17). This immunotherapy has appeared to be a highly effective treatment for metastatic melanoma (19). There are three types of T-cell transfer therapy: tumor-infiltrating lymphocytes (TILs) therapy, specific cancer T-cell receptor (TCR) therapy and chimeric antigen receptor (CAR) T-cell therapy (20,21).

TILs may be found in and around tumors, preventing and slowing their growth (5). People whose tumors contain TILs have been shown to have a better prognosis than those who do not due to their ability to recognize tumor cells (15,21). However, they may not be present in the necessary amount so that they can overcome the tumor's effects and kill it. As a result, TILs that best recognize autologous tumor cells are selected and treated with several substances to make them grow (5). This is the basis for TIL therapy.

Even though this strategy is highly useful to eradicate advanced cancer in humans, it is mainly limited to melanoma because it usually generates a very strong immune response and has a high number of TILs (5,19,21). Therefore, the administration of

genetically engineered T-cells expressing tumor-specific antigen receptors (TCRs and CARs) may be a more promising approach for types of cancer (19,21).

This approach is based on the ability of these receptors to attach antigens present in cancer cells and enhance the immune system response to destroy them. However, when comparing TCRs and CARs, TCRs present some limitations regarding the presentation and recognition of epitopes.

TCRs can recognize either cell-surface or intracellular epitopes that are being presented by the MHC molecules (10,19). In humans, the presentation is performed by human leukocyte antigen (HLA) (21,22). This means that its function is fully dependent on antigen processing and presentation, being a highly restricted targeting process (10,22), especially because it is normally downregulated in solid tumors, such as PDAC (21). Moreover, TCRs always need that the HLA background matches the patient's haplotype (22), meaning that the HLA allele must be common among the presentation and treatment cells (19).

Lastly, CARs are commonly formed by antibody fragments attached to a signaling domain of the TCR and T-cell co-stimulatory domain, thus recognizing cell-surface antigens without being restricted by HLA presentation (10,18,19,21). Furthermore, they present better flexibility than TCRs and can bind not only to proteins, but carbohydrates or glycolipids as well (21,22).

For the CAR T-cell therapy, the patient's T cells are modified so they express a receptor that is specifically designed to bind proteins located in the membrane of cancer cells (10,15). As it has already been said, ACT is a promising strategy that can turn out to be highly effective for PDAC treatment, especially the CAR variety because of its several advantages. The current research regarding this therapy will be discussed in detail in the Results section.

5. AIM OF THE STUDY AND HYPOTHESIS

Pancreatic cancer, and especially pancreatic ductal adenocarcinoma (PDAC), is an extremely fatal disease with an average 5-year survival rate of 10%. Symptoms do not appear until the tumor has already grown too much or has spread to other organs. Therefore, its diagnosis commonly takes place when it is an advanced or metastatic disease and treatment via surgical resection is no longer an option. At this point, it is an incurable disease and only palliative care can help the patient's health. To sum up, new or improved therapies are needed in order to increase the survival rate of PDAC patients.

This project hypothesizes that CAR-T cell therapy is a highly promising and effective therapy to treat PDAC. CAR T-cells recognize antigens predominantly only located in cancer cells, so the patient's immune system is stimulated.

The main aim of this literature study will be to investigate and compress the current state of knowledge in both PDAC and CAR T-cell therapy. Moreover, different antigens will be described to determine their efficacy, comparing results obtained from clinical and preclinical studies.

A scientific review will be conducted to determine if the CAR T-cell therapy may end up being useful and efficacious in obtaining beneficial outcomes when treating PDAC in the future.

6. MATERIALS AND METHODS

The main tool used during the literature research for this report was Pubmed, which is a free access database that contains citations and abstracts of biomedicine and health fields, as well as other related topics such as life sciences. Its information comes from Medline, life science journals, and online books. The search terms employed, alone and in combination, included:

- Pancreatic cancer
- PDAC
- CAR
- Chimeric antigen receptor
- Adoptive cell therapy

The articles were filtered by time (2015 - present), having a total of 159 results. The first 50 results and the ones appearing in their "similar articles" sections were examined. Those whose title and/or abstract did not seem to go according to the project's topic were excluded. Finally, 38 articles were used for this literature project.

Additionally, 6 official divulgation webpages from research institutions were also visited:

- Johns Hopkins Medicine
- Asociación Española Contra el Cáncer
- American Cancer Society
- National Cancer Institute
- Global Cancer Observatory
- ClinicalTrials.gov

7. RESULTS

7.1. CAR T-cell therapy

CAR T-cell therapy was conceived in 1993 and is based on the transduction of the patient's collected T cells with genetic material that codifies for a chimeric antigen receptor (CAR) by using lentiviruses, retroviruses, RNA electroporation, or transposons (3,10,23). The whole procedure is described in Figure 1. As there are a wide variety of cancers, each one related to specific antigens, it is first necessary to identify the cancer's nature so the gene added codifies for the corresponding CAR (3). This process can effectively help the immune system in lysing and destroying cancer cells.

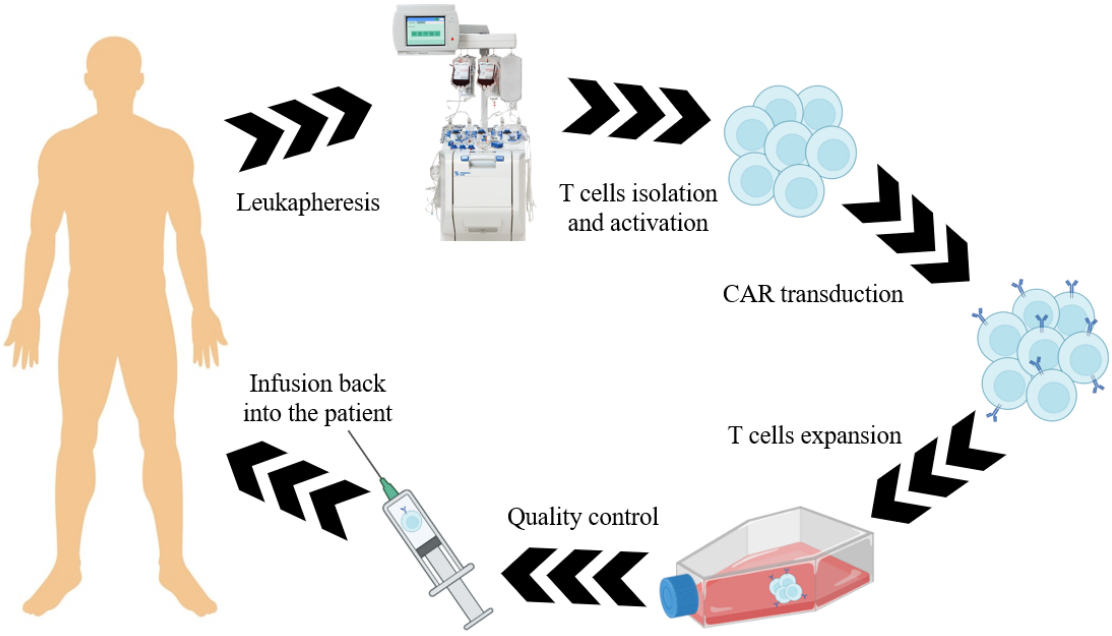

Figure 1. CAR T-cell therapy manufacturing workflow. Firstly, the patient's leukocytes are harvested by the leukapheresis procedure. Then T-cells are isolated by the Fluorescence-Activated Cell Sorting (FACS) or column methods and activated by magnetic beads (CD3 and CD28 antibodies). Afterward, T cells are transduced with the Chimeric Antigen Receptor (CAR) gene by using lentiviruses, retroviruses, transposons, or RNA electroporation. Later, transduced T cells are expanded in culture medium. Quality control is performed before these transduced T cells are infused back into the patient. Modified from (15) *and* (18).

Since 1989 four generation CARs have been developed. The first generation is formed by a single-chain variable antibody fragment (scFv) as binding antigen domain, attached to CD3ζ, which induces an antigen-specific T-cell immune response, thanks to a spacer domain. The second one is created by adding a co-stimulatory receptor signaling domain, such as 4-1BB, CD28, or OX40, that enhances T-cell response, survival, and proliferation (10,16,18,21,23,24). For the development of the third one, another co-stimulatory molecule must be added. However, its efficiency was similar to the second one, so it was

not commonly used (21). Fourth generation one adds cytokine releasing genes, such as IL-12 or IL-15, to improve T-cells survival in an immunosuppressive tumor microenvironment (10,16,18,23,24) or suicide genes, so that they can be turned off if necessary, improving safety (18).

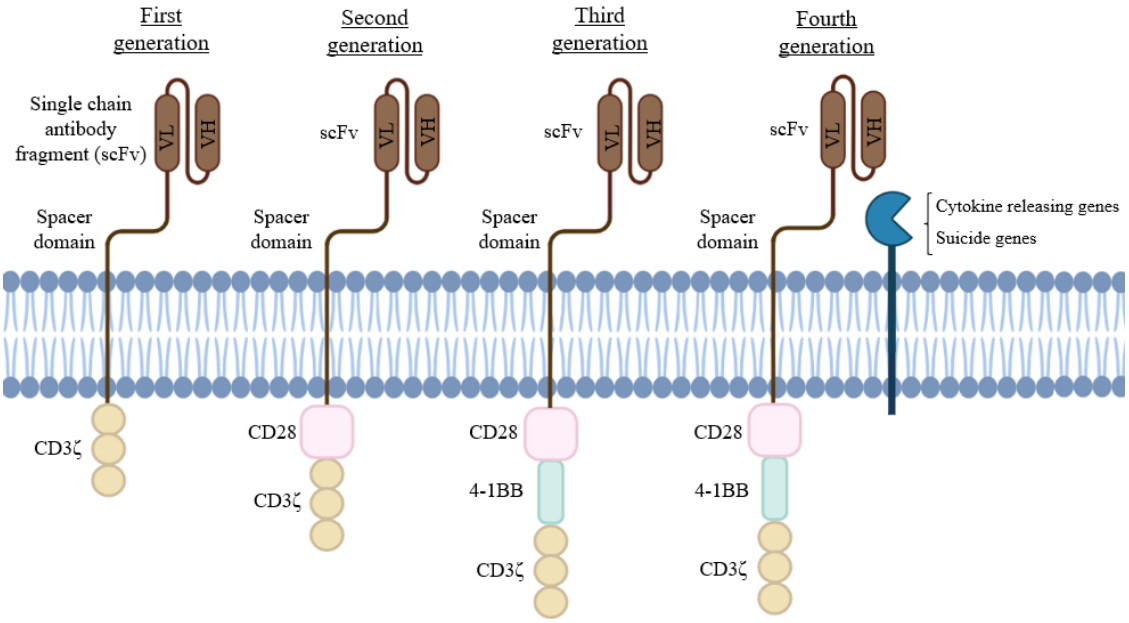

Figure 2. Structure of the four generations engineered of the CAR molecule. They all contain the antigen-binding domain (scFv), the spacer domain, and the CD3ζ domain. The second generation adds a co-stimulatory receptor signaling domain, which in this representation corresponds to CD28. The third one adds another co-stimulatory domain, being 4-1BB in this case. The last one includes either cytokine releasing or suicide genes. Modified from (18).

This therapeutical method has already proven to be useful and has been approved as a treatment of lymphomas, leukemias, and multiple myeloma (3,5,10,18,24). It appears to be a promising modality for solid tumors, like PDAC, as well.

7.2. Preconditioning

As PDAC has an immunosuppressive TME with a dense stroma, it is difficult for CAR T-cells to reach the target tumor cells and survive in that environment. Preconditioning, also known as lymphodepletion, helps reduce that immunosuppression by killing both patient's T cells and other immune cells that promote the tumor development, such as Tregs or MDSCs. This benefits CAR T-cells' survival since more cytokines and nutrients are available for them. Preconditioning may also result helpful for improving endogenous immune functions like antigen-presenting cell activation, facilitating the CAR T-cells binding to the antigens in tumor cells (21).

This lymphodepletion process is achieved by treatment with chemotherapy before CAR T-cell infusion. Some drugs that have been tested for this purpose are gemcitabine, 5-fluorouracil (5-FU), FOLFIRINOX (5-FU, oxaliplatin, and irinotecan), and cyclophosphamide (CP), among others (21). When treating pancreatic cancer, FOLFIRINOX showed the best outcomes by looking at overall and progression-free survival, and objective response rate, although tolerance may vary among patients (20).

7.3. Antigen targets

Different antigenic molecules can be targeted with CAR-T therapy. The ideal ones should be highly expressed on tumor cells and present very low or no expression on normal cells, reducing on-target/off-tumor toxicities (15,19,21). Moreover, this expression's patterns should be common for the wide majority of patients. Another feature that also enhances the molecule's value as a target is that it is related to the cancer's aggressiveness and/or that acting against it provides favorable immune responses (21). PDAC's related antigens that have been tested as targets are mesothelin (MSLN), carcinoembryonic antigen (CEA), CD24, CD133, mucin 1 (MUC1), human epidermal growth factor receptor type 2 (HER2), claudin 18.2 (CLD18.2), fibroblast activation protein (FAP), prostate stem cell antigen (PSCA) and natural killer (NK) receptors (Figure 3) (10,15,21,23,24).

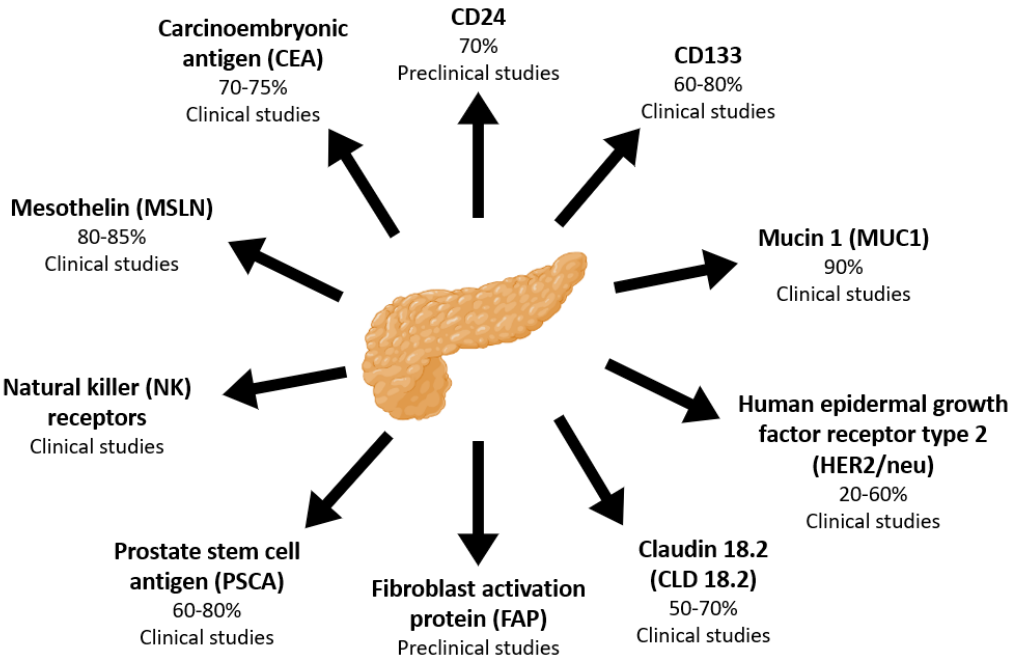

Figure 3. CAR antigen targets to treat PDCA. The percentage in which the antigen is present in pancreatic cancer cells is indicated in most of the targets, as well as if it is currently or has completed clinical or preclinical trials.

At the end of this section, the characteristics and current development of the CAR-T therapies based on each of these antigens will be summarized in Table 2. It will allow a better comparison of the state of development, efficacy, and toxicity among the antigens. Table 2 also includes information regarding potential side effects and combinatorial therapies, which will be introduced in the following sections.

7.3.1. Mesothelin (MSLN)

MSLN is a glycoprotein poorly expressed on the surface of normal mesothelial cells in the peritoneum, pericardium, and pleura, but it is overexpressed in some cancers such as PDAC (10,15,19,21,24). It acts as a differentiation factor on normal cells (10) and is involved in cell proliferation, cell signaling, adhesion, and metastasis in cancer ones, leading to a worse prognosis (21). 80%-85% of PDAC contain MSLN-expressing tumor cells, and 25-100% of mesothelial cells express the antigen (21,24).

MSLN is first formed as a precursor protein of 69kDa, and then it is cleaved into a C-terminal membrane-bound protein and an N-terminal soluble MSLN-related peptide (SMRP) (10,21,24). The latter can be measured in serum and can be used to detect the presence of MSLN-expressing tumor cells (21). Some immunotoxins, like LMB-100 or SS1, recombinant immunotoxins, like SS1P, and vaccines, like CRS-207, have been proven to target the membrane-bound MSLN *in vivo* showing antitumor activity (10,19,21). Consequently, MSLN appears to be an efficient target for CAR T-cell therapy.

This antigen has been tested as a CAR target in some pre-clinical and clinical studies. In a clinical trial conducted by Beatty et al., CAR T-cells were administered to a PDAC patient via mRNA electroporation and achieved a stable disease without serious adverse events (25). Tolerability to the treatment of PDAC with CAR T-cell therapy was established in these studies, as well as proving the beneficial outcomes of its use. However, patients from another clinical trial obtained mostly progressive diseases after this treatment, so it was terminated before reaching phase II (26).

7.3.2. Carcinoembryonic antigen (CEA)

CEA is a glycoprotein that is naturally low expressed in the colon, but its levels increase in some gastrointestinal cancers, like PDAC (10,21,24). It may also appear in blood, so it is used as well as a biomarker in clinical analysis (10). It has been involved with cell adhesion, and its presence is related to metastasis, thus a poorer prognosis. It is expressed approximately in 70-75% of pancreatic cancers (21,24).

CEA has been tested in some preclinical studies. Chmielewski et al. observed in mice a significant reduction of the tumor's size and its eradication in 67% of them, without any autoimmune impact (27). Clinical trials are currently underway or completed. On a phase Ib trial with previous chemotherapy treatment and intravenous IL-2, CAR-T infusions were administered for 6 weeks. It was observed that half the patients presented a stable disease afterward. Nonetheless, toxicities led to some breathing problems (28).

7.3.3. CD24/CD133

CD24 and CD133 are glycoproteins present in a subpopulation of cancer stem cells (CSCs), which are involved in tumor proliferation, invasion, metastasis, and recurrence (10). The cells carrying these antigens are the ones mainly responsible for those carcinogenic properties of SCS (10,21,23).

CD24 is present in approximately 70% of pancreatic cancers (24). Even though only 0.2-0.8% of cancer cells present CD24, its significant tumorigenic potential makes it a good target for CAR T-cell therapy (10). Mice with primary and metastatic PDAC xenografts with only a subset of CD24-presenting cells were used in a preclinical trial performed by Maliar et al. (29). They determined that targeting CD24 led to a clear reduction of the tumor size and an extended survival time. Moreover, the tumor was eliminated in half of the models after 2 months. There still is no evidence of any ongoing clinical trial.

CD133 is expressed in 60-80% of pancreatic cancers (15). In a phase I clinical trial conducted by Wang et al. there were several metastatic patients, including 7 with pancreatic cancer (30). It was demonstrated that they either showed stable disease, disease progression, or partial remission. However, some side effects appeared, like leukopenia, which refers to a low number of leukocytes.

7.3.4. Mucin 1 (MUC1)

MUC1 is a glycoprotein normally expressed in epithelial cells. Accumulation of aberrant glycoforms, like Tn or STn (O-glycosylation), modify cell adhesion and motility, increasing tumorigenesis and metastasis (31). This aberrant Tn-MUC1 is present in the early stages of many cancers, such as 90% of PDAC patients (10,21,24), and has been related to a poor prognosis and low survival rate (10,31).

Posey et al. targeted Tn-MUC1 in xenografts models of pancreatic cancer using a mAb 5E5 CAR-based (31). Tumor-specific cytotoxicity and growth control were observed, as well as a 100% survival rate. Some clinical trials are currently underway, but no results have been published yet (26).

7.3.5. Human epidermal growth factor receptor type 2 (HER2/neu)

HER2 is a tyrosine kinase receptor and oncogene, part of the human epidermal growth factor receptor, that is involved in cell growth, differentiation, and metastasis (10). It has low expression in normal tissue, but has been observed its overexpression in some cancers, like breast one (10,22). However, although in pancreatic cancer its expression is controversial, some studies support its presence in 20-60% of PDACs (10,21,24).

Maliar et al. observed in a preclinical trial with mice that CAR therapy targeting HER2 led to a significant reduction in the tumor size (29). In this study, they also concluded that targeting CD24 2 months after HER2 CAR improves considerably the survival rate. HER2 as antigen also has entered clinical trials, where patients either achieved partial response, stable disease, or disease progression. Some adverse events were observed, like transaminitis, which is due to an increased secretion of transaminases by the liver. Nevertheless, these adverse events were reversible, so it was resolved as a safe and feasible treatment (32).

7.3.6. Claudin 18.2 (CLD18.2)

Claudins are a family of proteins involved in tight cell junctions, which are important for sustaining cell polarity and adhesion. Claudin 18.2 is an isoform of Claudin 8 that is found in normal tissue in gastric mucosa cells; however, tumorigenesis alters cell polarity and leads to its overexpression in other organs (23,33). In PDAC, 50-70% of them express this molecule (33).

There are ongoing clinical trials targeting CLD18.2, but no results have been published (26). Zhan et al. studied both gastric and pancreatic cancer patients that achieved partial or complete response, stable disease, or progression of the disease (34). They established safety and tolerability, as no adverse events related to CAR therapy were observed.

7.3.7. Fibroblast activation protein (FAP)

FAP is a serine protease expressed in myofibroblast cells of the pancreatic stroma. Desmoplasia is related to a dense fibrotic stroma located around cancer cells that contain mainly collagen and cancer-associated stromal cells (CASC). FAP acts as a marker of a subset of CASC (16,21,35).

As it has been exposed, the massive therapeutic resistance of PDAC is partly consequence of this desmoplastic response. Moreover, tumor growth, invasion, metastasis, and angiogenesis are potentiated as well by this phenomenon. Therefore, the focus has been placed on FAP as a target in order to disrupt the synthesis of new stromal components by directly killing cells from the stroma that are surrounding the tumor (15,35).

In a preclinical study, Lo et al. demonstrated that targeting FAP inhibited tumor growth in mice, reducing both desmoplasia and angiogenesis (35). Moreover, mice did not show any bone marrow destruction or changes in body weight. On the other hand, Tran et al. observed lethal bone toxicity and cachexia after targeting FAP, as a consequence of its normal expression in bone marrow stromal cells (BMSCs) (36). These opposite outcomes may be explained by the different specificities of the antibodies used, since Lo et al. only aimed to target those cells with higher levels of FAP on their surface (35).

7.3.8. Prostate stem cell antigen (PSCA)

PSCA is a glycoprotein whose function has not been fully elucidated, although it is thought to be involved in cellular signaling (23,37). In normal tissues, it presents a low expression pattern, but in some malignancies, like PDAC, it is highly overexpressed (21,37,38). 60-80% of PDAC tumors present this antigen, mainly appearing in the early stages of the disease (21,24).

In a preclinical study by Katari et al. with PDAC patient-derived T cells, it was demonstrated that targeting PSCA with CAR therapy was an effective and safe approach, as only PSCA-expressing tumor cells were killed (37). Abate-Daga et al. performed a study on a humanized mouse model of pancreatic cancer and observed that second-generation CAR (CD28) had a more potent antitumor activity than the third one (CD28 and 4-1BB) (38).

It should be taken into consideration that PSCA is expressed on a basal level in the kidney, so the CAR used should not be very potent because it would cause many on-target/off-tumor toxicities (38).

7.3.9. Natural killers (NK) receptors

NK are lymphocytes that can kill both tumor and virally infected cells. Its function is highly controlled by activating and inhibitory signals promoted by receptors on their surface (21,39,40). Activating receptors include NK group 2 (NKG2D), DNAM-1, and natural cytotoxicity receptors (NCR) (40).

The receptor NKp46 or NCR1 has been proved to be the major activating receptor, so it is highly important to its lethal function in a non-MHC restricted way (40). It recognizes antigens whose expression in pancreatic cancer is upregulated (41). In a preclinical study, Tal et al. concluded *in vitro* and *in vivo* that NKp46-CAR presents anti-tumor activity (40).

On the other hand, NKG2D -CAR is able to target a wide range of tumors with high selectivity (42). Demoulin et al. demonstrate *in vivo* antitumor efficacy in a xenograft model of pancreatic cancer (39). Ongoing clinical trials are testing NKG2D against a variety of tumors, such as pancreatic ones, after proving its safety and tolerability in hematological ones without preconditioning (39,42).

Table 2. Tumor cells' antigens that can act as targets of engineered CAR T-cells and their prevalence on pancreatic cancers. Preclinical and clinical trials are exposed, describing characteristics of their study such as the patients or cells involved, the existence of preconditioning and combinatorial therapies, the structure of the CAR T-cells used, and how each gene was transduced, and the positive and negative outcomes.

Antigen target	Preval.	Trial type	Stage/cells cancer	Pre-conditioning	CAR T-cell generation	Method transduction	Combinatorial therapies	Effectiveness		Side effects	
								General	Detailed	General	Detailed
MSLN (25,44)	80-85%	C	Metastatic pancreatic cancer expressing MSLN+	CT (Fludarabine and CP)	n.d.	n.d.	CT (IL-2)	Not antitumor efficacy Limited safety	Progressive disease	SAEs	Hypoxia, lymphopenia, fatigue, infection, psychosis, and sodium, phosphate, neutrophils platelet, granulocytes, and white cells count decreased
			CT refractory metastatic pancreatic cancer	CT (Gemcitabine/ cisplatin and FOLFOX6)	Second (4-1BB and CD3ζ)	RNA electroporation	-	Antitumor efficacy Tolerability Acceptable safety	Stable disease. 40% decrease in tumor cells after 15 days	No SAEs. No evidence of off-tumor on-target toxicity	Jejunal obstruction, abdominal pain, and lymphocytosis
CEA (27,28)	70-75%	P	Pancreatic cancer cells (Panc02 CEA+)	-	Second (CD28 and CD3ζ)	Retrovirus	-	Antitumor efficacy Safety	Reduction of tumor and 67% long-term eradication. Tumor regression lasted for 20 weeks	No autoimmune effects	n.d.
		C	CT refractory metastatic pancreatic cancer	CT (5-FU, leucovorin, irinotecan and FOLFIRINOX)	Second (CD28 and CD3ζ)	Retrovirus	CT (IL-2)	Antitumor efficacy Acceptable safety	Durable efficacy. Survival time of 23 months, compared with the median survival time of 5 months of non-treated	SAEs (Grade III)	Fevers, chills, tachycardia, hypotension, diarrhea, dehydration, fatigue, abdominal distension, edema, myalgias, decreased appetite, thrombocytopenia, electrolyte dysfunction, coagulopathies, and elevations in liver function tests
CD24 (29)	70%	P	Human pancreatic cancer cell line (Capan-1) and xenograft mice	-	Second (CD28 and CD3ζ)	Retrovirus	CT (IL-2)	Antitumor efficacy Specificity	Reduction of tumor. >50% disease-free more than 2 months later Prolonged survival when only a subpopulation of tumor cells were CD24+. Higher specificity than HER2 as a target	Possible off-tumor on-target toxicity	n.d.

Antigen target	Preval.	Trial type	Stage/cells cancer	Pre-conditioning	CAR T-cell generation	Method transduction	Combinatorial therapies	Effectiveness		Side effects	
								General	Detailed	General	Detailed
CD133 (30)	60-80%	C	Metastatic refractory pancreatic cancer	CT (CP and nab-paclitaxel)	Second (CD137 and CD3ζ)	Lentivirus	-	Antitumor efficacy Regulated dose Acceptable safety	Progressive disease, stable disease, or partial disease. Reduction of tumor lasted for 4 months. CAR T-cells persistent for a long period	SAEs (Grade IV) and mild (Grade II) hematologic toxicities.	Lymphopenia, thrombocytopenia, anemia, nausea, anorexia, mucosa hyperemia, and CRS. Caution to patients with biliary obstruction
MUC1 (31)	90%	P	Pancreatic cancer cell lines (Capan-2, Panc-1, PL45, Hs766T)	-	Second (4-1BB and CD3ζ)	Lentivirus	-	Antitumor efficacy Specificity	100% survival, whereas non-treated had approximately 36%	n.d.	n.d.
HER2 (29,32)	20-60%	P	Pancreatic cancer cell line (Capan-1) and xenograft mice	-	Second (CD28 and CD3ζ) Third (CD28, CD137, and CD3ζ)	Retrovirus	CT (IL-2)	Antitumor efficacy Safety	Prolonged survival when most tumor cells were HER2+. Second generation works better	No SAEs	n.d.
		C	Unresectable, relapsed, or metastatic pancreatic cancer	CT (CP and nab-paclitaxel)	Second (CD137 and CD3ζ)	RNA electroporation	-	Antitumor efficacy Acceptable safety	Stable disease. Progress free survival longer than 5 months	SAEs (Grade III)	Febrile syndrome, skin pruritus, upper gastrointestinal hemorrhage, increase of CRP, IL-6, and transaminase, fatigue, nausea/vomiting, myalgia/arthralgia, and lymphopenia
CLD 18.2 (34)	50-70%	C	Advanced pancreatic cancer	CT (fludarabine and CP, with or without nab-paclitaxel)	n.d.	n.d.	-	Antitumor efficacy Tolerability Acceptable safety	Partial response and stable disease	SAEs (Grade IV)	Decreased lymphocytes, neutrophils, white blood cells, and CRS
FAP (35,36)	-	P	Murine 4662 PDA cells and KPC mice	CT (Gemcitabine)	Second (4-1BB and CD3ζ)	Retrovirus	-	Antitumor efficacy Safety	Reduction of tumor. Temporary disruption of tumor stroma	No SAEs	n.d.
			Pancreatic cancer cell line (HPAC) and xenograft mice	-	Third (CD28, 4-1BB, and CD3ζ)	Retrovirus	CT (IL-2)	Limited antitumor efficacy Non-safety	-	SAEs (Grade V) associated with antigen specificity	Bone marrow hypocellularity, cachexia, and necrosis. Morbidity and mortality

Antigen target	Preval.	Trial type	Stage/cells cancer	Pre-conditioning	CAR T-cell generation	Method transduction	Combinatorial therapies	Effectiveness		Side effects	
								General	Detailed	General	Detailed
PSCA (37,38)	60-80%	P	Pancreatic cancer cell lines (Panc02.03, Panc02.13) and xenograft mice	-	Second (CD28 and CD3ζ)	Retrovirus	-	Antitumor efficacy Limited safety	100% reduction of tumor, and 40% eradication of tumor. Greater reactivity than MSLN as a target	Possible off-tumor on-target toxicity	n.d.
			Pancreatic cancer cell lines (Capan-1, CFPAC, PL45, and ASPC-1)	-	First (CD3ζ)	Retrovirus	CT (IL-2)	Antitumor efficacy Specificity Safety	Efficient and rapid kill of PSCA+ tumor cells	No evidence of off-tumor on-target toxicity	n.d.
NK receptors (39,40)	-	P	Pancreatic cancer cell line (Capan-1) and xenograft mice	-	First (CD3ζ)	n.d.	-	Antitumor efficacy Safety	Effective without preconditioning.	No SAEs	n.d.
			Pancreatic cancer cell line (PANC-1)	-	First (CD3ζ) Second (4-1BB and CD3ζ)	RNA electroporation	-	Antitumor efficacy	Reduction of tumor. First generation works better.	n.d.	n.d.

n.d. not defined
P: preclinical study
C: clinical study
CT: chemotherapy
CP: cyclophosphamide
SAE: serious adverse event
CRS: cytokine release syndrome
CRP: C reactive protein

7.4. Potential side effects and toxicities

When talking about toxicities related to the administration of the CAR T-cells, these can be caused by the specificity of each engineered CAR T-cell. This is mainly because the antigens targeted may not only be expressed in cancer cells, but also in normal ones, some on-target/off-tumor effects can appear. Examples of the side effects related to this kind of toxicities may be observed in some trials explained in the "Antigen targets" section (30,32,36). For example, Feng et al. described transaminitis as a side effect in one patient of their study targeting HER2/neu (32).

Nevertheless, CAR T-cell therapy, in general, may also cause some side effects whose seriousness depends mainly on the overall previous health of the patient, how advanced the cancer is, and the administered dose. As these effects can end up being life-threatening, patients are watched closely after the treatment (3).

Firstly, as a consequence of the leukapheresis process, blood calcium levels can drop causing numbness and tingling. After the CAR T-cell infusion other side effects may appear like anaphylaxis, allergic reactions, weakened immune system, and low blood cell or mineral levels (3,10).

Nevertheless, the most typical side effect is cytokine release syndrome (CRS). This involves extremely high proinflammatory cytokine levels because of all the physiological and CAR T-cells releasing them into the blood. Fever, nausea, cardiac dysfunction, weakness, and trouble breathing are some of the symptoms that can appear as a result of this syndrome (3,5,10). This is typically treated with steroids, vasopressors, ventilatory support, supportive care, and tocilizumab (anti IL-6R antibody) (21,22).

Moreover, problems in the nervous system may arise, known as the CAR T-cell-related encephalopathy syndrome (CRES) or immune effector cell-associated neurotoxicity syndrome (ICANS) (15). This leads to some side effects like headache, confusion, seizures, shaking, or changes in consciousness (3,10,15). These are thought to be consequences of the high cytokine levels, specifically of IL-6, or simply because the CAR T-cells have a direct toxic effect if they infiltrate into the central nervous system (CNS) (10,15). Treatment with corticosteroids may be used to treat these neurotoxicities, although they reduce the effectiveness of CAR T-cells therapy (15).

7.4.1. Solutions to CAR-associated toxicities

As it has been exposed, there are a wide variety of side effects that may appear as a consequence of the CAR T-cell therapy. To address them in order to decrease the treatment's overall toxicity, and to increase its safeness, several solutions have been proposed.

Engineering the CAR T-cells so that they can target two different antigens. This way, they would only fully act on cells expressing both antigens (15,18,21). As the antigens are specifically selected so their presence in cancer cells is as high as possible and significantly lower in normal cells, this approach would decrease the probabilities of attacking normal cells. Therefore, this prevents many on-target/off-tumor effects, but maintains effectiveness (15,18).

These off-tumor effects have also been proposed to be addressed by using inhibitory CARs (iCARs) (18,21). These would have receptors targeting antigens that are expressed in normal cells, but not in cancer cells. The receptor (PD-1 and CTLA-4) would trigger an inhibitory response when binding to these antigens, protecting the normal cell from dying. Moreover, as the binding is transitory, CAR T-cells would be able to attack other cancer cells (18).

Addition of suicide genes into the CAR vector to be able to eradicate the CAR T-cells if they get activated in undesired cells. For example, the herpes simplex virus thymidine kinase (HSV-TK) and the inducible caspase 9 (iCasp9) (15,18,21,22). Another strategy also leading to the CAR T-cells death involves the addition of a depletable receptor, such as EGFR or CD20, expressed on the cell surface. They would act as targets for depleting antibodies (18,21).

Finally, it is also possible to control the CAR T-cell activation by the presence of another molecule, by blocking the antigen-recognition domain (15,18,21), or in hypoxic conditions (15). In the former, switchable CARs (sCARs) and multi-chain CARs (mcCARs) need to be in presence of an antibody-based molecule or a small molecule, respectively, to get activated. In the second one, that domain is protected by a substrate peptide that is only removed when the TME matrix metalloproteinases cleave it (18). Lastly, CARs can be designed so that they are sensitive to oxygen, only being active in hypoxic conditions as in the TME (15).

7.5. Combinatorial therapies with CAR-T

As it has already been observed, TME is really immunosuppressive, making it difficult for CAR T-cells to survive. In addition to the preconditioning approaches previously described, several combinatorial therapies have been proposed so that this problem is better overcome. For example, immunotherapies like immune checkpoint inhibitors (PD-1 or CTLA-4) as it also affects the immune system but differently, so it has a synergic effect when used together with CAR T-cell therapy. Moreover, radiation also potentiates the immune response (10,21).

Cryoablation, radiation, chemotherapy, radiofrequency ablation, and surgical debulking are treatments that can be used to reduce the bulk of the tumor, which correlates with better outcomes (10).

Efficacy can also be enhanced by taking advantage of chemokines. Adding chemokine receptors in CAR T-cells improves their migration to the tumor site, since these molecules are released by cancer cells (10).

8. DISCUSSION

Pancreatic cancer is a highly fatal disease with a 5-year survival rate of 10% (3,8). Specifically, 90% of pancreatic cancer cases correspond to PDAC, which is an extremely aggressive type affecting the exocrine gland (1,3). People suffering from it are difficult to diagnose since symptoms are quite non-specific and do not appear at early stages. Moreover, the tumor cannot be usually detected through a routine physical exam (3,5,8).

Consequently, 80% of patients already have advanced or metastatic disease at the time of diagnosis (1,11). At this stage, they are commonly unsuitable for surgical resection. Chemotherapy would be the only treatment available, and the aim is mainly to palliate the symptoms. Therefore, most PDAC patients face an incurable disease (7,8,10). New therapies are needed to treat pancreatic cancer and extend the survival rate of patients.

Nowadays, CAR T-cell therapies are already approved by the FDA (Food and Drug Administration) as treatment for some lymphomas and leukemias (3). For example, Kymriah is used as treatment for patients with acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL). It contains tisagenlecleucel as active principle that targets the CD19 antigen in tumor cells (5). A clinical trial from Maude et al. studied this drug in patients with relapsed or refractory B-cell ALL. They observed a relapse-free survival rate of 80% at 6 months and 59% at 12 months (43). Considering these positive outcomes, CAR T-cell therapy arises as a promising strategy for treating PDAC.

In this therapy, T cells are collected from the patient and engineered so they express a chimeric antigen receptor (CAR) that binds to an antigen present on the surface of tumor cells (10,15). Selection of the antigen target is of high importance in order to reduce on-target/off-tumor toxicities. Optimal ones are expressed on the membrane of most tumor cells in most cancer patients, and not in normal cells or healthy people (15,19,21).

Several antigens have been tested as targets for CAR T-cell therapy for PDAC. In this literature project, MSLN, CEA, CD24, CD133, MUC1, HER2/neu, CLD18.2, FAP, PSCA, and NK receptors are described (Table 2). As was mentioned, a higher prevalence of the antigen in pancreatic cancer patients relates to lower on-target/off-target toxicities. Therefore, the ones that would be preferable to use as targets according to this statement are MUC1, MSLN, CD133, and PSCA. Moreover, FAP is mainly only present in pathological cells, and NK receptors recognize with high specificity tumorigenic antigens, so both would also be a desirable target.

Preclinical and clinical trials have been conducted following different protocols regarding preconditioning, the structure of the CAR T-cells, gene transduction method, and combinatorial therapies, among other variables. All the antigens described in Table 2 have been used as CAR T-cell targets in preclinical trials. These experiments were mainly conducted with cell lines from human pancreatic cancer (Capan-1, PANC-1, HPAC, etc) and their transplant into mice (xenografts).

Additionally, positive outcomes observed in these studies have allowed some engineered CAR T-cells to enter clinical trials. The antigen targets that have completed any clinical phase are MSLN, CEA, CD133, HER2, and CLD 18.2. Nevertheless, ongoing clinical trials are being conducted targeting MUC1, HER2, CLD18.2, PSCA, and NK receptors (26). Commonly, patients enrolling in these studies have metastatic or advanced pancreatic cancer. Moreover, their cancer tends to be refractory, meaning that it is no longer positively responding to other treatments, like chemotherapy.

Some conclusions can be drawn from the experiments reviewed in Table 2. Firstly, preconditioning is only done in some studies, being more common to skip this step. Among the studies that do carry out preconditioning, several chemotherapy agents are used. The most used drugs in this process are cyclophosphamide (CP), nab-paclitaxel, and fludarabine. Nevertheless, it does not seem to make much of a difference when reducing toxicities.

It is worth mentioning that from the leukapheresis process all kinds of T cells are collected. However, it was recommended by Tal et al. to isolate and use some T-cell helper populations like Th1 instead of Th17 or Tregs. Selecting the correct T cells for engineering the CAR T-cells can already improve the patient's immunity against the tumor (40).

The CAR T-cell generation most commonly used is the second one, with either CD28 or 4-1BB as a co-stimulatory domain. It was suggested that aggregation of the 4-1BB domain enhances the CAR T-cell persistence as a consequence of the amelioration of T-cell exhaustion (43). Moreover, CD137 has also been used as a co-stimulatory domain as in the anti-CD133 CAR T-cell produced by Wang et al. They also observed a prolonged presence of their cells *in vivo* (30), so this domain may also act improving persistence.

CAR gene needs to be activated before being transduced. All experiments mentioned in this project followed the same procedure which involves the addition of bead

immobilized anti-CD3 and anti-CD28 antibodies. These beads have been proven to be strongly effective when expanding T helper cells like the recommended one Th1. As for the method of gene transduction, several strategies can be applied. Among all the experiments mentioned in this project, the use of retrovirus is the most typical one (27–29,35–38).

Before infusing CAR T-cells back into the patient, all experiments conducted some quality control tests to determine if the number of T-cells that actually contained the CAR gene was acceptable. Afterwards, they were commonly infused intravenously in 1-2 cycles of 2-4 doses each. In parallel to the administration of the CAR T-cells, some experiments consisted of combinational therapies in which the chemotherapy agents IL-2 or its synthetic version aldesleukin were administered to potentiate the immune system of the patients (28,29,36,37,44).

Different outcomes in terms of efficacy and toxicity have been analyzed and are presented in Table 2. Most of the trials exhibited some level of antitumor efficacy in terms of reduction or eradication of the tumor (MSLN, CEA, CD24, CD133, FAP, PSCA, and NK receptors) or prolonged survival time (CEA, CD24, CD133, MUC1, and HER2). However, as happens with other therapies, the level of effectiveness was not the same for all patients in each study. For example, when targeting CLD 18.2 patients achieved either partial response or stable disease. Therefore, it must be taken into account that the results obtained from receiving this treatment will vary at the interpersonal level, since the immune system of each person acts differently.

Moreover, the degree of efficacy is not equal for all CAR T-cell therapies. For example, studies targeting HER2 and PSCA concluded that the efficacy of the treatment was improved when most of the tumor cells presented the antigen, as would be expected. Nonetheless, targeting CD24 was more efficient if only a subpopulation of the tumor cells expressed it. Hence, the whole CAR T-cell procedure must be specifically designed to appropriately suit the antigen targeted and the characteristics of the tumor and its cells.

It is worth mentioning the results obtained from one clinical trial where MSLN was used as a target. In this study, patients enrolled did not see any improvement in their disease state, in fact, it progressed while they were being monitored. The extremely lower antitumor efficacy achieved from the administration of those CAR T-cells prevented the study to enter phase II (44).

The level of the overall safety of the CAR T-cells administration was assessed based on the number and degree of serious adverse events (SAEs) experienced by patients during the time they are being monitored. A SAE includes life-threatening events, requires hospitalization, or results in either a significant disability, a congenital anomaly, or death (26).

Generally, SAEs took place in not a high number of experiments. However, all experiments showed some kind of toxicity, even if it was not a serious one. Some common side effects observed were decreased number of lymphocytes and platelets, fatigue, CRS, abdominal pain, nausea, and a reduced appetite.

A preclinical study from Tran et al. where CAR T-cells were engineered to target FAP did observe SAEs of the highest possible level (grade V). They determined that the administration of the CAR T-cells to mice led to bone marrow toxicities that resulted in morbidity and mortality, concluding that their therapy was not safe at all (36).

Taking into account the prevalence of the antigens, and both efficacy and toxicity from all the CAR T-cell therapies tested and reviewed in Table 2, conclusions are drawn. Relatively low expression levels in tumor cells from pancreatic cancer patients are seen for HER2 (20-60%) and CLD 18.2 (50-70%) antigens. Therefore, other proteins would be more suitable to be targeted as a higher number of tumor cells would be attacked. On the other hand, in order to use MSLN and FAP as targets, more studies should be carried out because of the lack of antitumor efficiency and safety, respectively, observed in some experiments. Both efficacy and safety from the clinical trial targeting CD133 could be improved, since some patients still presented progressive disease, and grade IV SAEs were observed.

The rest of the antigens described in this project seem to be appropriate to be targeted with engineered CAR T-cells. However, trying to further focus the search for the best antigen is still possible. All MUC1, PSCA, and NK receptors have shown beneficial outcomes without high levels of toxicity in preclinical studies. They are currently being tested in clinical trials, so it will take some time until the results are published. Nevertheless, CEA arises as a promising target since clinical trials have already been completed and both antitumor effectiveness and safety were acceptable. Therefore, it is a highly optimistic approach that could be approved as a PDAC treatment in a short time, although more studies are still needed to ensure its value.

9. CONCLUSION

New treatments for PDAC patients are needed to increase the still low survival rate that makes it a highly fatal disease. The input of CAR T-cell immunotherapy in the medical industry has already had many positive outcomes when targeting hematological tumors. Consequently, several antigens in pancreatic cancer cells are being tested as targets in preclinical and clinical trials. Many experimental factors determine the level of success of each study and must be carefully selected, including the prevalence of antigen target, its toxicities and SAEs, construction of the CAR T-cell and its transduction methodology, preconditioning and combinatorial therapies, stage of progression of the disease and individual differences from patients. Therefore, more trials should be conducted before any CAR T-cell is approved as a pancreatic cancer treatment.

Nonetheless, the positive results that have been published so far indicate that CAR T-cell therapy is a strongly potent and beneficial approach. Particularly, it can be extremely profitable for those patients with pancreatic cancer in such an advanced stage that would be facing an incurable disease if only current treatments were available.

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