LEONOR CASTRO DA FONSECA

APPLICATION OF NANOTECHNOLOGICAL DELIVERY SYSTEMS IN PANCREATIC CANCER THERAPY

Orientador científico: Professor Doutor Pedro Fonte
Co-orientação: Professora Doutora Catarina Reis

Universidade Lusófona de Humanidades e Tecnologias

Escola de Ciências e Tecnologias da Saúde

Lisboa

2018

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Dissertação defendida em provas públicas na Universidade Lusófona de Humanidades e Tecnologias no dia 28 de Março de 2019, perante o júri, nomeado pelo Despacho de Nomeação nº: 25/2019 de 29 de Janeiro, com a seguinte composição:

Presidente:

Professor Doutor Luís Monteiro Rodrigues

Vogais:

Professora Doutora Ana Sofia Macedo - Arguente

Professora Ana Mirco

Professora Dulce Santos

Orientador:

Professor Doutor Pedro Lopes da Fonte

Universidade Lusófona de Humanidades e Tecnologias

Escola de Ciências e Tecnologias da Saúde

Lisboa

2018

"In the middle of difficulty lies opportunity"

Albert Einstein

Acknowledgments

First, I want to thank professor Pedro Fonte for having welcomed me and for accepting to be my supervisor in the area in which I have been most interested since I started this research stage, nanotechnology. There was no one more competent in this matter than you, and I only must thank you for all the support, help, and understanding you have always given me. Thank you for advising me, for opening my horizons and for encouraging me to move on. Thank you for believing in me.

I also want to thank professor Catarina Pinto Reis for inviting me and encouraging me to follow the path of research, which has given me the opportunity to discover the magic of being part of something that can revolutionize the future of science. I have learned to value my skills, I have become more autonomous and I have acquired the capacity to accept that we can't always achieve success in everything we do.

To my colleague and great friend Rafael Hartmann, I would like to thank you for always being by my side, always and always supported me by whatever decision I made. It was my balance many times and it turned out to be one of the key people for all this to be possible. Throughout this long journey, there were moments of despair, sadness, and frustration, but we never gave up! We are not brothers by blood, but we are brothers by heart. I am very happy and proud to finish this adventure with you!

I would like to thank Filipe for all his support in the last seven years. Without it, none of this would have been possible, for it was often my strength, my harbor, the person who made me never give up on my goal. For your patience, for your words at the right time, for your love, for being always present and for believing in me even when I did not, a sincere and huge thank you.

And now I leave the most important to the end, my parents. The biggest foundation of my life. To my father, I thank you for the guidance you gave me to follow this path, without any certainty that it would work, thank you for your support and thank you for believing in me and my choices, even when you do not agree. To my mother, I am grateful to be my greatest haven and my greatest confidant. Thank you for getting me up when I fell, and I failed, and you always told me that it would be okay. To both, thank you for your unconditional love and for always saying that I leave you proud to be who I am.

Abstract

The aim of this dissertation is to understand the applications of nanotechnology in the delivery of drugs in pancreatic cancer therapy. It was necessary to realize the pathogenesis of pancreatic cancer, as well as the reasons why conventional therapies currently used do not provide a significant increase in patient survival.

Pancreatic cancer is one of the most lethal cancers in the world, mainly because of the inability to diagnose it in a timely manner and the lack of effectiveness of existing treatments. In this way, the development of new innovative therapies has become fundamental.

In the last decade, nanomedicine has gained increasing prominence as a new approach for the delivery of drugs, since it allows the transport of the drugs to the specific region and in established concentrations, reducing in this way the systemic toxicity. Several studies are related to different types of nanoparticles (lipid, polymer and metal), highlighting the advantages and disadvantages of each of them, as well as some drugs already marketed using nanotechnology.

A model administration system was developed for the delivery of Parvifloron D. The characteristics selected were related to the properties of the drug to be encapsulated, with diameters of 200nm, negative zeta potential, IdP of 0.200 and connection efficiency of 97%. The toxicity of the formulation was also evaluated, and it was concluded that the lower concentration nanoparticles (30 μM) provided a growth inhibition of 3.2% *Saccharomyces cerevisiae*, and at the highest concentration (53 μM) of 15.6%.

These results lead us to believe that through further studies it may be possible to use this optimized formulation as a suitable and promising carrier of Parvifloron D, and thus contribute to a therapeutic alternative for pancreatic cancer.

Keywords: Pancreatic cancer; Nanomedicine; Nanoparticle; Drug delivery; Parvifloron D.

Resumo

O objetivo desta dissertação prende-se com a compreensão das aplicações da nanotecnologia na vectorização de fármacos na terapia do cancro do pâncreas. Para tal foi necessário perceber a patogénese do cancro do pâncreas, bem como os motivos pelos quais as terapias convencionais atualmente utilizadas não proporcionam um aumento significativo da sobrevivência dos doentes.

O cancro do pâncreas representa um dos cancros mais letais no mundo, devido sobretudo à incapacidade de diagnosticá-lo em tempo hábil e à falta de eficácia dos tratamentos existentes. Desta forma, o desenvolvimento de novas terapias inovadoras tornou-se fundamental.

Na última década, a nanomedicina tem ganho cada vez mais destaque como uma nova abordagem para a vectorização de fármacos, uma vez que possibilita o transporte dos fármacos até à região específica e em concentrações estabelecidas, reduzindo desta forma a toxicidade sistémica. São abordados diversos estudos relacionados com diferentes tipos de nanopartículas (lipídicas, poliméricas e metálicas), salientando as vantagens e as desvantagens de cada um deles, bem como alguns fármacos já comercializados com recurso à nanotecnologia.

Foi desenvolvido um sistema de administração modelo para vectorização de Parviflorona D. As características selecionadas foram de encontro às propriedades do fármaco a encapsular, sendo que obtivemos diâmetros de 200nm, potencial zeta negativo, IdP de 0,200 e eficiência de ligação de 97%. A toxicidade da formulação também foi avaliada, e concluiu-se que as nanopartículas de menor concentração (30 μM) proporcionaram uma inibição de crescimento de 3,2% de *Saccharomyces cerevisiae*, e na maior concentração (53 μM) de 15,6%.

Estes resultados levam-nos a acreditar que mediante mais estudos possa ser possível utilizar esta formulação otimizada como sistema de veiculação adequado e promissor da Parviflorona D, e desta forma contribuir para uma alternativa terapêutica para o cancro do pâncreas.

Palavras Chave: Cancro pancreático; Nanomedicina; Nanopartícula; Veiculação de fármacos; Parviflorona D.

Table of contents

List of figures

List of tables

List of abbreviations and acronyms

ALP	Alkaline phosphatase
ANS	Autonomic nervous system
Apo B	Apoprotein B
BRCA	Breast cancer gene
CE	Connection efficiency
CEA	Carcinoembryonic antigen
CCK	Cholecystokinin
CgA	Chromogranin A
CT	Computed tomography
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention
FDA	Food and drug administration
GLUT	Glucose transporter
HER	Human epidermal growth factor receptor
hTERT	Human telomerase reverse transcriptase
RNAi	RNA interference
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen activated protein kinase
MCNs	Mucosal cystic neoplasms
miRNA	microRNA
MR	Magnetic resonance
mRNA	Messenger RNA
NETs	Neuroendocrine tumors
NSCLC	Non-small cell lung cancer
PAMAM	Polyamidoamine
PanIN	Pancreatic intraepithelial neoplasm
PCL	Polycaprolactone
PEG	Polyethylene glycol
PET	Positron emission tomography
PdI	Polydispersity index

PLA	Poly-(lactic acid)
PPI	Polypropyleneimine
PvD	Parvifloron D
rhTNF	Recombinant human tumor necrosis factor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCA	Serous cystadenoma
siRNA	Small interfering RNA
TfR	Transferrin receptor
TNM	Tumor-node-metastasis
WHO	World health organization
YPD	Yeast extract-peptone-dextrose
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophane

Introduction

Pancreatic cancer is one of the leading causes of cancer death worldwide and is more common in men and has a 5-year survival rate of less than 5%. This is due to the lethal nature of this type of cancer, since it can rapidly spread to the lymphatic system and distant organs, being mostly asymptomatic up to stage IV. The presence of imperceptible metastases when the diagnosis occurs, together with the inefficiency of the treatments that currently exist due to the intrinsic resistance of the tumor cells to the chemotherapeutic agents, contribute to the high mortality rate. [1,2]

Chemotherapy is currently the most common treatment option, although it allows for minimal survival. Currently, gemcitabine is the first-line chemotherapy agent, prolonging overall survival in only 6 to 12 weeks. However, its benefits are compromised by its low half-life and low relative concentration around the tumor tissue. In this way, new and effective therapies are urgently needed. [3,4,5,6]

Nanotechnology is based on a multidisciplinary science that addresses the development and preparation of systems with a nanometer scale. This science comprises the use of particles with a size between 1-1000nm, which depending on their size, can alter properties such as stability, reactivity, and the ability to interact with other molecules and systems. [7,8]

In this way, this type of technology gives us the possibility to investigate new methods that can be applied positively in the various scientific areas. The application of nanotechnology in the field of medicine emerges as a new field of interdisciplinary research, which in the last decade has been increasingly expressed, since it has added value both in the diagnosis and in the treatment of various pathologies. [9,10]

Several studies have been carried out involving nanotechnology and pancreatic cancer, already existing several marketed products based on nanotechnology as oncology therapy. Different types of nanoparticles, such as lipid, polymeric and metallic, have been used to understand the best formulation to obtain more efficacy in pancreatic tumor tissue.

One of the main challenges is the toxicity associated with this type of formulations, as well as their stability. However, the studies that exist refer to the encapsulation of existing chemotherapeutic agents, such as gemcitabine, the first-line agent in the treatment of pancreatic cancer.

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

Currently, about 60% of the antitumor medications used clinically come from natural products. There is a class called terpenoids, which have medicinal properties, especially anticancer, since most of them induce the death of tumor cells, targeting the apoptotic pathways. [11,12]

In this dissertation, the development of a formulation of serum albumin nanoparticles (the most abundant protein in blood plasma, biocompatible, biodegradable and non-toxic), with specific characteristics to vector the main compound of *Plectranthus ecklonii* called Parvifloron D (PvD). [13,14]

This compound inhibits the proliferation of cancer by apoptosis in some cancer cell lines, such as human myeloid leukemia, human melanoma, and breast cancer, thus becoming a target of interest for pancreatic cancer. PvD demonstrates lack of specificity and some cytotoxic effects in non-cancer cell lines, so developing a formulation that can effectively encapsulate it may indicate a solution to deliver this drug into pancreatic cancer tissue without undesirable effects. [15]

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

Chapter 1. Pancreas

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

1.1 Anatomophysiology of pancreas

The pancreas is an acinar tubular gland, located in the retroperitoneal space, between the large curve of the stomach and the duodenum, arranged transversely in the upper wall of the abdomen. It has an elongated and flat structure, which measures about 15 centimeters in length and weighs between 85g and 100g.[16]

This organ consists of the head, which is in contact with the duodenal loop and separated from the body of the pancreas, through an isthmus, a restricted zone, bounded by two cracks, by the body, slightly oblique part from below to and from the right to the left, disposed frontally to the aorta and the inferior vena cava, and by the tail, which is in contact with the spleen and is lined by the parietal peritoneum, as shown in **Figure 1**.[17]

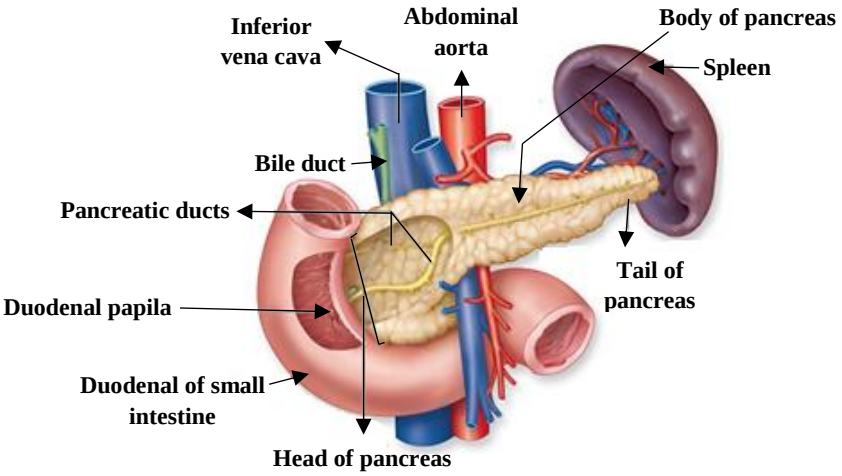

Figure 1. Structure and connections of the pancreas. [17]

The pancreas is a mixed gland, with endocrine and exocrine functions. The exocrine pancreas plays a key role in the digestive process. The stomach secretes food partially digested into the duodenum in the form of chyme, consisting of food bolus, hydrochloric acid, gastric juice, and digestive enzymes (pepsins). It reaches the duodenum through the Wirsung duct, which leads to the Vater ampule, and the Santorini duct, which flows 3 to 4 cm above. Pancreatic acini **(Figure 2)** release pancreatic juice to complete the process of chyme digestion in the duodenum. [18]

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

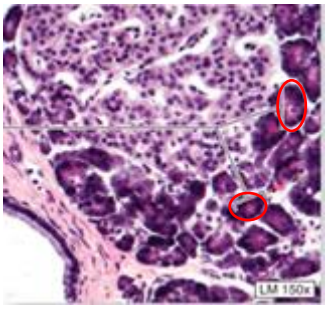

Figure 2. Histological image of a pancreatic acini. [18]

Pancreatic juice is composed of water, salts, bicarbonate, and numerous digestive enzymes, and through this constitution, namely the bicarbonate ions, it is possible to neutralize the acid present in the chyme to protect the intestinal wall and to create the proper environment for the good performance of pancreatic enzymes. [18]

The enzymes present in the pancreatic juice are:

- **Proteolytic enzymes**, whose function is the digestion of proteins. They are divided into exopeptidases (such as carboxypeptidase), which act on the chemical bonds between amino acids, from a terminal end of the protein, and into endopeptidases (such as chymotrypsin and trypsin), which degrade proteins by cleaving chemical bonds between the amino acids of the protein molecule.[19]
- **Glycolytic enzymes**, or amylases, contribute to the digestion of carbohydrates and sugars. These enzymes hydrolyze the α-bonds throughout the starch chain, that is, they transform all the carbohydrates that were not attacked by ptyalin and reach the duodenum in a mixture and elemental sugars (glucose and maltose), capable of crossing easily the intestinal mucosa, passing into the bloodstream; [20]
- **Lipolytic enzymes**, or lipases, whose function is to digest lipids or fats. The lipases hydrolyze the fats, transforming them into glycerol-free fatty acids, easily assimilated by the cells. In addition to this function, they also break down neutral fats or triglycerides into fatty acids and glycerin.[21]
- **Nucleases,** enzymes that promote the digestion of nucleic acids. Ribonuclease has the function of cleaving RNA molecules in the sugar ribose and in the nitrogenous bases adenine, cytosine, guanine, and uracil, while the deoxyribonuclease digests the DNA molecules in the sugar deoxyribose and in the nitrogen bases adenine, cytosine, guanine, and thymine. They are enzymes of two types (α and β) that catalyze the hydrolysis of the phosphodiesteric bonds.[22]

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

The endocrine pancreas controls the blood glucose homeostasis previously discussed. Glucose levels in the bloodstream should be controlled so that there is a constant supply of glucose to the cells. High levels of glucose can cause damage to the kidney as well as to other organs. The pancreas produces two antagonistic hormones that control this homeostasis, being glucagon and insulin. [23]

Glucagon is produced by α-cells and accumulates within the secretory granules from which it is released by exocytosis at the time of hypoglycemia. In the liver, this is recognized by hepatocytes and by the presence of a receptor that, when it binds to this hormone, causes a chain of phosphorylation reactions that activate the enzymes of glycogen lysis and glucose synthesis, causing a greater release of glucose into the bloodstream. It also stimulates adipose tissue to metabolize triglycerides into glucose. It plays an important role in facilitating amino acid uptake, active phagocytosis process, among others. [24]

Unlike glucagon, insulin has the function of lowering blood glucose after a meal by stimulating the absorption of glucose by the liver, muscle, and adipose tissues. Insulin is produced in β-cells, and when there is a peak of glucose in the blood, insulin is released to systemic circulation, increasing the glucose uptake by the cells by binding to the membrane receptor tyrosine kinase. This receptor activates the exocytosis of GLUT4 transporter molecules specific glucose tolerance. When the extracellular concentration of glucose is reduced, GLUT2 is transported back into the cell by endocytosis and stored in vesicles for later use.[25]

Insulin also stimulates glycolysis, that is, increases the intracellular use of glucose and facilitates its transformation into glycogen (in the liver or muscles) or into triglycerides (adipose tissue). In addition, insulin has the function of stimulating lipid metabolism, favoring the passage of free fatty acids, from plasma to adipocytes, which convert them into triglycerides, reducing the mobilization of fats and inhibiting their oxidative dissolution, as well as acts on the metabolism of proteins, facilitating the transport of amino acids to the cells. [16, 25, 18]

Insulin secretion is mediated by glycemia through a negative feedback mechanism. B cells are very sensitive to blood glucose levels, so that by massively flowing through the membrane, it causes a series of biochemical reactions that end with depolarization of the cell. By activating a system of microtubules and microfilaments, the flow of Ca^{2+} ions promote the excretion of this hormone. [27]

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

The regulation of pancreatic function is performed by the autonomic nervous system (ANS) and the endocrine system. The ANS divides into sympathetic and parasympathetic. Sympathetic innervation becomes active when situations of stress, emergencies or exercise occur. Sympathetic neurons stimulate the alpha cells of the pancreas to secrete glucagon into the bloodstream. In turn, glucagon stimulates the liver to initiate cleavage of glycogen into small molecule glucose molecules. [28]

Glucose is then released into the bloodstream to achieve especially the cardiac and skeletal muscle. At the beta cell level, sympathetic innervation also acts to reduce the secretion of pancreatic juice and insulin, which would counteract the intended effect. The parasympathetic innervation becomes active during the rest and digestion, acting by stimulating the release of insulin and pancreatic juice. [28]

The endocrine system uses two hormones to regulate digestive function, such as secretin and cholecystokinin (CCK). Secretin is produced by the cells of the lining of the duodenum, in response to the arrival of the chyme. This hormone stimulates the secretion of bicarbonate and water by the pancreas and inhibits the formation of gastrin by the stomach. CCK is also produced by the same cells that produce secretin in response to the presence of proteins and fats in the chyme. This hormone travels through the bloodstream and binds to pancreatic acini to stimulate these cells to produce and secrete pancreatic juice, rich in digestive enzymes, that break down the protein molecules into peptones and reduce the lipid molecules in microdroplets soluble and assimilable by intestinal cells. [29,30]

1.2 Diseases of the pancreas: non-oncologic

There are several pancreatic pathologies, including acute or chronic pancreatitis and hereditary pancreatitis. The main problem of these pathologies is their diagnosis, due to the inaccessibility of the organ itself. Initially, the medical team begins by performing a physical examination using palpation but is often inconclusive since the pancreas lies deep in the abdomen near the spine. [31]

Subsequently they performed hematological examinations to verify if there are signs of inflammation that lead to a diagnosis, but that can lead to a false conclusion. Radiographic examinations are the best way to evaluate the structure of the pancreas, namely computed tomography (CT), endoscopic ultrasound, and magnetic resonance

(MR). There are also tests to evaluate the pancreatic ducts such as endoscopic retrograde cholangiopancreatography and magnetic resonance cholangiopancreatography. There are cases in which it is only through surgical exploration that one can confirm the diagnosis, but this method is the last approach due to its invasive character. [31]

1.2.1 Acute pancreatitis

This pathology is defined as an inflammation of the pancreas which is usually associated with high abdominal pain, which can be painful and its durability long. In addition to these symptoms, nausea, vomiting, diarrhea, swelling and fever may still occur. In the United States, this pathology is caused mostly by gallstones. Other causes include chronic alcohol consumption, heredity, trauma, drugs, infections, electrolyte changes, high lipid levels, hormonal changes, or unknown factors. Most patients with this pathology recover completely after pharmacological treatment. [32]

1.2.2 Chronic pancreatitis

Chronic pancreatitis is associated with the destruction of the organ itself in the long term. It is a pathology that affects mostly men between 30 and 40 years of age. Initially, it can be confused with the acute one since the symptoms are similar, with high abdominal pain and diarrhea being the most prevalent. As the disease progresses, patients may present themselves with malnutrition and considerable weight loss. If the pancreas is destroyed in the late stages of the disease, patients may develop diabetes mellitus due to lack of insulin production by the organ itself. The most common cause of chronic pancreatitis in the United States is alcoholism, and there are other causes, such as cystic fibrosis for example.[33]

The treatment of this pathology depends mainly on the symptoms that the patient presents. In most cases, the therapy is focused on pain control and nutritional support. Pancreatic enzyme oral supplements are used to aid the digestion process. For patients who develop diabetes mellitus, a supply of insulin is needed to control blood glucose levels. For alcohol consumers, abstinence from alcohol is essential. [31,33]

Chapter 2. Pancreas cancer

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

2.1 Types of cancer

Pancreatic cancer is one of the leading causes of death worldwide and is considered the 7th leading cause of cancer-related death. In 80% of cases, this develops in the exocrine part. About 75% of cancers in the exocrine pancreas are located inside the head of the pancreas, 15 to 20% in the body and only 5 to 10% in the tail of this organ. [34,35]

For pancreatic cancer located in the exocrine part ductal adenocarcinoma is the most diagnosed, accounting for about 95% of cases. This lesion produces an intense stromal reaction postulated that has the function of resisting chemotherapy. [36,37]

This type of cancer usually begins in the pancreatic ducts, being less frequent to develop from the cells that produce the pancreatic enzymes, in which acquires the name acinar cellular carcinoma. The latter is morphologically prominent by significant acinar cell differentiation, by cytoplasmic granules and prominent single-nucleus. [38]

Figure 3 demonstrates the evolution of normal pancreatic duct epithelium to pancreatic adenocarcinoma through early events (shortened telomerase, KRAS mutation, p16 loss) and at a more advanced stage (p53 loss, SMAD4 / DPC gene loss). This scheme is ilustred that the cancer of the pancreas is a result of hereditary line germination somatic mutations are acquired in cancer-related genes (oncogenes, tumor suppressor genes, cell cycle genes, genes for apoptosis and genome maintenance). Also, cell turnover, telomerase shortening and genomic instability may be responsible for the progression of pancreatic epithelial cells in tumor cells. [39]

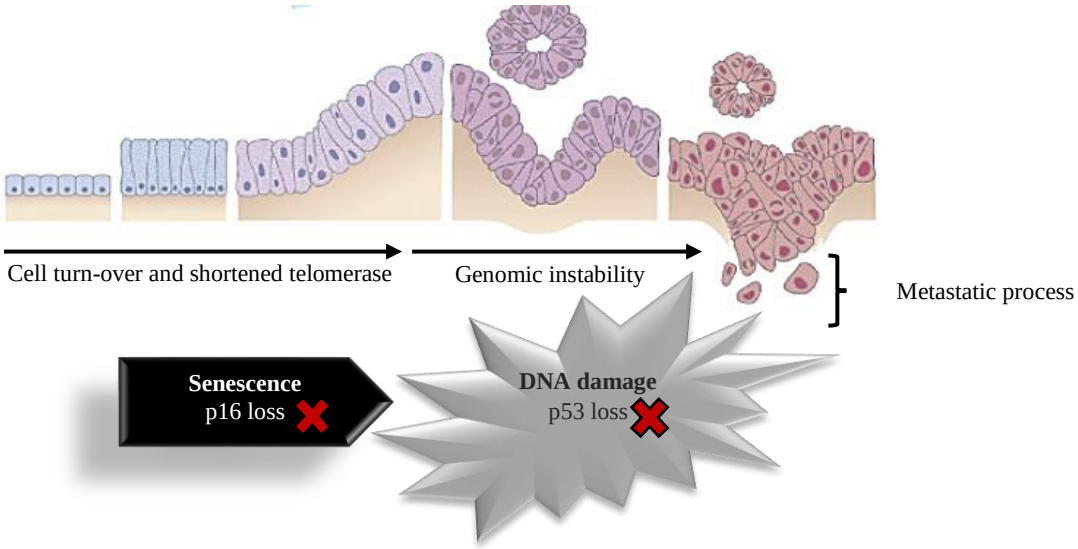

Figure 3. Progression to pancreatic cancer. [39]

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

Pancreatic intraepithelial neoplasm (PanIN) is usually located in the small pancreatic duct and is divided into 3 groups by epithelial atypia, PanIN-1 (minimum atypia), which also has 2 subgroups, i.e. PanIN-1A (flat type) and PanIN-1B (papillary type), PanIN-2 and PanIN-3 (limited atypia). This type of neoplasm is associated with invasive carcinoma and chronic pancreatitis, and HER-2/neu expression is 82% in PanIN 1A, 86% in PanIN 1B and 92% in PanIN-2. [40]

In addition to the above, there are also cystic neoplasms of the pancreas. Pancreatic cysts are common, some of which are curable precursors of a possible ductal adenocarcinoma. Within these neoplasms we firstly have the intraductal papillary mucinators, located in the exocrine pancreas. This type of lesion is defined by non-invasive productions of papillary mucin and arise in the larger pancreatic ducts. Because they are large they are easily detected in imaging tests and detected in time can prevent the appearance of a more invasive cancer. [36,37,41,42]

Secondly, mucosal cystic neoplasms (MCNs), which are related to well-defined tumors with mucin-producing cysts and septation with distinctive ovarian-like stroma without communication with the ductal system. It is usually a single lesion consisting of a thick fibrous wall. This type of neoplasm can be invasive and non-invasive. Noninvasive MCNs are subdivided into MCNs with low grade dysplasia, moderate dysplasia, or high-grade dysplasia. [36,40,43]

Third, we have the serous cystadenoma (SCAs, in which the cysts are mostly benign and slow-growing, coated by non-mucinous epithelium. Finally, solid pseudopapillary neoplasia, very rare. This type of lesion usually has a favorable prognosis, although it is mostly malignant. [37,43,44,45,46]

Tumors of the endocrine or neuroendocrine pancreas (NETs) are uncommon, accounting for less than 5% of all pancreatic cancers. These tumors may be benign or malignant, and at the microscopic level they may be quite similar, not often being noticeable. For the most part, they are only diagnosed as malignant when they spread outside the pancreas. There are several types of NETs:

- **Functional NETs:** about 50% of the neuroendocrine tumors produce hormones that are released into the bloodstream and thus trigger some symptoms. Each type of tumor is called a hormone that secretes.

  - **Gastrinomas** are derived from gastrin-producing cells. About 50% of these malignant ones;
  - **Insulinomas** are derived from insulin-secreting cells. Most are benign;
  - **Glucagonomas** are derived from cells that produce glucagon. Most are very severe;
  - In addition to these there are still **somatostatinomas** (somatostatin), **VIPomas** (vasoactive intestinal peptideomas) and the **PPomas** (pancreatic polypeptideomas);
- **Nonfunctional NETs:** This type of tumor does not produce enough hormones to cause symptoms, so they are more likely to develop cancer because they are asymptomatic.
- **Carcinoid tumors:** rarely has onset in the pancreas, being more common in other parts of the digestive system. They usually produce serotonin (5-HT) or its precursor, 5-HTP.

Finally, we have **pancreatoblastomas**, which are rare solid cell neoplasms and where their location is still not well described. They consist of multiple cellular compounds, such as the acinar component. [47,48,49]

2.2 Cancer stages

The American Joint Committee on Cancer has created a tumor-nodule-metastasis classification system to evaluate the stage and type of pancreatic cancer. The evaluation items are: tumor size and its relation to the vitality of the blood vessels, used to characterize the tumor from TX to T4; the extension of the lymph node surrounding region determines nodal classification ranging from NX to N1; the presence and / or absence of identifiable metastases in distant organs designates the metastatic category as M0 or M1, respectively (**Tables 1 and 2**).[50]

Table 1. International classification of malignant tumors for pancreatic cancer. [50]

Primary tumor (T)		Regional lymph nodes (N)		Distant metastasis (M)	
TX	Primary tumor cannot be assessed	**NX**	Regional lymph nodes cannot be assessed	**M0**	No distant metastasis
T0	No evidence of primary tumor	**N0**	No regional lymph node metastasis	**M1**	Distant metastasis
Tis	Carcinoma *in situ*	**N1**	Regional lymph node metastasis		
T1	Tumor limited to the pancreas, 2 cm or less in greatest dimension				
T2	Tumor limited to the pancreas, more than 2 cm in greatest dimension				
T3	Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery				
T4	Tumor involves the celiac axis or the superior mesenteric artery; unresectable primary tumor				

Table 2. International classification of malignant tumors: stages of pancreatic cancer. [50]

Stage	Primary tumor	Regional lymph nodes	Distant metastasis
0	Carcinoma *in situ*	No regional lymph node metastasis	No distant metastasis
IA	Tumor limited to the pancreas, 2 cm or less in greatest dimension	No regional lymph node metastasis	No distant metastasis
IB	Tumor limited to the pancreas, more than 2 cm in greatest dimension	No regional lymph node metastasis	No distant metastasis
IIA	Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery	No regional lymph node metastasis	No distant metastasis
IIB	Tumor limited to the pancreas; Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery	Regional lymph node metastasis	No distant metastasis
III	Tumor involves the celiac axis or the superior mesenteric artery; unresectable primary tumor	No regional or regional lymph node metastasis	No distant metastasis

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

IV	Tumor limited to the pancreas; Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery; Tumor involves the celiac axis or the superior mesenteric artery; unresectable primary tumor	No regional or regional lymph node metastasis	Distant metastasis

2.3 Pathogenesis of pancreas cancer

Advances in the knowledge of the pathogenesis of pancreatic cancer have been increasing in the last decade, which is a plus for developing more effective diagnostic methods and therapies. Multiple subsets of genes have been discovered that undergo activation or deactivation during the progression of this type of cancer. Activation of oncogenes (point mutation, amplification) and inactivation of tumor suppressor genes are responsible for the process of initiating the development of pancreatic cancer. Although all the genetic changes described above are complex, the recognition that they represent a basic set of processes that are essential for understanding pancreatic cancer. [36,51,52]

Somatic mutations, genetic alternations, and the germ line are responsible for pancreatic cancer. There are sixteen identified mutated oncogenes, including KRAS, TP53, CDKNA2A, SMAD4, MLL3, TGFBR2, ARID1A, SF3B1, EPC1, ARID2, ATM, ZIM2, MAP2K4, NALCN, SLC16A4, MAGEA6. [37,40,39]

The KRAS oncogene undergoing a mutation, most often at codon 12, but may also occur at codon 61 and 13, results in 90% of cancerous pancreatic cells and 20% of these are whole body cells. This oncogene negatively affects cell life and functions, such as cell differentiation and proliferation. [41]

A mutation in KRAS induces the formation of ductal precancerous formations and causes hyperplastic multifocal focus in the pancreatic duct. It is also responsible for the activation of many signaling pathways, such as the activated P13K-AKT pathway, which affects cell life and cell circulation; the MEK and ERK1 / 2 pathway, which upon activation affects angiogenesis, cell proliferation, cellular apoptosis, carcinogenic cell migration and cell cycle regulation; the notch pathway, which activated, affects cell proliferation, cell differentiation and cellular apoptosis, and the Hedgehog pathway,

which causes metastases. Activation of STAT3 has also been identified in patients with pancreatic cancer, and the inhibitors of this oncogene are already used in the treatment of this oncological pathology. [53]

MiRNAs are involved in the development of pancreatic tumors and perform oncogenic functions. Among the 1,000 that exist, pancreatic cancer functions are miRNA-196a, miRNA-190, miRNA-186, miRNA-200b, miRNA-15b, miRNA-95, miRNA-21, miRNA-155, miRNA -221 and miRNA-222. [54]

Tumor suppressor genes have the function of protecting the cell cycle or promoting apoptosis of tumor cells. A mutation of p53 (protein with functions in the maintenance G2-M, regulating the G1-S control point of the phases of the cell cycle, inducing apoptosis, regulating the senescence, repairing the DNA, and altering the cellular metabolism), that is, the inactivation of this protein by missense conjugated mutations with loss of the remaining allele, is common in about 75% of cases ductal adenocarcinoma. The functional loss of this protein also allows survival and cell division in the presence of DNA damage, which promote the accumulation of other anomalies. [36,55]

Mutations and deletions in DPC4 (locus 4), LKB1 (kinase B1, present in the liver) and INK4a are found in 95% of pancreatic cancer cases, and deletion MKK4 (mitogen-activated protein kinase 4) is observed in patients with cancer in the pancreas, DPC4 triggers metastases even though it is not present in pancreatic cancer, and the mutation of the LKB1 gene causes Peutz-Jeghers syndrome, associated with pancreatic cancer. [39]

2.4 Risk factors, signs and symptoms

Risk factors are related to any characteristic, action or habit that contributes to the development of a cancerous process. Some of these factors, such as smoking habits, can be changed. However, there are others, such as age or family history, that are unalterable. Presenting one or several risk factors characteristic of a pathology does not mean that the disease develops. Smoking is known as the major risk factor for the development of pancreatic cancer, although the exact mechanism of cause and effect has not yet been fully clarified**.** [56]

There are some factors that have not yet been scientifically clarified, namely diets rich in red and processed meats and low in fruits and vegetables, physical inactivity, coffee, and alcohol, in which recent studies have shown a link between overuse of alcohol and pancreatic cancer, although nothing is definitive at this time. It is only known that alcohol abuse can lead to chronic pancreatitis and cirrhosis, which are known risk factors for this type of cancer. Pancreatic cancer is also more common in type II diabetic people, but the reason is still unknown. In relation to risk factors that are not changeable, there are several, such as age, in which the upper risk in age > 45 years, gender, where the risk is slightly higher in men than in women and race in that African-americans are more susceptible than Caucasians. [50]

Genetics and family history of disease are also risk factors that are not alterable and are very supported by the literature. About 5% to 10% of pancreatic cancer patients have a family history of the disease. Individuals with the BRCA2 mutation who are at increased risk of developing breast and ovarian cancer are now recognized as having a greater susceptibility to developing pancreatic adenocarcinoma. [57,58]

In addition to these genes, there are others with variants associated with increased risk of pancreatic cancer, such as BRCA1, MLH1, MSH2, PRSS1 (associated with familial pancreatitis), STK11, PALB2, ATM, CDKN2A, APC, MSH6 and PMS2. [56]

In general, pancreatic cancer develops without any specific early sign or symptom, and any manifestation will depend on the location of the tumor within the gland. Approximately 50% of patients have jaundice, which is more common when the tumor is in the head of the pancreas, due to obstruction of the adjacent biliary system. [50]

Apart from this, there are others such as abdominal discomfort, nausea, and weight loss. When tumors metastasize to other organs, duodenal obstruction, gastrointestinal bleeding, and obstruction of the pancreatic duct may result in steatorrhea. Hyperglycemia and diabetes mellitus have been more associated with early manifestations of the disease. Patients with advanced pancreatic cancer may also present ascites, pain, and depression. Following the most frequently encountered analyzes are liver function, hyperglycemia, and anemia. [57]

Table 3. Risk factors, signs, and symptoms of pancreatic cancer.

Risk factors	Signs & Symptoms	
Smoking	Abdominal discomfort	Hardened stools
Age (>45)	Weight loss	Diabetes
Diabetes	Nausea	Ascites
Obesity	Jaundice	Mid-back pain
Chronic pancreatitis	Indigestion	Depression

2.5 Epidemiological data: in Portugal and worldwide

Pancreatic cancer is one of the leading causes of death in developed countries and one of the most lethal causes worldwide. Based on data provided by GLOBOCAN in 2012, this type of cancer causes more than 330,391 deaths per year (4.0% of all deaths) and is ranked as the 7th leading cause of cancer death in both sexes. The estimated 5-year survival rate is less than 5%, and the incidence and mortality correlated with increasing age were more common in males (> 25%) than in females. In 2018, the number of deaths increased to 432,242 (4.5%) according to GLOBOCAN (**Figure 4**). [34,35,59]

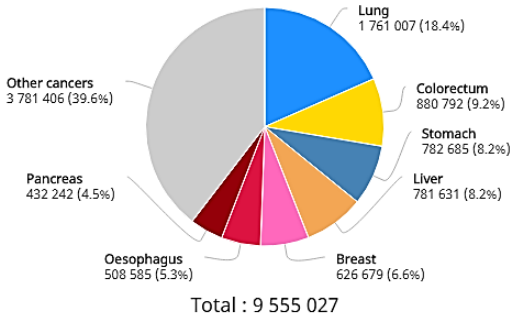

Figure 4. Estimated number of cancer deaths, worldwide, both sexes, all ages (2018)
Source: WHO. [59]

In recent decades, pancreatic cancer-related mortality has increased in both developed and developing countries, such as Europe, the United States, Japan, and China. The incidence of pancreatic cancer varies greatly across regions and populations, with the highest rates in 2012 being recorded in North America (7.4 per 100,000 people) and Western Europe (7.3 per 100,000 population). On the other hand, the lowest rates were

recorded in Central Africa and South and Central Asia (1.0 per 100,000 people). This is since the latter are developing countries and people often do not have access to health care because they are sick before they die. [60–62,63]

About 55.5% of the new cases were registered in the more developed countries, and about 41.0% were recorded in Asian countries.[34] According to a study conducted between 2010 and 2014 in the United States, the incidence rate of new cases increases with age, being higher above 65 years (27.8%). In that same study, a prevalence of 64,668 people living with pancreatic cancer was recorded in the United States, where the estimated 5-year survival rate was about 8.2%. [62,64]

In Portugal, the number of deaths from pancreatic cancer in both sexes was around 4% in 1983 and 5.3% in 2013. In 2018, the estimated number of deaths from pancreatic cancer in both sexes is about 5.5% (**Figure 5**). It was found that, despite medical and scientific advances, this cancer remained highly lethal. [65,66,67]

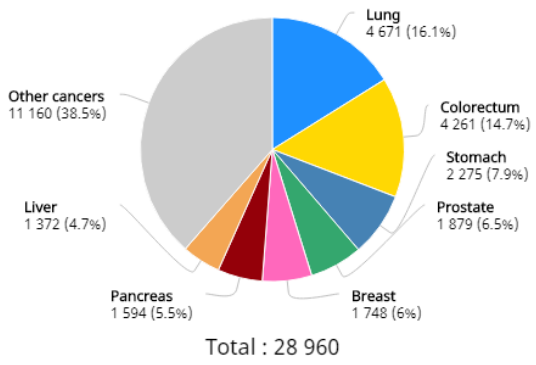

Figure 5. Estimated number of deaths in 2018, Portugal, all cancers, both sexes, all ages. Source: WHO. [67]

2.6 Diagnosis

In addition to the physical examination, imaging tests may also be done to look for areas suspected of having cancer, to determine if the tumor is localized or if it is already metastasized, in evaluating the efficacy of treatment, as well as looking for signs of cancer recurrence after treatment. Within the imaging tests we have computed tomography that allows detailed cross-sectional images to be obtained after the injection of intravenous (IV) contrast and is widely used in the diagnosis of pancreatic cancer because it shows the injured organ in a very clear way. This is an excellent imaging test

to see if surgery can be a good therapeutic option. When a pancreatic tumor is suspected, a needle biopsy is performed using endoscopic ultrasound to guide the needle to the specific site, although computed tomography can also be used, but it is not as usual. [39]

Another imaging test that can analyze the pancreatic ducts is called cholangiopancreatography. This test aims to assess whether the ducts are blocked, narrower or dilated due to the presence of a tumor. Somatostatin receptor scintigraphy may be very useful for finding pancreatic neuroendocrine tumors. Positron emission tomography (PET) is performed through an injection containing a slightly active form of sugar, which has affinity mainly with cancer cells. A specific camera is used to create an image of areas of radioactivity in the body. This imaging test is sometimes used to look for spread of exocrine pancreatic cancer. Since the NETs grow slowly, they do not present well in PET examinations. [68]

Finally, angiography, another imaging test, is a test that aims to evaluate blood vessels. A small amount of contrast dye is injected into an artery to delineate the blood vessels and then taken to x-ray. This method of diagnosis can demonstrate whether blood flow in a specific area is blocked or compressed by the presence of a tumor. It may also show abnormal blood vessels. This test may be helpful in assessing whether pancreatic cancer has grown through the walls of certain blood vessels. [68]

In addition to imaging tests, it is critical that blood tests be done to help with more accurate diagnosis or to determine treatment options. If pancreatic cancer is in the exocrine part, then hepatic function tests (bilirubin levels) are performed. In addition to this, tumor markers can also be searched for in the blood, with CA 19-9 (most common) and carcinoembryonic antigen (CEA) being the most commonly identified in this type of cancer. However, these tumor marker tests are not elevated in all people with pancreatic cancer, and some people who do not have pancreatic cancer may have elevated levels of these markers for other reasons. This examination alone is not decisive.[50]

For pancreatic neuroendocrine tumors, blood tests are carried out to evaluate pancreatic hormone levels, namely insulin, gastrin, glucagon, somatostatin, pancreatic polypeptide, and vasoactive intestinal peptide. In addition, the levels of chromogranin A (CgA) and glucose and c-peptide (for insulinomas) can still be evaluated.[69]

In carcinoid tumors, a blood test can be performed to look for the existence of serotonin, produced by many of these tumors. Urine can also be tested for serotonin and related chemicals, such as 5-HIAA and 5-HTP. All the previously mentioned methods

may suggest that there is pancreatic cancer, but generally the only way to be sure is to remove a small sample from the tumor and to analyze it microscopically. This process is called a biopsy and can be performed in different ways such as through the skin (percutaneous), endoscopic (endoscopic) or surgery (surgical), the latter being less frequent these days.[39]

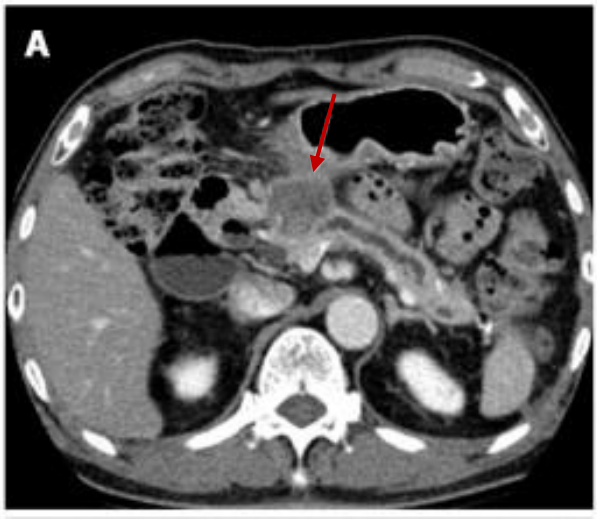

Figure 8. Computed tomography scan of a 73 year old male patient with pancreatic cancer in the pancreas head with about 3 cm (arrow). [70]

Chapter 3. Conventional treatment of pancreas cancer

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

3.1 Chemotherapy

Systemic chemotherapy is used to alleviate symptomatology and prolong the survival rate of patients. However, metastatic pancreatic tumors are highly chemoresistant, with response rates to multiple chemotherapeutic agents, such as antimetabolites, alkylating agents, antibiotics, and anthracyclines, in monotherapy and combination therapy, less than 20%. Gemcitabine replaced 5-fluorouracil as a first-line treatment for metastatic pancreatic cancer because the overall survival time was higher and clinical improvements in the main common symptoms, namely, pain, functional impairment, and weight loss. However, gemcitabine only provides a 6-week increase in mean survival, ranging from 4.5 months to 6 months. [71]

Currently, this drug is used not only in monotherapy but also in combination therapy with other chemotherapeutic agents, i.e., antimetabolites (fluorouracil, pemetrexed), topoisomerase I inhibitors (irinotecan, exatecan), platinums (cisplatin, oxaliplatin) and taxanes (paclitaxel, docetaxel). [72]

A phase III clinical trial demonstrated that adding oxaliplatin to gemcitabine resulted in an increase in response rate and progression-free survival, providing clinical benefits. However, it failed to demonstrate a higher overall survival rate. Another phase III clinical trial demonstrated that the addition of erlotinib (epidermal growth factor receptor 1 inhibitor (EGFR1) to gemcitabine provided a 1-year survival rate (23% vs 17%) compared to gemcitabine monotherapy. [73,74]

In 2011, FOLFIRONOX, a combination of 5-FU, leucovorin / folinic acid, oxaliplatin and irinotecan, demonstrated a superior survival rate in patients with pancreatic cancer, compared to gemcitabine as sole agent, which led to the adoption of this drug as therapy of choice in this type of cancer. However, the toxicity profile was not inconsequential, demonstrating an increased risk of myelosuppression, fatigue, vomiting, diarrhea, and thrombocytopenia. For this reason and even though this drug has improved the overall survival rate, it remains low, more effective, and effective treatments are needed for the treatment of pancreatic cancer. [72,75]

3.2 Radiotherapy

Radiotherapy is often used in combination with systemic chemotherapy, offering no benefit alone after pancreatic cancer surgery. However, a randomized phase III study showed that patients benefited more from the combination of chemotherapy and radiotherapy than with isolated chemotherapy alone. [76,77]

Radiotherapy has a substantial advantage over local control and improves the rate of resectability after downstaging. However, mean survival rates do not improve in patients with non-resectable pancreatic cancer. A novel and specific technique called stereotactic radiotherapy, which uses imaging guidance to deliver toxic radiation in direct doses to tumors, has shown partially improved survival outcomes. However, in comparison to tumors, the overall survival of patients with pancreatic cancer is still unsatisfactory, as is the toxicity associated with this treatment method, and further studies are needed to determine the role of radiotherapy in resectable pancreatic tumors. [78]

3.3 Hormonal therapy

Somatostatin and its analogues, peptide hormones, can act as inhibitors of tumor cell growth, by triggering signal transduction, which negatively controls cell growth, or through downregulation, responsible for tumor growth. [79]

Szende *et al*. demonstrated that somatostatin can induce tumor regression through a mechanism of cell death program. [80]

However, somatostatin monotherapy has no therapeutic benefit in the treatment of pancreatic cancer. Ebert *et al*. demonstrated that using a somatostatin analogue called octreotide (at high dose), the median survival in patients with advanced pancreatic cancer was 6 months, compared to the low dose which was only half. [81,82]

In addition to somatostatin and its analogs, estrogens may also be used in the treatment of this type of cancer, since pancreatic carcinomas have estrogen receptors. Rosenberg *et al*. reported that patients receiving a combined octreotide-tamoxifen regimen demonstrated an average survival benefit (12 months) compared to monotherapy. [83,84]

The use of leuprolide (Lupron), a luteinizing hormone agonist, alone or in combination with somatostatin, demonstrated in vitro and in vivo activity in hamsters

with pancreatic cancer. With these results, Zaniboni *et al.* performed a phase II clinical trial where they tested the leuprolide-tamoxifen combination, but the results were disappointing, achieving only a mean survival of 5 months. In short, the impact of hormone therapy on this type of cancer is quite limited. [85–87]

3.4 Immunotherapy

The use of this therapy has been growing considerably, particularly in pancreatic cancer. The microenvironmental immunosuppressive tumor in pancreatic cancer is an important regulator in the process of disease progression and is responsible for the ineffectiveness associated with conventional therapy, i.e. resistance to chemotherapy. Usually includes a fibrotic stroma that has a considerable stromal density and acts as a barrier that prevents the delivery of cytotoxic drugs, also limiting the access of T cells to tumor cells. [88,89]

In addition, extensive infiltration of myeloid cells composed of macrophages and immature / suppressor myelogenous cells derived from myeloid cell derivatives (MDSCs) accumulated during the progression of pancreatic cancer can lead to T cell dysfunction. Thus, depletion of macrophages or MDSCs results in the increased infiltration and activation of $CD8^+$ T cells, improving the fight against tumor cells. [89,90] Zhang *et al*. concluded that myeloid cells are necessary at multiple stages of pancreatic carcinogenesis, and therefore, depletion of myeloid cells during the development of pancreatic cancer can prevent the formation of tumors. [90]

These authors also identified that myeloid cells act as conductors of immune check-point ligand PD-L1 expression in tumor cells by the activation of epidermal growth factor / mitogen-activated protein kinases signaling. PD-L1 is an immuno-inhibitory molecule that suppresses T-cell activation, leading to tumor progression. Thus, by inhibiting MAPK, tumoral vulnerability can be increased by PD-1 / PD-L1 blockade and may be a new therapeutic approach in the treatment of pancreatic cancer. [89,90]

3.5 Surgery

There are two types of surgery for pancreatic cancer. Potentially curative surgery, which is used when the results of diagnostic tests suggest that removal or resection of the entire tumor is possible, or palliative surgery that can be performed if imaging tests show that the tumor is too widespread to be completely removed. The latter is used to relieve symptoms or to prevent certain complications, such as blockage of the bile duct or intestine, and is not a surgery with a curative potential. However, since most cases of pancreatic cancer are only diagnosed when metastases already exist, that is, in stage IV, the resection surgery does not apply, since the cancer is no longer limited to the pancreas.[72]

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

Chapter 4. Nanomedicine

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

4.1. Definition, advantages and disadvantages

Nanomedicine is defined as the application of nanobiotechnology to medicine, based on the use of materials and devices at the nanoscale for diagnosis and delivery of drugs. In addition to these applications, nanobiotechnology is also being developed for the implementation of nanosurgery. Such a technological advance can be useful for nanoscale systems administration the human body and detect or treat various pathologies. [91]

Over the last decade, extraordinary progress has been made in the application to cancer, which is currently the most important pathology in nanomedicine investment. Nanobiotechnology plays a pivotal role in the discovery of tumor biomarkers and several drugs are being developed for the treatment of various types of cancer, some of which are already approved. [92]

Applying nanomedicine to the treatment of pancreatic cancer, the subject of this dissertation, there are numerous advantages compared to conventional therapies. These advantages relate to controlled and sustained release of drugs; lower systemic toxicity, since the delivery of the drug is directed, through the conjugation of ligands on the surface of the nanoparticles; reduced number of administrations, as there is an increase in drug time and concentration at the local level; and better stability and anticancer activity. [93]

One of the major problems for the ineffectiveness of pancreatic cancer treatments is that pancreatic tumor cells are resistant to gemcitabine, a pyrimidine antagonist, used as a first line treatment in chemotherapy.[92]

Through nanomedicine it may be possible to circumvent this problem by increasing the effectiveness of this type of treatment and also by exploring alternative therapies. [94]

Nanoparticles have dimensions 100 to 10000 times smaller than human cells and can remain stable in physiological environment and passively maintain the target in pancreatic tumor cells through increased permeability and retention effect, which is due to their size, causing them to leak out of blood vessels, and to attach to the carcinoma. [8]

However, they are not only advantages. Its non-physiological chemical surface can cause non-specific cellular segmentation and consequently precipitation, which leads to the occurrence of cellular damage. [94]

Nanoparticles consisting of polymers or natural substances, such as modified polyesters, polysaccharides, and proteins (e.g., albumin), have numerous functionalities and a promising importance with regard to localized chemotherapy, since they can be used to actively target tumor cells through of a combination with specific recognition elements, such as monoclonal antibodies, already used in the treatment of oncological diseases. [95]

The nanostructured materials are processed forms of crude nanomaterials that provide specific shapes or with certain predefined functionality, and which are divided into polymers, such as dendrimers, micelles, etc., and non-polymers, such as carbon nanotubes, metal nanoparticles, among others (**Figure 7**). [96]

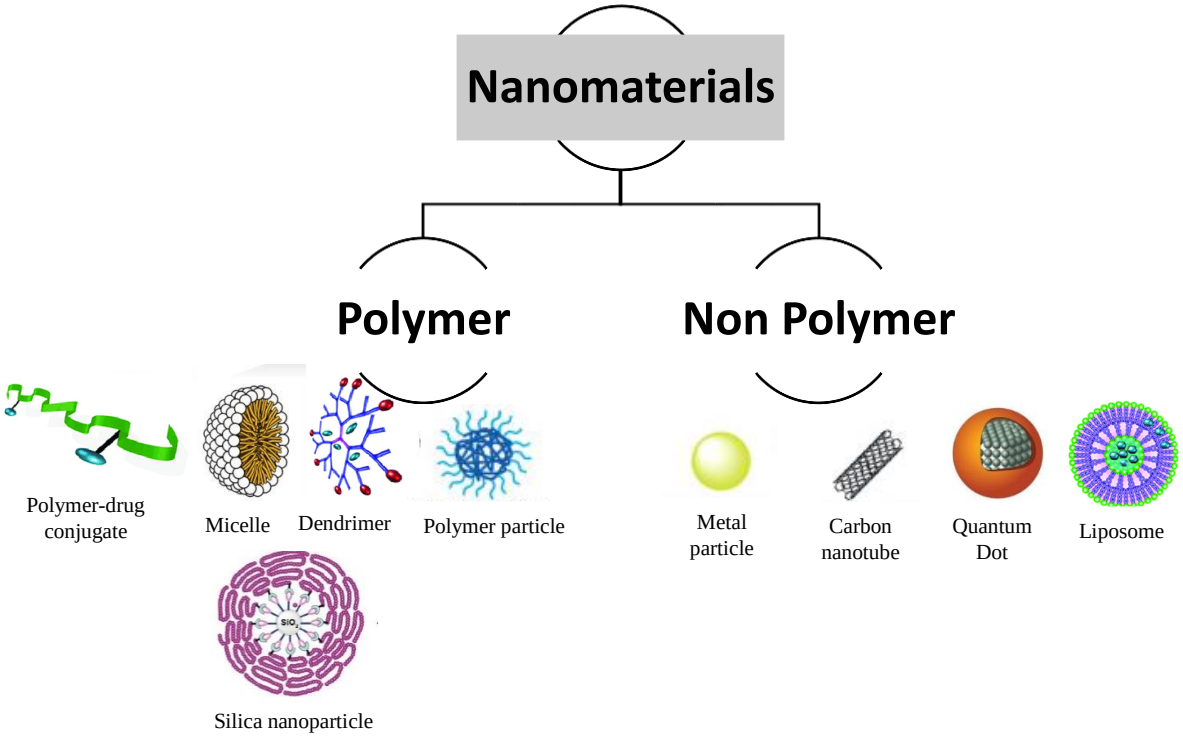

Figure 7. Types of nanoparticles for drug delivery in cancer therapy. [96]

4.2 Methods of preparation of nanoparticles

The method used in the preparation of nanoparticles and the choice of the ideal polymer are dependent on the physicochemical properties of the drug to be encapsulated, as well as the intended release characteristics of the therapeutic target, the route of administration for which it is intended, biocompatibility the particle and the delivery system. [97]

The choice of these conditions is modeled by the possibility of achieving a high encapsulation efficiency, a high yield of the product and an *easy scale-up.* There are several methods of preparing nanoparticles, which fall into two main types: "*bottow-up"*, in which nanoparticles are formulated according to their molecular size, and *"top-down"* where physic-chemical methods are applied. [98,99]

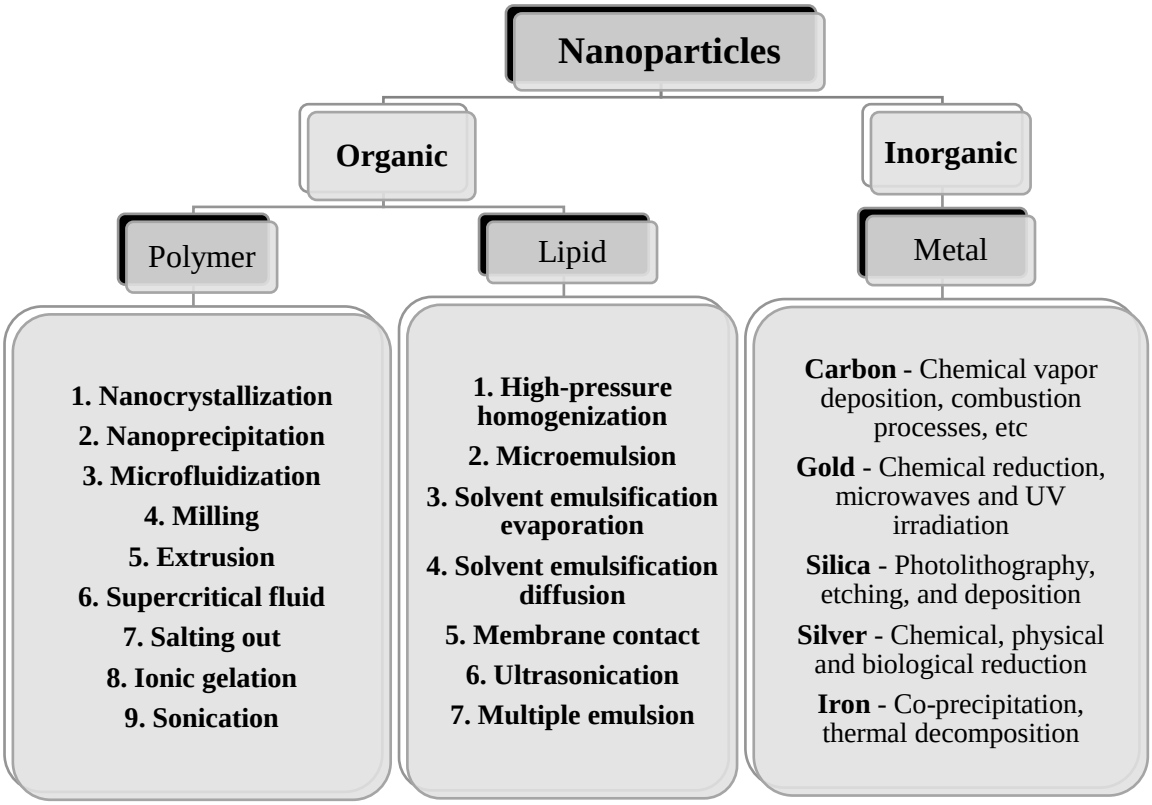

Figure 8. Methods of nanoparticles production; [100]

Polymeric nanoparticles are the most studied model in the pharmaceutical field, which allowed their greater development with respect to the preparation methods. These can be classified into two groups, and their classification depends on the fact that the nanoparticles are formed simultaneously to the polymer, which requires polymerization reactions, or to be obtained directly from a preformed polymer. [98,101]

The first group, where polymerization reactions are required, comprises the methods of emulsion polymerization (nanosphere formation), interfacial polymerization and interfacial polycondensation, the first being quoted the fastest [98,101]

The emulsion polymerization method is divided into two categories, distinguished using continuous organic or aqueous phases. In the case of a liquid phase, the monomer

is dispersed with agitation in a continuous phase in which it is immiscible and can be made in systems without emulsifier or oil-in-water or water-in-oil emulsions. [98,101]

Interfacial polymerization includes distinct phases of preparation where the monomer is added in an oil phase of a previously prepared microemulsion, very useful when it is desired to encapsulate highly water-soluble molecules, such as peptides and nucleic acids, and in the formation of nanocapsules. Interfacial polycondensation is also used in the formation of polymeric nanoparticles, with the aid of a lipophilic monomer in the presence or absence of a surfactant.[98]

The second group, where the nanoparticles is obtained from a preformed polymer, may be previously formed synthetic, using solvent emulsification / evaporation methods, solvent nanoprecipitation or interfacial deposition, solvent emulsification / diffusion and salting-out. The solvent emulsification / evaporation method consists of two phases, wherein the first comprises the emulsification of the polymer in an aqueous phase, and the second comprises the evaporation of the solvent, which induces the formation of nanospheres by precipitation of the polymer. [101]

Nanoprecipitation involves the precipitation of a preformed polymer into an organic character solution, and then diffusion of the organic solvent into aqueous medium in the presence or absence of a surfactant, which results in polymer precipitation into nanospheres. The solvent emulsification / diffusion method involves the dissolution of a polymer in partially water miscible medium and simultaneously saturated therein. [101]

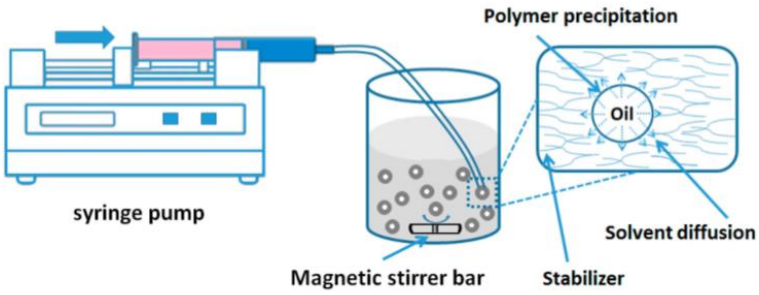

Figure 9. Preparation of nanoparticles by nanoprecipitation method. [101]

The salting-out method is a change from the above-described method since it comprises separating this partially miscible water solvent from the aqueous solution through salting-out, where the polymer is dissolved in the organic solution (oil phase), generally miscible in water and the aqueous phase is comprised of surfactant and a

solution saturated with an electrolyte (for example, the magnesium acetate tetrahydrate normally used in a ratio of 1:3 (polymer to salt)), which should not be soluble in the organic solvent. The oily phase is emulsified in the aqueous phase by a strong shear force through a mechanical overhead stirrer. In summary, the difference between the two methods is that in the latter there is no solvent diffusion stage due to the presence of the electrolyte. [101,102]

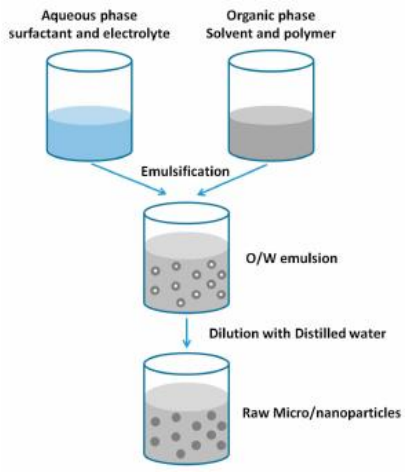

Figure 10. Preparation of nanoparticles by salting-out method; [102]

4.3 Nanoparticles for pancreas cancer treatment

In recent years, much has been done to improve not only the quality of life, but also to extend the life span of pancreatic cancer patients. There are numerous nanotechnology-based formulations with chemotherapeutic agents already known in different phases of clinical trials, and some of them already in phase III, close to being approved and marketed.[92]

The future of pancreatic cancer treatment also undergoes interference-based RNA (iRNAs) therapy. This therapy is progressing, proving to be a viable alternative in relation to conventional treatments, about the specificity, the toxicity, and the overcoming of existing resistance to numerous drugs. RNAi is an endogenous process that acts by silencing the genes that can cause any type of degradation of the mRNA once the RNA sequence used is known. SiRNAs have been assigned to ensure propagation both in vitro and in vivo. SiRNAs are involved in the silencing of post-transcription genes for specific mRNAs. The RNA-induced silencing complex acts as a guide to cleave mRNAs through

a sequence complementary to siRNA and can be recycled repeatedly to cleave additional mRNAs. [103,104]

It is important to note that the introduction of chemically synthesized siRNAs into the cell may give rise to the activation of this previously described process, which may be useful as a gene therapy to suppress specific genes associated with various pathologies. However, the vectorization of siRNA into the target cell still represents an obstacle to this innovative therapy, mainly due to the administration of siRNA *in vivo*, because it presents lack of stability and vulnerability to degradation, due to its highly negative (anionic) charge. [105]

However, nanoparticles in the form of liposomes, lipid polymers and dendrimers may be the key to this problem. These delivery systems can encapsulate the siRNA, protecting it from degradation and elimination by the immune system, as well as delivering the same to the specific target cell. [106,107,108]

Thus, in this chapter, nanoparticles will be addressed as well as the means of delivery of already known drugs, such as lipid nanoparticles, polymer nanoparticles, metallic nanoparticles, or magnetic nanoparticles, as well as delivery systems that can encapsulate siRNA, as previously mentioned. Both methods have increasingly been shown to be the future in the treatment of pancreatic cancer.

4.3.1 Lipid nanoparticles

Among the various lipid formulations based on nanotechnology, the most widely used liposomes are extensively studied. However, they are not the only ones. Lipoplexes, (lipid-based assemblies of non-covalently associated charge-load interactions) are used in genetic targeting studies. [109]

The following sub-chapters will address examples of lipid nanoparticle types drug delivery medium as well as siRNA.

4.3.1.1 Liposomes

Liposomes are small spherical particles consisting of one or more phospholipid bilayers with an internal hydrophilic compartment through the introduction of

phospholipids in aqueous solution. Depending on the design, they can vary between 10 nm and micrometers. The use of liposomes for delivering drugs has had a huge impact on medicine. Drugs with high toxicity or low bioavailability benefited from the stabilization and biodistribution added by the liposomes. [75,110]

A study in which the efficacy and safety of nanoparticles of liposomal cisplatin (lipoplatin) conjugated to gemcitabine in patients with pancreatic refractory cancer was established, demonstrated that there was a partial response (increase of 50% reduction in the sum of products of the diameters perpendicular lesions lasting at least four weeks) and that the mean survival from the start of treatment was four months. The bi-weekly dose of treatment was 100mg/m^2 of lipoplatin and 1000mg/m^2 of gemcitabine and was well tolerated without evidence of neurotoxicity or nephrotoxicity. A liposomal formulation containing Raf-1 antisense oligonucleotides also demonstrated improved antitumor activity against human pancreatic tumors in an athymic mouse model. [111]

4.3.1.2 Modified liposomes

A study realized in 2006 reported the therapeutic potential of the nanoparticles complexed with siRNA. In this study, the authors used PEG-modified liposomes and contained on their surface-active targeting moieties to improve tumor bioavailability. These modified liposomes were to be administering apoprotein B siRNA to non-human primates. Single administration at systemic level resulted in a significant reduction in mRNA and apoprotein B (apo B) protein expression as well as a reduction in total cholesterol. In this study it was also possible to determine that the complex formed by the modified liposome and siRNA was non-toxic and allowed the sustained blockade of apo B for 11 days. [106]

These complexes were also used in another study to carry the siRNA so that it is mutated into a gene involved in promoting the aggressive growth of orthotopic liver tumors. Together, the results of these studies highlight the therapeutic potential of the use of nanoparticle-siRNA complexes in the treatment of human diseases. [112]

4.3.1.3 Cationic lipid polymers

The advantage of using cationic lipid polymers to deliver siRNA stems from their ability to traverse the cell membrane, promote leakage of the endosomal compartment and target genes with good biocompatibility. However, there are also drawbacks. As the induction of a response by interferon and may occur undesired interactions with negatively charged serum proteins. Cationic lipid polymers can interact with negatively charged siRNA through ionic interactions. Thus, the self-assembled lipoplexes confer protection to the siRNA, preventing it from undergoing enzymatic degradation, increasing its cellular uptake by endocytosis, increasing the release of siRNA from the endosomal/lysosomal compartment, and promoting its accumulation in the cytosol. [108]

Currently there are various formulations of commercially available cationic lipid polymers, such as **Lipofectin®, Lipofectamine®** (Invitrogen), **Dharmafect® (Dharmacon®), RNAifect®** (Qiagen) and **TransIT TKO®** (Mirus). These formulations were studied as transfection reagents for *in vitro* administration of siRNA, and it was concluded that the lipid / siRNA ratio affects the colloidal properties of lipoplexes (size and zeta potential), facilitates the cellular internalization of lipoplexes and dissociates nucleic acids in the cytosol. [113]

4.3.1.4 Liposomes conjugated to antibodies or peptides

Currently, nanoparticles conjugated to antibodies or peptides with the goal of targeting the siRNA to target cancer cells have been the goal of many investigators. [108] The transferrin receptor (TfR), folate receptor and Arginine-Glycine-Aspartic ligands are some of the examples of cell surface target receptors used, since there are several studies that prove that they are highly expressed in various types of tumors. [114,115]

Pirollo *et al.* used liposomes which contained a TfR antibody conjugated to its surface, carrying siRNA against HER-2. They compared the use of this same siRNA delivery system, compared to one without TfR antibody, and concluded that there was greater delivery of siRNA in pancreatic tumors in a murine xenograft model in which TfR antibody was contained. In addition, they also concluded that lipocomplex-siRNA had

the ability to inhibit HER-2 expression in pancreatic tumor cells and increase sensitivity to gemcitabine, a promising advance in the treatment of pancreatic cancer. [116]

4.3.2 Polymer nanoparticles

Nanoparticles based on natural polymers are usually biocompatible, biodegradable, and non-toxic, although there are often stability problems when delivered to the various biological membranes. Within natural polymers, chitosan, gelatin, albumin, and alginate are the most commonly used. However, synthetic polymers may also be used in the form of a preformed polymer, and polyesters such as polycaprolactone (PCL), poly (lactic acid) (PLA) or monomers that can be polymerized *in situ*. There are a few advantages in using such nanoparticles in drug delivery, such as biocompatibility, increased stability of any less toxic volatile pharmaceutical agent, are readily manufactured in large quantities, non-immunogenicity, and non-toxicity, among others. [117]

Polymeric nanoparticles can be vesicular systems (nanocapsules) or matrix systems (nanospheres). Nanocapsules are systems in which the drug is confined to a cavity surrounded by a single polymer membrane. Nanospheres are systems in which the drug is dispersed by a polymer matrix. Within polymer nanostructures beyond polymer nanoparticles, there are dendrimers, micelles, and conjugated drugs. [118]

In the next subchapter we will cover micelles, albumin nanoparticles, and dendrimers, three types of polymer nanoparticles used in clinical trials under development for the treatment of pancreatic cancer.

4.3.2.1 Micelles

An example of polymeric nanoparticles is the micelles, consisting of PEG as the hydrophilic compound and the hydrophobic modified polyaspartate which binds the drug. Paclitaxel, an antimicrobial chemotherapeutic agent used in solid tumors, has limited efficacy due to its poor water solubility. Using a micelle formulation, it was possible to overcome this limitation of paclitaxel by encapsulating it into the micelle which is water soluble. This study concluded that with this formulation it was possible to achieve improved antitumor activity due to the EFR effect. [119]

Hamaguchi *et al.* conducted a phase I clinical trial to determine the maximum tolerated dose, dose-related toxicity, and pharmacokinetics of NK105, a micellar carrier system for paclitaxel delivery. Pancreatic cancer patients receiving IV infusion of NK105 were recruited, and the results were encouraging. The patients well tolerated this system, and none of the patients developed significant hematological toxicity. A partial response was seen in a patient with metastatic pancreatic cancer, who received 150 mg/m^2, but only in hepatic metastases (reduction in size of 90%), but the effect on the pancreas was not reported specifically. [120]

4.3.2.2 Albumin nanoparticles

Albumin is a carrier protein with broad interest in drug delivery as it is biocompatible, biodegradable, non-toxic and non-immunogenic. Many authors consider it an ideal material for producing nanoparticles for drug delivery. The accumulation of albumin nanoparticles can occur either by passive (active EPR) or active segmentation, due to the high affinity with cellular receptors overexpressed in tumor cells. [121]

Significant amounts of drug can be encapsulated in its matrix, since there is a high number of binding sites present in the albumin molecule. This protein can also improve the solubility of lipophilic drugs. The conjugation between a compatible and specific ligand and the surface of the albumin nanoparticle, confers active cleavage and enables target delivery to a receptor present in the target cells, by covalent attachment.[122]

A phase I / II study for pancreatic cancer demonstrated the maximum tolerated dose for Nab-paclitaxel (paclitaxel-linked albumin nanoparticles) combined with gemcitabine. The authors also reported a better overall survival (12.2 months) compared to gemcitabine alone. [14]

Another phase III clinical trial for metastatic pancreatic adenocarcinoma demonstrated that the addition of Nab-paclitaxel to gemcitabine not only significantly improved mean survival (8.5 months) compared to treated patients with gemcitabine alone (6.7 months) which significantly reduced neuropathy and neutropenia, associated with cremaphor used to dissolve paclitaxel, which allows a higher dose of paclitaxel administered. [123]

Some authors constructed multifunctional albumin nanospheres (called C225-GEM / MANs), where albumin acted as an outer shell, while the inner core imprisoned

magnetic nanoparticles of Fe_3O_4 conjugated to gemcitabine. The anti-EGFR mab C225 functioned as an active agent that directed the delivery system to the target site. [124]

4.3.2.3 Dendrimers

Dendrimers are highly branched macromolecules, and the functional groups present on their surface can be modified to increase biocompatibility and decrease toxicity. It is possible to complex them with siRNA through electrostatic interactions, such as polycyclic dendrimers (polyamidoamine (PAMAMs) or polypropyleneimine (PPI)), which have a high density of positive charges to their surface. For PAMAMs, which have primary amine groups on their surface and tertiary amine groups within them, they can complex with siRNAs. Thus, the complex formed promotes the cellular uptake of siRNA and the tertiary amine groups initiate the proton sponge effect, to increase the endosomal release of siRNA. [125]

The *in vitro* characterization and anti-cancer effect of siRNA delivered by PAMAM dendrimers against human telomerase reverse transcriptase (hTERT) was also done in mouth cancer. Liu *et al.* concluded that siRNA-hTERT dendriplexes, applied by intratumoral administration, inhibited cell growth and apoptosis in vitro, as well as inhibited tumor growth in vivo in the xenograft model. It also found that there was a decrease in the expression of hTERT proteins in tumors. [126]

4.3.3 Magnetic nanoparticles

Magnetic nanoparticles are part of a group of materials based on nanotechnology with a strong impact in the fields of analytical chemistry, biomedicine, and bioengineering. These nanoparticles came to aid in the diagnosis and treatment of various pathologies, the magnetic separation of biological entities contributed to the development of diagnoses, the magnetic nano delivery systems contributed to the release of drugs, while the magnetic nanoparticles controlled by radiofrequency came to provide a new approach to treatment of cancer. [127]

The only limitations of this type of nanoparticles are biocompatibility and immunogenicity, but both can be controlled by suitable coatings of particles. Iron oxide nanoparticles represent significant advantages such as price, since they are

cheap to produce, physical and chemical stability, biocompatibility, and are environmentally safe. [128]

The use of this type of nanoparticles for the treatment of pancreatic cancer is discussed in the next subchapter.

4.3.3.1 Iron oxide nanoparticles

The most widely used iron oxide magnetic nanoparticles are iron oxide in the core surrounded by a biocompatible surface coating that demonstrates stability in the physiological environment. With the ligation of functional ligands to the surface of the nanoparticle, a greater functionality of the nanoparticle is possible, namely the vectorization of a drug to a target cell. Several studies have already been carried out in which the surface of nanoparticles of iron oxide was modified using cytotoxic drugs, such as doxorubicin (DOX), catechin-dextran and paclitaxel. [94]

The study in which they conjugated catechin-dextran nanoparticles to **Endorem®** (FDA approved drug) demonstrated that there was an increase in the intracellular concentration of the drug compared to the free drug. This formulation was placed in a human pancreatic cancer cell line (MIA PaCa-2) under a magnetic field and caused 98% apoptosis. These results were suggestive that this conjugation enhanced the antitumor activity of **Endorem®** and provided a novel delivery system specific for cell tumors driven by magnetic fields.[94]

4.3.3.2 Gold nanoparticles

The three most important properties of the gold nanoparticles that aroused the researchers' interest were that they were easily prepared, had low toxicity, and easily conjugated to molecules of biological interest. A clinical trial using CYT-6091 (colloidal gold nanoparticles with surface-bound recombinant tumor necrosis factor and PEG) in patients with advanced solid pancreatic tumor has been shown to selectively target tumor tissue. There were, however, associated adverse effects, such as lymphopenia, hypoalbuminemia, electrolyte disturbances, and disorders in liver enzymes, but not very marked. This study did not specify any overall survival. These results suggest that the CYT-6091 formulation selectively targets pancreatic cancer, which may be beneficial in

delivering chemotherapeutic agents, but further studies are needed to specifically understand the effect in terms of survival. [92]

In another study the gold nanoparticles have been shown to be biologically viable and highly adaptable for conjugation to any compound having a high functionality. Conjugating gold nanoparticles with gemcitabine demonstrated that there was significant inhibition of pancreatic cancer cell growth *in vivo* and *in vitro* with virtually no toxicity.[3]

4.4 Toxicity problems

The toxicity associated with this type of delivery system may occur due to the composition, size, or load of the nanoparticles. In general, the particles of smaller dimensions present greater toxicity in relation to the larger ones, the comparison being made when they have the same chemical and crystalline structure and composition. However, very small particles are less efficient because the clearance system more easily removes them. [129,130]

The main concerns related to NPs are the fact that they are carcinogenic. Often the encapsulated or encapsulating material causes increased production of reactive oxygen species (ROS), which can lead to mutations in the DNA. There are also reports that exposure to nanoparticles is associated with asthma, bronchitis, Alzheimer's disease, and Parkinson's. Several events related to vascularization, such as blood clots, are associated with nanoparticles administered parenterally. Cationic liposomal nanoparticles can interact with the extracellular matrix, serum proteins and lipoproteins, with resulting aggregation and / or oxidative stress that can cause non-target tissue damage.[131]

The metallic nanoparticles have a large surface due to their size, low surface charge at physiological pH, and easily aggregate in solution due to their inherent metallic character. In some cases, aggregation is a concern because it can decrease long-term stability.[132]

The accumulation of dose-dependent silver nanoparticles was observed in the brain and other organs, suggesting a systemic distribution after oral administration. There was also a significant increase in ALP and cholesterol indicating hepatotoxicity. [130]

Gold nanoparticles can cross the placenta and cause damage to fetal development but are also involved in the induction of reactive oxygen formation and initiation of autoimmunity. [133,134]

Since there are several materials used in the construction of nanoparticles, there are an infinite number of combinations of interactions with a high potential for harmful interactions that must be studied and considered to ensure the life and well-being of patients.

4.5 Walkthrough on pipeline products

About 150 drugs under development are based on nanotechnology. Some of them are already approved and the rest are in the various stages of development. [91]

In 1995, research around liposomes resulted in the first approved drug of **Doxil®** by the US Food and Drug Administration (FDA). In January 2012 there were 33 more approved nano-drugs and marketed worldwide. [135,136]

In liposomes, in addition to **Doxil®**, which is based on doxorubicin hydrochloride encapsulated in stealth liposomes (100 nm), consisting of N- (carbonyl-methoxypolyethylene glycol 2000) -1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium phosphatidylcholine, fully hydrogenated soy phosphatidylcholine and cholesterol, and which has multiple myeloma and ovarian (IV) cancer as a therapeutic indication, other nano-drugs have been approved. More recently, in 2012, **Marqibo®**, vincristine was encapsulated in sphingomyelin / cholesterol liposomes (100nm) (60/40, molar, and it was indicated in the treatment of acute lymphoblastic leukemia. [135,137]

In 2015, FDA approved a nanoliposomal formulation of irinotecan called **Onivyde®**. It is an indicated topoisomerase I inhibitor, in combination with (5-FU) and leucovorin, for the treatment of metastatic pancreatic cancer following gemcitabine-based therapy. [138,75]

There are other nanoformulations based on polymers approved by the FDA. Among them **Eligard®**, approved in 2002, classified as nanoprotectic when evaluated by the same criteria applied to liposomes. This nanoproduct is based on nanoparticles composed of PLGA (DL-lactide / glycolide; 1/1, molar) copolymer incorporated with leuprolide acetate, a synthetic analogue of GnRH or LH-RH, used in advanced prostate cancer. [139]

In 2012, **Opaxio®** was approved and it consisted of solid nanoparticles composed of polyglutamate covalently bound to paclitaxel, indicated for glioblastoma. It acts by releasing the antineoplastic drug within the solid tumor by the enzymatic

hydrolysis of the polyglutamate and is another example of a polymer-based nanoformulation. [137]

Another example of nano-drugs are the drug-protein conjugates. One of them is **Kadcyla®**, an immunoconjugate approved in 2013 by the FDA for the treatment of metastatic breast cancer. **Abraxane®**, an example of a drug-protein conjugate consisting of albumin-bound paclitaxel, has also been approved by the FDA for use in patients with metastatic breast cancer and non-small cell lung cancer (NSCLC). [140,141]

No less important, metal-based nanoformulations, such as **NanoTherm®**, approved since 2013, are indicated for gliobastoma, prostate and pancreatic cancer (intratumoral). It consists of a formulation consisting of nanoparticles of superparamagnetic iron oxide coated with aminoasilane. It acts by thermal ablation, injecting the nanoparticles exposed to the magnetic field, causing them to oscillate, thus generating a heat wave directly inside the tumor tissue. [142]

Finally, there are virosomes. The world's first genetically-engineered therapies were approved in the People's Republic of China and the Philippines. In 2003, **Gendicine®** was approved in the PRC, a recombinant Ad-p53 gene therapy for squamous cell carcinoma of the head and neck. [143,144]

In 2007 in the Philippines, **Rexin-G®,** described as the "first targeted molecular genetic injectable medicine", was approved, blocking the endogenous cyclin G1 protein, disrupting the cell cycle. This drug is used for all metastatic solid tumors, unlike the previous one, which can't be used if they are metastatic. [145,146]

However, there are also drugs that are still in development. One example is **Thermodox®**, which is in phase III, a doxorubicin bound to liposomes, like **Doxil®**, but is formulated with thermal sensitivity lipids that degrade the bilayer when exposed to high temperatures. [75]

Another example is NKTR-102, a PEGylated etirotinotecan drug, which has been extended in phase III clinical trials. The results were promising, demonstrating that after prolonged exposure of tumor cells to the topoisomerase I inhibitor, the therapeutic response was improved. [147]

Recently a phase I assay was performed with CYT-6091, a recombinant human tumor necrosis factor linked to colloidal gold, for the treatment of solid tumors. In this assay, rhTNF was conjugated to gold nanoparticles using a biocompatible PEG ligand

and it was discovered that the dose of rhTNF administered after gold immobilization could be 3 times greater than the isolated rhTNF without causing toxicity. [148]

Several clinical trials are underway for combination therapies based on gemcitabine, a deoxycytidine nucleoside analogue whose antiproliferative properties are dependent on various inhibitory actions on DNA synthesis, blocking cell cycle progression at the G1 / S phase boundary for treatment of pancreatic cancer. The combination of gemcitabine and **Abraxane®** for stage II pancreatic cancer is in phase II, gemcitabine with erlotinib for advanced localized cancer is in phase I, among others. [149]

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

Chapter 5. Experimental studies

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

5.1 Introduction

The objective of this experimental study was the development of polymeric nanoparticles with specific characteristics that could be used as a therapy for pancreatic cancer.

The choice of the best production of these nanoparticles was based on the possibility of subsequently encapsulating a compound of natural origin named Parvifloron D, an abietane diterpene extracted from *Plectranthus ecklonii* species, belonging to the family *Lamiaceae.*[15]

This interest in the use of this drug has arisen since some studies confirm that this diterpene has strong cytotoxic properties against several human tumor cell lines and that its antiproliferative effect is generally associated with an increase in the intracellular level of reactive oxygen species (ROS) that play a crucial role in the apoptosis process, which may be advantageous for promising therapy for pancreatic cancer. Parvifloron D is a polymorphic drug and exhibits very low water-soluble characteristics, as well as an apparent lack of selectivity for cancer cells.[150]

The delivery of this cytotoxic agent through nanotechnology may be the key to a targeted delivery to pancreatic tumor tissue with less undesirable effects.

5.2 Materials and methods

5.2.1 Evaluation of the best nanoparticles producing method

Parvifloron D has low solubility and lipophilic characteristics, and for this reason a biocompatible and hydrophilic nanomaterial was selected, with the main objective being to achieve optimized bioavailability and stability.

Albumin was chosen as the encapsulating material because this protein has an active target and a special activity in the liver-pancreas system and is the most abundant protein in the blood plasma. [5,6,151,152]

It is a biodegradable, non-toxic, non-immunogenic protein, and is very easy oncology because of its ability to transport hydrophobic drug molecules under aqueous conditions by physical encapsulation. In addition to having a significant role of unimproved solubility, this protein can also extend circulation time, stimulate prolonged drug release, and accumulate at tumor sites, thereby maximizing therapy and minimizing toxicity. [153,154]

The albumin nanoparticles can be physically stable, and their negatively charged surface allows a homogeneous suspension, thus avoiding flocculation.[155]

Desolvation was the method chosen to produce nanoparticles. This technology is suitable for a wide range of polymers, especially those sensitive to high temperature, such as albumin. The main advantage of this method is that there is no increase in temperature. The last step of this method is the addition of an agent whose function is the stabilization of the newly formed nanoparticles, with glutaraldehyde being the most used agent. [156,157,158,159]

However, there is evidence to show that the same have toxic effects such as dermatitis and eye irritation and nose, with allergic rhinitis associated. In 2005, the cytotoxicity of surgical tape used in the treatment of dissections of the aorta, composed by albumin and glutaraldehyde, was studied, and although the glutaraldehyde ensures a strong adhesion to tissues and synthetic materials, use it as an agent of reticulation as his release, can result in both cytotoxic effects *in vitro* and *in vivo*. For this reason, we tested alternative crosslinking methods including ultraviolet (UV) radiation, addition of glucose and the combination of both.[160]

The crosslinking agent was not the only concern we had in the production of albumin nanoparticles. Other conditions were also tested to confirm whether we could improve our formulation, excluding the worst-case scenario until we reached the best conditions for the formation of BSA nanoparticles.

Thus, we tested different rpm in the formation of particles (100 and 500rpm), different crosslinking times (30min and 24hrs) and, since our active compound (Parvifloron D) is hydrophobic, we had to add an organic solvent to the BSA solution we tested different types of organic solvents (hexane, acetone, DMSO and ethanol) and, consequently, different ratios of BSA: organic solvent (1:1, 2:1 and 3:1 BSA: Organic solvent, respectively).

Briefly, 50 mg of BSA was dissolved to 2.0 mL of purified water. Subsequently, this solution was added dropwise to absolute ethanol solution under magnetic stirring. After the desolvation process, the crosslinking agent was added to induce crosslinking of particles. The crosslinking process was carried out under agitation of the suspension over a period. The same preparation technique was used in all other groups, although each group had different conditions according to the results achieved.

After the optimum rpm was set, four cross-linking groups were added after desolvation: A) glutaraldehyde in water was added to induce crosslinking of particles; B) placed in the UV chamber under UV light (wavelength 254 nm) for 30 minutes (the distance between the light source and the sample was 15 cm); C) glucose was added; D) addition of glucose was followed by immediate exposure to the UV source for the same time period and same intensity and distance, as mentioned in point C). [160]

After selecting both the rpm and the crosslinking agent, the latter was tested at different time periods and agitation. The last step was the addition of different organic solvents to different proportions, to be possible to add the active compound, to verify the best solution to achieve the best formulation.

5.2.2 Toxicity tests on *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is referred to as a non-pathogenic unicellular eukaryotic organism that has significant genetic characteristics and protein homologies like mammals and has been used as a model for numerous toxicological studies. In addition it is easy to grow and maintain in laboratory, grows rapidly in a liquid medium, tests are quick and affordable and easy to genetically manipulate. [161]

This yeast is also very useful in toxicity tests involving mechanisms related to oxidative stress, since it has the capacity to grow both in the presence and absence of oxygen. Through this assay, we intend to study the potential cytotoxicity of BSA nanoparticles by testing the effects on the growth of S. cerevisiae when in contact with different concentrations of BSA nanoparticle samples. [161,162]

Saccharomyces cerevisiae (ATCC® 9763 ™) were cultured in yeast extract peptone dextrose (YPD) medium containing 1% yeast extract, 0.5% peptone and 2% glucose in disposable cuvettes. Subsequently, untreated cultures were used as a negative control and serum albumin nanoparticle samples were analyzed at different concentrations (3.0 to 5.3 μM). An ultrasonic bath (Bandelin SONOREX Super RK510) was used to homogenize the nanoparticle suspensions for 3 minutes. After this process, the yeast cultures were added to the samples. The remaining procedure was performed as described by *Roberto* et al. [162]

Before each absorbance measurement in the spectrophotometer (Thermo Scientific Model Evolution 300 BB, UK) at 525 nm, the cultures were vortexed. The logarithmic growth curve for each sample, as well as for the control group, was performed

by correlating the logarithm of the cell concentration (number of cells / ml) with the incubation time. Each group was tested in four replicates (n = 4). For each sample and corresponding concentration, growth inhibition was calculated from the linear slope in the logarithmic phase of the growth curve of *Saccharomyces cerevisiae.*[162]

A stability assay of the formulation in the YPD medium, the optimal yeast growth medium, was also performed to understand whether it would have any influence on the formulation at different concentrations of the yeast. The difference in sample preparation was that they were untreated, such as the negative control.

5.3 Results and discussion

5.3.1 Evaluation of the best nanoparticles producing method

To select the best method of production of nanoparticles, as well as the ideal conditions for a subsequent encapsulation of the active compound (PvD), different parameters were evaluated. Throughout the tests the worst hypotheses were excluded until the desired conditions were reached, and our goal is to have a particle size between 100-300 nm and a zeta potential close to zero.

The zeta potential characterizes the electric charge of the surface of a particle and is a parameter that plays a fundamental role in the various properties of the nanoparticles. In addition to the stability of the formulation, the release rate, circulation in the bloodstream and absorption in the membranes are also affected by ZP. [163]

In this case, as our goal is for the formulation to be administered parenterally, which will cause the particles to circulate in the bloodstream, the more neutral the charges are, i.e. the ZP value closer to zero, greater than biocompatibility from the point of view of immunity.

Thus, when all parameters described above (particle size, zeta potential and crosslinking efficiency (EC (%)) were analyzed, with different rpm (100 and 500rpm), we observed that with 500rpm, with the crosslinking group C) (glucose), with interconnection time of 30 min and with acetone as an organic solvent, we obtained better results. The choice of the interconnection time of 30 min was due to the EC% decrease drastically as well as the zeta potential suffering a huge increase over time. Finally, when

testing different ratios of BSA: organic solvent, the chosen ratio was 3: 1 BSA: organic solvent.

Figure 11. Appearance of selected nanoparticles.

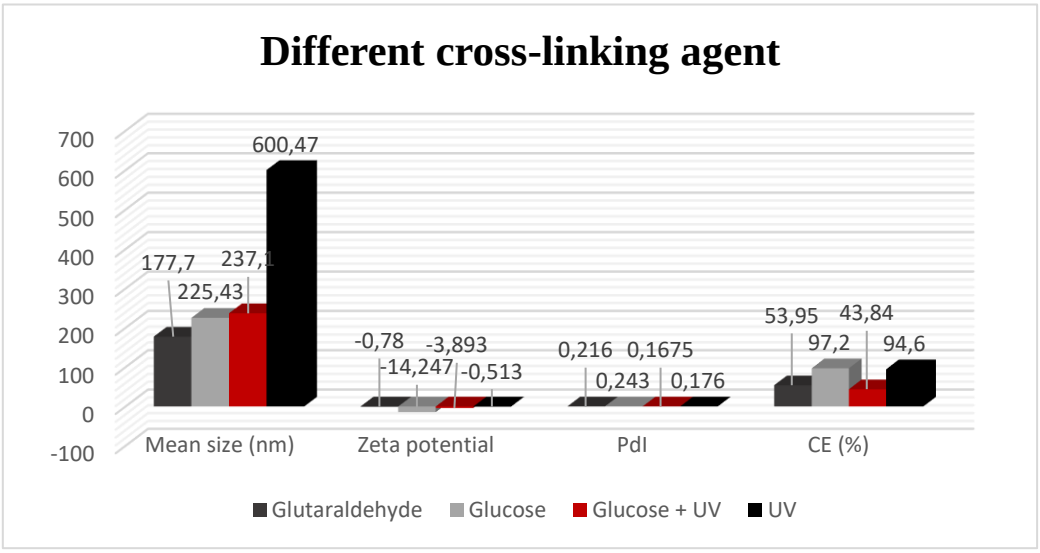

Figure 12. Results of the study of the formulation of BSA nanoparticles: Different cross-linking agent.

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

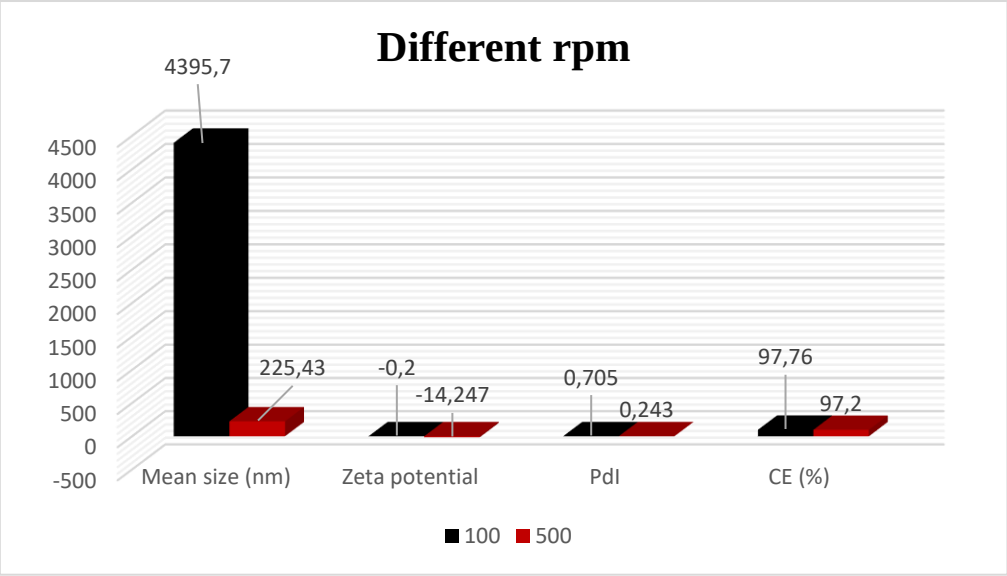

Figure 13. Results of the study of the formulation of BSA nanoparticles: Different rpm.

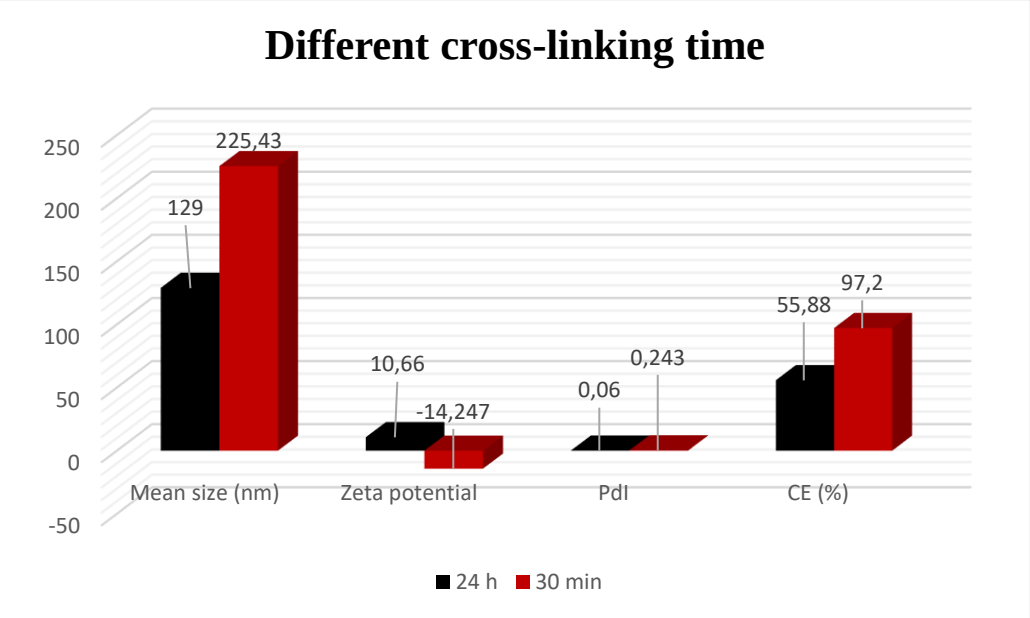

Figure 14. Results of the study of the formulation of BSA nanoparticles: Different cross-linking time.

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

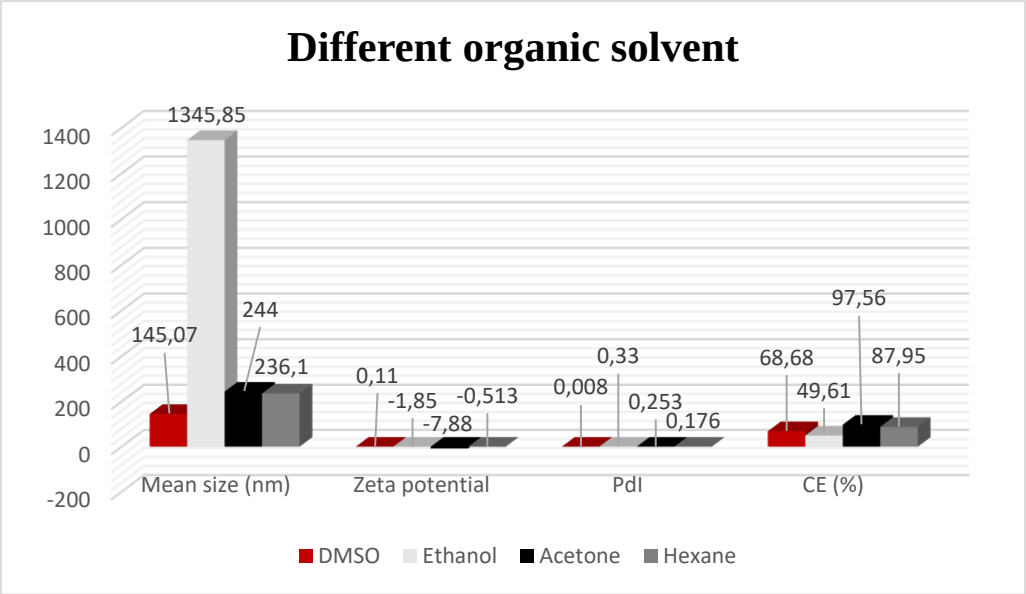

Figure 15. Results of the study of the formulation of BSA nanoparticles: Different organic solvent.

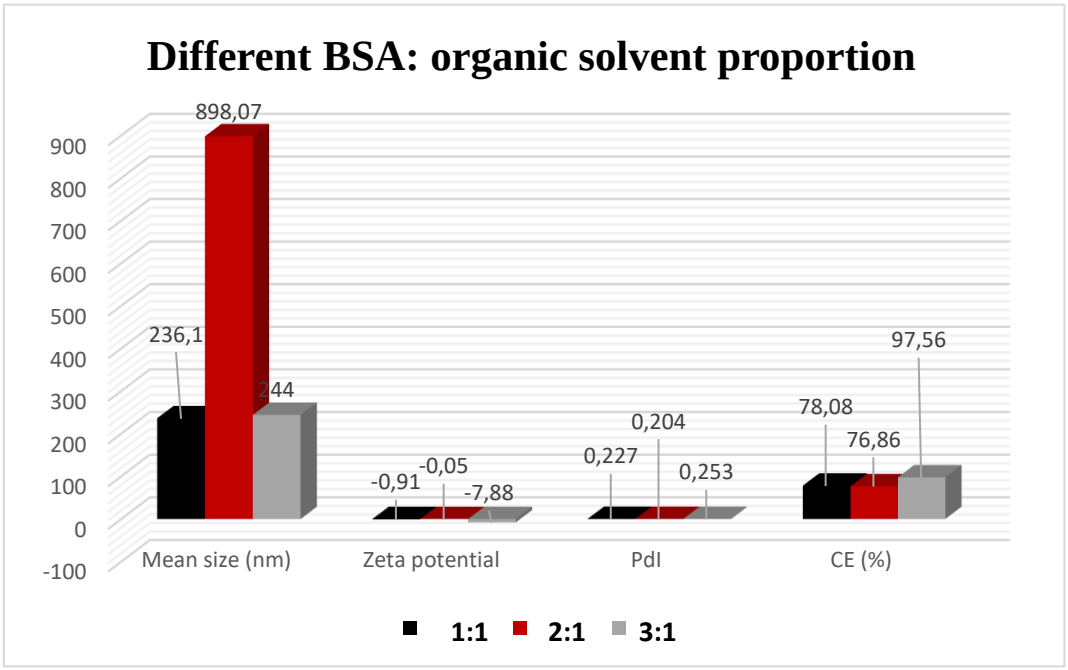

Figure 16. Results of the study of the formulation of BSA nanoparticles: (Different BSA: organic solvent proportion).

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

5.3.2 Toxicity tests on *Saccharomyces cerevisiae*

The percentage value of yeast growth inhibition was calculated by determining the linear slope in the log phase for each of the samples (**Table 4**). Exposure of the BSA nanoparticle formulation to *Saccharomyces cerevisiae* cultures demonstrated that at the lowest nanoparticle concentration (3,0 μM) there was a growth inhibition of 3.2%, and at the highest concentration (5,3 μM) of 15.6%. The negative control was assumed to produce zero percentage inhibition of growth (100% yeast growth) in all studies.

Table 4. Growth of Saccharomyces cerevisiae cultures exposed to BSA nanoparticles at concentrations between 3.0 and 5.3 μM. The growth of Sac. cerevisiae is shown as the natural logarithm of cell concentration, expressed as the number of yeast cells / ml. The time interval of 2-5 hours was considered as logarithmic growth phase (n = 4, mean ± SD).

Concentration (μM)	**Exposure period (h)**	**Ln (Cells/mL)**	**Concentration (μM)**	**Ln (Cells/mL)**
3,0 μM	2,5	14,66	3,9 μM	14,54
	3	14,79		14,72
	3,5	14,96		14,91
	4	15,14		15,05
	4,5	15,23		15,21
	5	15,34		15,26
Concentration (μM)	**Exposure period (h)**	**Ln (Cells/mL)**	**Concentration (μM)**	**Ln (Cells/mL)**
4,6 μM	2,5	14,68	5,3 μM	14,89
	3	14,86		15,06
	3,5	15,02		15,30
	4	15,18		15,46
	4,5	15,29		15,56
	5	15,47		15,69

Additionally, the stability of the formulation in the YPD medium was studied to understand if the medium could have any influence on the results of the cytotoxicity, and observing **Figure 17**, it can be verified that the stability of the samples did not oscillate considerably.

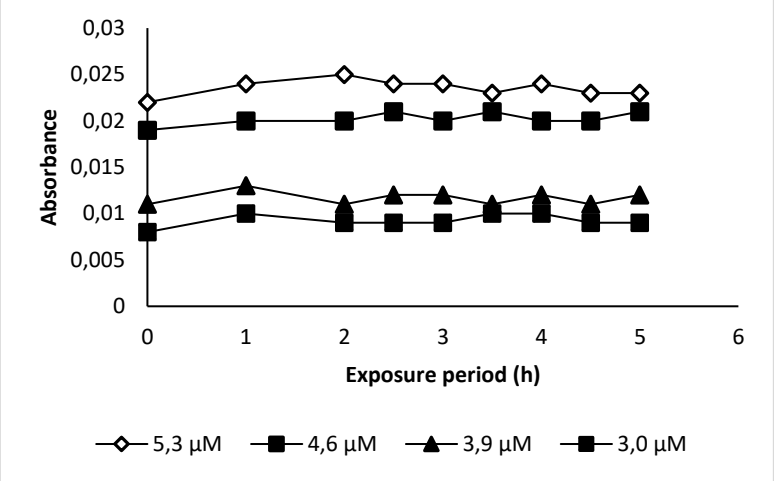

Figure 17. Study of stability: Absorbances as a function of the time of exposure of the formulation to the medium.

Chapter 6. Conclusions and future perspectives

Leonor Castro da Fonseca

Escola de Ciências e Tecnologias da Saúde, Universidade Lusófona de Humanidades e Tecnologias

6. Conclusions and future perspectives

Pancreatic cancer is one of the oncological pathologies with the highest associated mortality rate, the main reason being the stage IV diagnosis, where metastasis already exists. [164]

There are some factors that can be used for prognosis, such as tumor size, lymph node involvement and resection margin status. The treatment of this type of cancer is still a continuous challenge, since chemoresistance is very associated with this type of cancer disease. [165,166]

Pancreatic cancer results from the interaction between genetic and environmental factors, which may be associated with genetic susceptibility and, therefore, it is essential that new therapeutic options are developed. [167]

The therapies so far are mostly based on nonspecific chemotherapy agents. However, over the last few years there has been a progression that has led to increased interest in the development of molecularly designed, targeted therapies for the treatment of pancreatic cancer. Thus, nanotechnology was considered a good strategy to encapsulate new molecules that would allow tumor-directed administration of cytotoxic drugs. [168,169]

Clinical trials were performed involving albumin, colloidal gold, iron oxide, micelles, liposomes, among others, and demonstrated that nanoparticles can be used in conjunction with chemotherapeutic agents and others as they increase their efficacy and reduce their toxicity, very present in non-specific chemotherapy. Despite the efficacy demonstrated *in vivo* and *in vitro* studies in animal models, more research is needed to understand the long-term side effects of nanoparticle use, particularly in terms of systemic toxicity. [92]

In the future, if a specific drug with cytotoxic activity can be encapsulated for pancreatic tumor cells, in non-toxic nanoparticles that can overcome immunogenicity and act only on target cells with high specificity, all the current obstacles associated with the treatment can be solved, increasing the overall survival rate of pancreatic cancer patients.

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