Università degli Studi di Torino

Scuola di Dottorato in

Biotecnologie Molecolari e Scienze per la Salute

Dottorato in

Medicina Molecolare (XXXIV ciclo)

Applying meta-analysis to develop novel anti-tumor strategies

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Table of Contents

List of Tables

List of Figures

Chapter 1: General Introduction

Pancreatic cancer (PC) remains a highly fatal malignancy and is projected to become the second leading cause of cancer death, surpassing colorectal cancer before 2030 in the USA (Rahib et al., 2021). PC is more common in developed countries compared to developing countries. The highest incidence was reported in North America and Europe, while the lowest was in Asia and Africa (Sung et al., 2021). Almost 80% of PC patients present with either regional (unresectable) or metastatic disease at the time of diagnosis in the USA; hence, five-year-overall-survival is around 10-11% (Siegel et al., 2022). Overall-five-year-survival remains poor even for patients with localized resectable PC at the time of diagnosis. In 15-20% of patients who undergo radical resection of pancreatic cancer, the prognosis remains poor, with only 10-25% surviving five years after the surgery (Q. Li et al., 2022; Mizrahi et al., 2020).

PC is categorized into three types: pancreatic ductal adenocarcinoma (PDAC), pancreatic neuroendocrine tumors (rare), and cystic neoplasm (less than 1% of pancreatic cancers). Among them, PDAC accounts for more than 90% of cancers in the pancreas (Mostafa et al., 2017; Sarantis et al., 2020). Most PDAC arise from precursor lesions, termed pancreatic intraepithelial neoplasia, that progress in a stepwise process through the acquisition of genetic alterations and culminate in the development of over PDAC. A hallmark of PDAC is the point mutation of the KRAS oncogene and used for the early molecular characterization of patients (Luo, 2021). KRAS mutation is characteristic of the early stages of PDAC. The Hallmark of the later stage of carcinogenesis in PDAC is the mutation of TP53 and SMAD4 in PDAC (Hayashi et al., 2021; Mizrahi et al., 2020). Almost 70% of PDAC patients with aggressive staging (Grade III and IV) have reported TP53 mutations, whereas 50% of PDAC patients report inactivation of SMAD4 (Cicenas et al., 2017; Hayashi et al., 2021). Mutation in TP53 in PDAC patients is one of the significant causes of metastasis (Hayashi et al., 2021; Morton et al., 2022; Voutsadakis, 2021).

Patients with PC generally present the following clinical features at the time of diagnosis that includes abdominal pain (40–60%), abnormal liver function tests (~50%), jaundice (~30%), new-onset diabetes (13–20%), dyspepsia (~20%), nausea or vomiting (~16%), back pain (~12%) and weight loss (~10%) (Mizrahi et al., 2020; Schmidt-Hansen et al., 2016). For disease detection, the carbohydrate antigen (CA) level 19-9 in serum is well-studied and recommended (Ballehaninna & Chamberlain, 2011). The sensitivity and specificity of CA19-9 in PDAC symptomatic patients is around 80%, making it a well-documented and validated serum biomarker (Ballehaninna & Chamberlain, 2012; Mizrahi et al., 2020).

Even though the survival rate for resectable surgery is 10-25%, surgery remains the only curable option. The first line of adjuvant chemotherapy for PDAC patients was gemcitabine monotherapy. A large clinical trial assessed the role of adjuvant chemotherapy with the combination of fluorouracil and folinic acid (leucovorin) after surgery and compared it with no chemotherapy after surgery (Neoptolemos et al., 2001, 2004). The clinical trials found that patients treated with combination drugs had significantly better survival than the control group (Neoptolemos et al., 2001, 2004). Another study found that patients treated with the combination of fluorouracil plus leucovorin, oxaliplatin, and irinotecan (mFOLFIRINOX) had significantly better survival rates compared to gemcitabine monotherapy (Conroy et al., 2018). Even after administering more intensified chemotherapy regimens as adjuvant therapy, FOLFIRINOX has only modestly improved survival rates (Conroy et al., 2018). Hence, there is a need for effective therapeutic targets.

Cancer immunotherapy has been revolutionized with immune checkpoint inhibitors (ICI) advancements. ICI are monoclonal antibodies that target inhibition of Cytotoxic T-Lymphocyte–Associated Antigen 4 (CTLA-4), Programmed cell Death protein-1 (PD-1), and Programmed cell Death protein Ligand-1 (PD-L1) (Robert, 2020). The usage of ICI in solid tumors has emerged as very promising in treating melanoma, lung cancer, breast cancer, renal cell carcinoma, and other types of tumors (Robert, 2020; Schizas et al., 2020). ICI in PDAC has shown unfavorable results (Mucciolo et al., 2020; K. C. Soares et al., 2012). Recent clinical trials have focused on the combination therapy of chemo and ICI in PDAC, showing promising results (Mizrahi et al., 2020; Mucciolo et al., 2020; Schizas et al., 2020).

Another approach of immunotherapy is targeting tumor-associated antigens (TAAs). TAAs are self-protein expressed in both tumor and non-malignant cells but aberrantly expressed by tumor cells in terms of amount, chemical feature, and location (Fortner et al., 2017; Gnjatic et al., 2006). TAAs can elicit multiple specific immune responses. Studies have identified novel TAAs in PDAC, such as alpha-enolase (Capello et al., 2011; Cappello et al., 2013; Cappello, Tomaino, Chiarle, Ceruti, Novarino, Castagnoli, Migliorini, Perconti, Giallongo, Milella, et al., 2009; Tomaino et al., 2011), carcinoembryonic antigen-related cell adhesion molecule 6 (Pandey et al., 2019), and mucin-4 (Gautam et al., 2020).

Risk factors associated with the development of PDAC and PC are obesity, type 2 diabetes, and tobacco use (Mizrahi et al., 2020). Obesity causes approximately 20% of cancers (Gallagher, Emily J., 2022; C. J. Lin et al., 2020; Swinburn et al., 2019). A large prospective study by the National Institutes of Health studied more than 0.5 million patients to understand the role of obesity in developing PC (Stolzenberg-Solomon et al., 2013). The study followed the participants for 10.5 years. The study was the first to establish the role of obesity in developing pancreatic cancer. The study found that there was an increased likelihood of developing PC when the patients were overweight or obese (defined by body mass index [BMI] ≥30 kg/m²) compared to patients with normal range BMIs with hazard ratios (HR) of 1·15–1·53 (Stolzenberg-Solomon et al., 2013).

Obesity plays a role in type 2 diabetes and cardiovascular disease (Gutiérrez-cuevas et al., 2021; Ng et al., 2021; Piché et al., 2020; Silveira Rossi et al., 2022). Combining all the data from type-2 diabetes, cardiovascular diseases, and cancers (2,5%, 31,4 and 18.6% of the global burden of death, respectively), it is clear that obesity is the most significant contributor to the global burden of death (Abbafati et al., 2020).

World Health Organization defines obesity as “abnormal or excessive fat accumulation that presents a health risk” (World Health Organization (WHO), 2000). Adipose tissue is fat-depositing tissue and endocrine and paracrine organs (Zorena et al., 2020). It secretes a wide variety of hormones known as adipokines (or adipocytokines) that signal the key pathways to maintain metabolic homeostasis.

Adipokines include Leptin, Adiponectin (AdipoQ), Resistin, TNF-α, IL-6, and many others (H. Cao, 2014). AdipoQ and Leptin have been associated with obesity (Singh et al., 2016), and their levels are decreased in obese and increased in lean people (Choi et al., 2020; Gariballa et al., 2019; Körner et al., 2005; Singh et al., 2016).

AdipoQ interacts with two types of receptors, namely AdipoR1 and AdipoR2. AdipoR1 is expressed in muscles, the liver, and the hypothalamus, whereas AdipoR2 is expressed in the liver, white adipose tissue, and vasculature (Yamauchi et al., 2014). Both receptors are homologous, with seven transmembrane domains (Yamauchi et al., 2003). AdipoQ triggers downstream signaling events even though AdipoR1 or AdipoR2 have no intrinsic phosphorylation or protein kinase activity [(Ruan and Dong, 2016). AdipoR1 activates AMP-activated kinase (AMPK), whereas AdipoR2 activates peroxisome proliferator-activated receptor (PPAR)α leading to increased insulin sensitivity (Yamauchi et al., 2014). AdipoQ stimulates the phosphorylation and subsequent activation of AMPK via AdipoR1, resulting in glucose consumption and lipid metabolism. AdipoQ via AdipopR2 activates and increases the expression of PPARα, thus increasing lipid and fatty acid metabolism and energy consumption (Yamauchi et al., 2014).

The role of AdipoQ and its receptors in cancer is still debated, as both negative and positive correlations with cancer were observed (Katira & Tan, 2016). A meta-analysis compared the AdipoQ levels in people with different cancers and healthy people in the random effect model. It was shown that circulating AdipoQ levels in cancer cases were significantly lower than in the controls (Wei et al., 2016). In colorectal cancer, lower levels of AdipoQ in patients' sera compared to healthy or no association with AdipoQ was reported (An et al., 2012; Joshi et al., 2014; Xu et al., 2011). The expression of AdipoQ and its receptors was significantly higher in the bladder and cortical tumors than in benign tumors (Babińska et al., 2019; Kashiwagi et al., 2020).

Higher AdipoQ levels in serum association with PDAC risk are contested (positive, negative, or no association) (Chang et al., 2007; Dalamaga et al., 2009; Phelip et al., 2011; Grote et al., 2012; Bao et al., 2013), although PDAC patients with worse survival after treatment had a higher concentration of AdipoQ in the immune complexome (Mandili et al., 2020).

The thesis aims to understand the role of AdipoQ and its receptors in cancers and PDAC. We also discuss the post-translational modification in TAAs as novel immunotherapeutic targets.

1.1 Aim of thesis

As discussed above, one of the therapeutic approaches in PC and other cancers is targeting TAAs. I got interested in understanding the role of post-translational modifications (PTMs) in TAAs eliciting a specific immune response, making them targets for immuno-oncology therapy. Chapter 2 of the thesis reported the review that we just published, which discusses the hypothesis that PTMs increase the immunogenicity of TAAs by rendering them more similar to foreign antigens breaking the tolerance established against self-TAAs in the absence of PTMs (Srivastava et al., 2022). In this review, we outlined the preclinical studies of acetylation, citrullination, and phosphorylation as vaccines in multiple cancers. We highlighted the clinical trials of glycosylated and phosphorylated proteins in onco-immunology therapies.

Another issue I tackled during the PhD Program was the role of AdipoQ in cancer, which is still controversial. AdipoQ level is high in PDAC patients with worse survival undergo chemotherapy. This led us to question, what is the precise role of AdipoQ and its specific receptors in cancer progression, and in particular, whether or not AdipoQ receptor expression has any impact on the overall survival of the patients. To get an idea of the possible role played by AdipoR1 and AdipoR2 in cancer, I undertook a bio-informatic approach by using the publicly available transcriptomic data of more than 10,000 patients along with their clinic-pathological information. In the study reported in Chapter 3 of the thesis (manuscript under preparation), I hypothesized that lower mRNA AdipoR1/R2 expression in tumor cells has a better impact on patient clinical features, including survival.

I undertook a computational approach to get a better understanding of AdipoQ and its receptors in PC in Chapter 4:. I employed consensus-Independent Component Analysis (c-ICA) to analyze 184 transcription profiles of pancreatic cancer patients. I found that c-ICA segregates the bulk expression profiles into statistically independent Transcriptional Components (TC). In Chapter 4 of the thesis, I described the findings that five TC associated with AdipoQ and its receptors play a role in survival outcomes (manuscript under preparation).

Chapter 2: Exploiting post-translational modifications of tumor-associated antigens to enhance the efficacy of cancer immunotherapy

This chapter aims to comprehensively understand the role of PTMs in TAAs in eliciting an immune response in multiple cancers. PTMs involves adding small chemical groups to amino acid residues in proteins after they are translated. I got interested in understanding PTMs, as they are associated with cancer progression, growth, and survival by altering the normal functions of proteins in tumor cells. TAAs are present in both tumor and normal cells, but they are over-expressed in tumor cells. They can elicit specific immune responses, which makes them an attractive target for cancer immunotherapy. In this review, which has been accepted for publication (Srivastava et al., 2022), I have addressed and discussed how the addition of acetyl, citrulline, and phosphorylation to proteins expressed by tumor cells makes them more antigenic and affects the immune response in multiple types of cancer. In addition, this review highlights the clinical trials using glycosylated and phosphorylated TAAs as a vaccine.

Post-Translational Modifications in Tumor-Associated Antigens as a Platform for Novel Immuno-Oncology Therapies

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Cancers 2023, 15, 138.

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Simple Summary

Tumor-associated antigens (TAA) are antigens present in tumor cells but are also expressed in normal cells. However, TAAs are aberrantly expressed by tumor cells and can elicit multiple specific immune responses. One key feature of TAA is the presence of post-translational modifications often absent in normal proteins. This article offers an overview of the role of post-translational modifications in TAA in eliciting a specific immune response, which candidates them as targets for immuno-oncology therapy. Both preclinical and clinical studies will be discussed.

Abstract

Post-translational modifications (PTMs) are generated by adding small chemical groups to amino acid residues after the translation of proteins. Many PTMs have been reported to correlate with tumor progression, growth, and survival by modifying the normal functions of the protein in tumor cells. PTMs can also elicit humoral and cellular immune responses, making them an attractive target for cancer immunotherapy. This review will discuss how acetylation, citrullination, and phosphorylation of proteins expressed by tumor cells render the corresponding tumor-associated antigen more antigenic and affect the immune response in multiple cancers. In addition, the role of glycosylated protein mucins in anti-cancer immunotherapy will be considered. Mucin peptides, in combination with stimulating adjuvants, have, in fact, been utilized to produce anti-tumor antibodies and vaccines. Finally, we will also outline the results of the clinical trial exploiting glycosylated-MUC1 as a vaccine for different cancers. Overall, PTMs in TAA could be considered in future therapies to result in lasting anti-tumor responses.

Keywords: TAA; cancer immunotherapy; post-translational modifications; acetylation; citrullination; phosphorylation; glycosylation

Graphical Abstract

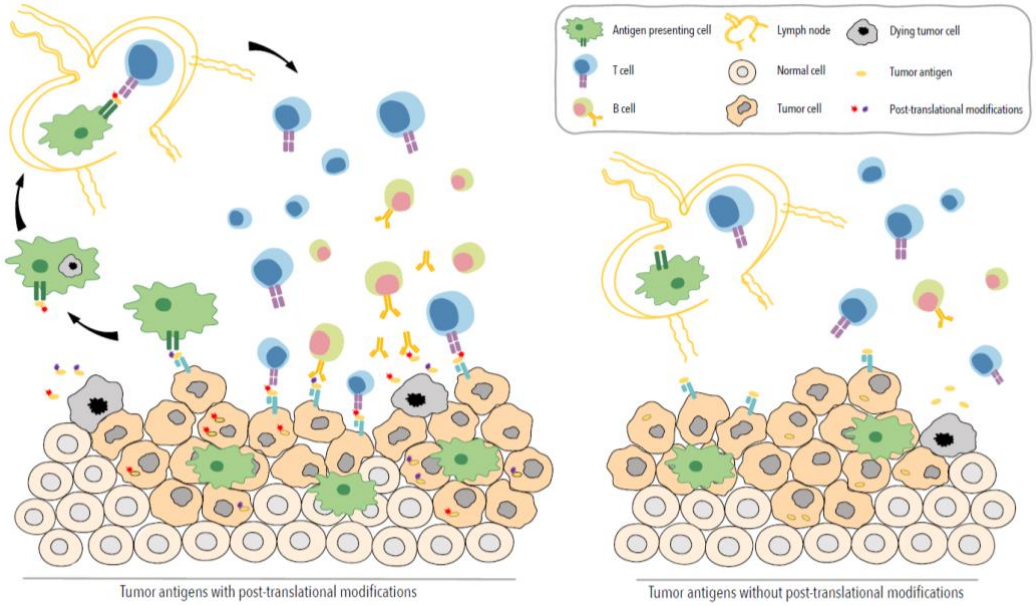

Figure 2-1: Graphical Abstract depicting the significance of the presence of post-translational modifications in tumor-associated antigen in breaking the tolerance

2.1 Introduction

Post-translation modifications (PTMs) add small chemical moieties or chemical modifications at individual amino acids in translated proteins. PTMs regulate protein stability, folding, function, and their interaction with other biomolecules (Hermann et al., 2022). The most characterized PTMs are phosphorylation, acetylation, glycosylation, citrullination, and ubiquitination (Ramazi & Zahiri, 2021). PTMs are frequently involved in many diseases besides cancer. Highly phosphorylated tau protein has been associated with neurodegenerative diseases, including Alzheimer's disease (Wegmann et al., 2021). In rheumatoid arthritis, protein hyper-citrullination is a hallmark of the disease, and autoantibodies to hyper-citrullinated proteins are typically detected in the patient's synovial fluid (Darrah, E., and Andrade, 2018). In type 2 diabetes (T2D), an increase in global glycosylation levels leads to the impaired release of secreted proteins from adipose tissue and also induces insulin resistance (B. Chatterjee & Thakur, 2018; Lim et al., 2014).

Cancer cell transformation profoundly changes gene assets and, consequently, protein expression and activation. Tumor antigens are classified into: i) tumor-specific antigens (TSA) and ii) tumor-associated antigens (TAA). TSA are proteins specifically expressed by tumors but not by normal cells. TSAs can be classified into wild-type TSA and mutated TSA or "*neoantigens*" (Haen et al., 2020). These latter are proteins with individual specificity and emerge from somatic mutations in the tumor genome (Peng et al., 2019). As each tumor displays an individual heterogeneity and mutational burden, neoantigens can be defined as truly tumor-specific. Wild-type TSA has been identified as Human-Leukocyte Antigens (HLA)-eluted peptides with wild-type sequences (compared with the relevant germline sequence) that, nonetheless, have a tumor-specific presentation, not represented on benign/normal tissues, and to which the immune system has not been previously exposed (Haen et al., 2020). TSA was also identified and characterized by using high-throughput multi-omics analyses, including next-generation sequencing or tandem mass spectrometry (MS/MS) (Haen et al., 2020).

Self-proteins expressed in both tumor and non-malignant cells but aberrantly present in tumor cells in terms of amount, chemical features, location, or time, is defined as TAA and can elicit multiple specific immune responses (Sahin et al., 1995). TAAs

were identified by using Serological Proteome Analysis (SERPA) or SErological analysis of Recombinant cDNA EXpression libraries (SEREX) (Le Naour et al., 2002). Between these two approaches to identifying TAA, SERPA - which specifically identifies the different isoforms of tumor proteins - is the more appropriate way to also identify the autoantibody response to PTM (Zaenker & Ziman, 2013). A key example of a TAA identified by SERPA is alpha Enolase (ENO1), which is overexpressed, acetylated, or phosphorylated in pancreatic cancer (Capello et al., 2011, 2015; Cappello, Tomaino, Chiarle, Ceruti, Novarino, Castagnoli, Migliorini, Perconti, Giallongo, & Milella, 2009; Tomaino et al., 2011; Zhou et al., 2010). Other examples of TAA that have been identified thanks to the analysis of the immune response elicited by them in cancer patients include acetylated and phosphorylated p53, phosphorylated insulin receptor substrate 2 (IRS2), cell division cycle 25b (CDC25b), citrullinated vimentin (Vim), and glycosylated mucin (MUC) protein (Brentville, Metheringham, et al., 2020; Karanikas et al., 1997; Kumai et al., 2014; K. Ohara et al., 2018; Zarling et al., 2014).

The choice of TSA or TAA is essential in any strategy aiming to unleash the anti-tumor T cell response by vaccine approaches (Saxena et al., 2021). Therapeutic cancer vaccines allow peptides derived from tumor proteins (TSA or TAA previously identified) to be presented by HLA molecules in order to activate the immune system to recognize and kill the established tumors expressing those proteins. These vaccines typically involve exogenous administration of selected tumor antigens combined with adjuvants that activate dendritic cells (DCs) as antigen-presenting cells or even DCs themselves previously loaded with the tumor antigen (Hollingsworth & Jansen, 2019). The aim of therapeutic cancer vaccines is to stimulate the patient's adaptive immune system against specific tumor antigens to regain control over tumor growth, induce regression of established tumors, and eradicate the minimal residual disease (Paston et al., 2021; Saxena et al., 2021).

The presence of PTM in TAA increases the immunogenicity as they may be considered foreign antigens by the immune system or break the tolerance established against the self-unmodified protein (see Figure 2-1). Immunogenic epitopes of TAA elicit immune responses, especially the production of autoantibodies (Fortner et al., 2017). In general, TAA phosphorylated epitopes are better presented than non-

phosphorylated epitopes by Human leucocyte Antigen (HLA) (Alpízar et al., 2017; Y. Li et al., 2010; Petersen, Wurzbacher, et al., 2009).

Some key examples of PTM-modified TAA and the relative induced-immune responses are listed in Table 2-1. All the studies reporting specific T cells or antibodies against the modified TAA always analyzed the same response induced by the un-modified antigen.

Table 2-1 Immune response elicited by PTM-modified TAA

PTMs	TAA	Type of Tumor in which TAA has been identified or to which the antigen is associated	Immune recognition (if any)	References
Acetylation	ENO1	Pancreas	NA	(Zhou et al., 2010)
	p53	Colon, Prostrate, Pharynx	CD4+T Cell	(Kumai et al., 2014)
Phosphorylation	MART-1	Melanoma, Leukemia	CD4+T Cell	(Depontieu et al., 2009)
	IRS2	Melanoma, Breast, Ovary, Colon	CD8+T Cell	(Engelhard et al., 2020; Zarling et al., 2006, 2014)
	β-Catenin	Ovary, Melanoma	CD8+T Cell	(Engelhard et al., 2020; Zarling et al., 2006)
	Breast cancer antiestrogen resistance 3	Melanoma	CD8+ T Cell	(Engelhard et al., 2020)
	p53	Head and Neck	CD4+T Cell	(K. Ohara et al., 2018)
	ENO1	Pancreas	Ab, CD4+T Cell	(Capello et al., 2015; Tomaino et al., 2011)
	CDC25b	Melanoma, Breast, Ovary, Colon, Leukemia	CD8+ T Cell	(Zarling et al., 2006, 2014)
	TNF receptor-associated protein (TRAP-1)	Lung	CD8 + T Cell	(M.-H. Lin et al., 2019)
	Vim	Colon	CD4+ T cells	(M. Ohara et al., 2020)

Citrullination	Vim	Melanoma, Lung	CD4 + T Cell	(Brentville et al., 2016, 2019; Brentville, Metheringham, et al., 2020; Symonds et al., 2021)
	Enolase	Melanoma, Lung	CD4 + T Cell	(Brentville et al., 2019; Brentville, Metheringham, et al., 2020; Cook et al., 2018; Symonds et al., 2021)
Glycosylation	MUC1	Breast, Ovary	CD8 +T Cell, Ab	(Boland et al., 1982; Nuti et al., 1993)
Sialylation	Silayl-Tn-Antigen	Breast, Ovary	Ab (IgM)	(Fung et al., 1990; Itzkowitz SH, Yuan M, Montgomery CK, Kjeldsen T, Takahashi HK, Bigbee WL, 1989; Longenecker et al., 1993; MacLean GD, Bowen-Yacyshyn MB, Samuel J, Meikle A, Stuart G, Nation J, Poppema S, Jerry M, Koganty R, Wong T, 1992)
SUMOylation	p53	Sarcoma	NA	(Rodriguez et al., 1999)
Methylation	Enolase	Pancreas	NA	(Zhou et al., 2010)

Below, we will discuss in greater detail each PTM relevant to cancer immunotherapy.

2.2 Acetylation

Lysine (K) acetylation is a reversible PTM, which converts a positively charged lysine into a neutral amino acid, changing the function and properties of the protein. Acetylation plays an essential role in transcription regulation by modifying the core

histone tails through lysine acetyltransferase (KAT) or lysine deacetylases (KDACs) (Figure 2-2) (Lee & Workman, 2007; Shahbazian & Grunstein, 2007). Acetylation in histone protein is a crucial regulator in the transcription process (Allfrey et al., 1964; Verdin & Ott, 2015). Due to technical difficulties, earlier acetylation was studied at the protein-to-protein level, which had restricted acetylation to be a nuclear PTM. With the advancement in enrichment methods and high-resolution mass spectrometry, studying acetylation at the proteome level became possible. These advanced methods led to identifying acetylation even in cytosolic and membrane proteins (Choudhary et al., 2009, 2014; Kim et al., 2006).

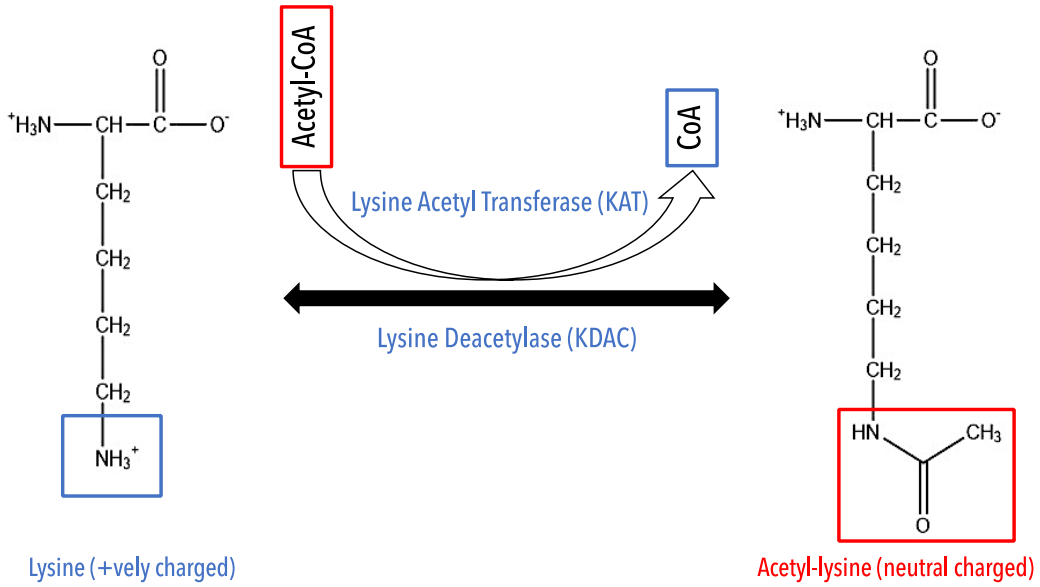

Figure 2-2:Schematic representation of the acetylation process

Altered acetylation levels in histone and non-histone proteins have been shown to play a role in tumorigenesis in numerous cancer types (Audia & Campbell, 2016; Calcagno et al., 2019; Guo et al., 2018; M. Hu et al., 2022; T. Li et al., 2014, 2018; S. Liu et al., 2019; Lv et al., 2013). Gene expression of proto-oncogene gets activated under hyperacetylation of histone. Histone acetylation is a reversible process for cancer therapeutics (Ramezankhani et al., 2021; Shanmugam et al., 2022). Five inhibitors of KDAC have been approved by the American Food and Drug Administration (FDA) for treating myeloma and T-Cell lymphoma (Bondarev et al., 2021). The successful approval of five agents has pushed scientific communities to test the efficacy of KDAC inhibitors in other tumors. Many agents targeting KDAC

have shown promising results in multiple clinical trials (Cancerología, 2006; Corporation, 2005; Hospital, 2011; Michael Luebbert, 2016; National Institute of Cancerología, 2007; Ramezankhani et al., 2021; Therapeutics, 2011). These results suggest that acetylation could represent a good target for tumor treatment, but any of these studies evaluated the potential presentation of acetylated histone epitopes or how the specific anti-tumor response was affected. Therefore, we did not include them in our discussion.

Besides histones, acetylation regards many other cytoplasmic proteins, such as the lactate dehydrogenase-A (LDH-A). In this case, acetylation inhibited its enzymatic activity, and acetylation of K5 in LDH-A in the pancreatic ductal adenocarcinoma (PDAC) cell line BxPC-3 was suggested to play a role in supporting tumor cell proliferation (D. Zhao et al., 2013). On the other hand, K5 acetylation of LDH-A also decreases lactate production, thereby restraining pancreatic cancer cell migration. However, in human pancreatic cancer samples, a significant decrease in the ratio of K5 acetylated LDH-A to total LDH-A protein was observed and acetylated LDH-A correlated with tumor stage; this suggested a possible role of LDH-A-K5 acetylation in the initiation of pancreatic cancer but not in its progression (D. Zhao et al., 2013). Linear Trap Quadrupole-orbitrap mass spectrometry identified 26 acetylation sites in ENO1 from PDAC and normal pancreatic duct cells, and of those, five were unique to PDAC cells (Zhou et al., 2010). ENO1 is a cytosolic or nuclear protein expressed on the membrane wall of bacteria to help in their invasion (Pancholi, 2001). In tumor cells, ENO1 is also highly expressed on the cell surface, but the mechanism by which it switches from cytoplasm to membrane is unknown (Capello et al., 2011). It is supposed that PTM could represent one of the molecular mechanisms for its membrane exposure.

A pilot study to test whether or not acetylated peptides could be immunogenic was performed with p53 peptides. CD4 T cells were stimulated *in vitro* with autologous DCs pulsed with acetylated or non-acetylated p53 peptides (Kumai et al., 2014). Three-fold increased cytokine production was observed when CD4 T cells were stimulated with acetylated p53 peptides compared to non-acetylated p53 peptides. This T cell response was inhibited by the addition of an anti-HLA-DR but not by

other anti-HLA class II (HLA-DQ, HLA-DP) antibodies, suggesting that the peptides (acetylated or non-acetylated) are mainly presented by HLA-DR molecules. Cancer patient peripheral blood mononuclear cells (PBMC) but not healthy donor PBMC were able to specifically produce IFNγ after seven days of stimulation with acetylated but not with non-acetylated p53 peptides (Kumai et al., 2014). These results demonstrated that tumor-associated acetylated peptides are good candidates for developing cancer vaccines.

2.3 Citrullination

Unlike other PTM, citrullination is an irreversible modification, which converts the positively charged amino acid arginine (Arg) to neutral citrulline by a family of peptidyl arginine deiminase (PAD) enzymes (Yuzhalin, 2019). The process is called citrullination or deamination (Figure-2-3); PAD replaces the primary ketamine (=NH) group in Arg with a ketone (=O) group, which was implicated in the recognition from the T cell receptor via HLA presentation (Alghamdi et al., 2019; Sakkas et al., 2014). The loss of positive charge affects the protein-protein interaction and the protein structure and may lead to protein denaturation. Citrullination is a standard process observed in cells under stress, nutrient starvation, and during apoptosis due to an increase in PAD expression (Yuzhalin, 2019).

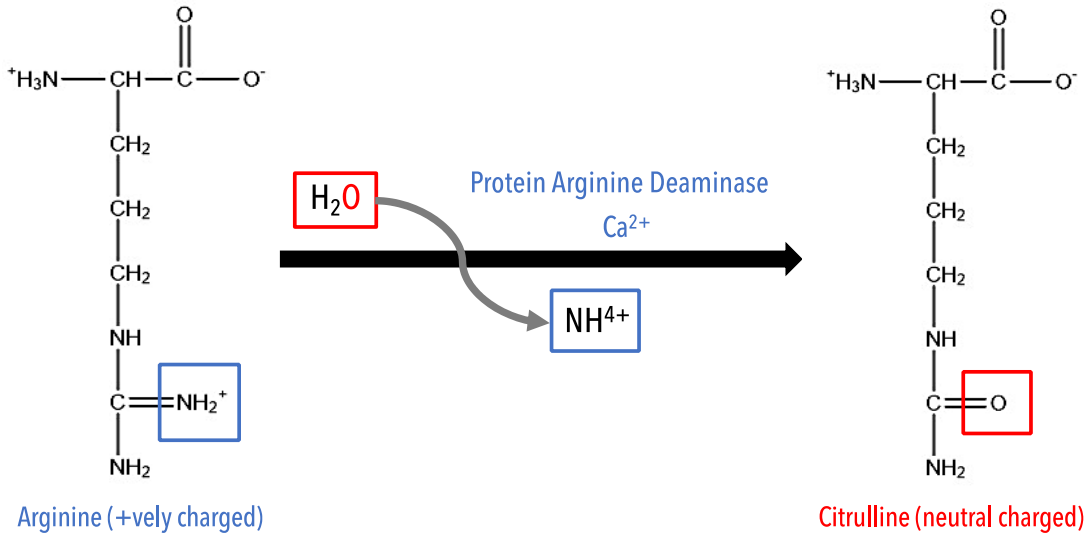

Figure-2-3:Schematic representation of citrullination

A number of studies have reported that hyper citrullination could be a factor in breaking immune tolerance and inducing autoimmune diseases like Type-1-Diabetes, Multiple Sclerosis, and Rheumatoid Arthritis (Alghamdi et al., 2019; Buitinga et al., 2018; Darrah, E., and Andrade, 2018; Sakkas et al., 2014; Schellekens et al., 1998; L. Yang et al., 2016). In Rheumatoid Arthritis, anti-cyclic citrullinated peptides in patient sera are biomarkers to identify the disease at its early stages (Darrah, E., and Andrade, 2018; Schellekens et al., 1998). Given the role of citrullination in autoimmune diseases and its ability to break immune tolerance, the hypothesis that citrullinated peptides could also be immunotherapeutic agents in cancer treatments was evaluated.

Citrullinated peptides from Vim and ENO1 elicited a specific immune response with strong IFNγ release in HLA-DR4 transgenic mice, whereas there was no response against non-citrullinated peptides (Brentville et al., 2016; Cook et al., 2018). CD4 T cells were the most involved in mediating the citrullinated-specific response but displayed cytotoxic activity by expressing granzyme and Fas Ligand and directly killing tumors expressing HLA-II (Brentville et al., 2016; Brentville, Vankemmelbeke, et al., 2020; Cook et al., 2018). Splenocytes from mice immunized with citrullinated Vim peptides released granzyme upon stimulation with specific stimuli. Immunization with citrullinated (Brentville et al., 2016; Brentville, Vankemmelbeke, et al., 2020; Cook et al., 2018) Vim and ENO1 peptides increase survival of HLA-DR4 transgenic mice implanted with B16F1 tumors expressing HLA-DR4, as well as Lewis lung carcinoma cells (LLC/2), ovarian cancer cells (ID8) and pancreatic cancer cells (Pan02) (Brentville, Metheringham, et al., 2020).

Proliferative response against citrullinated Vim and ENO1 peptides was observed in 67% of healthy donors of PBMC (Brentville et al., 2019). Only 28% of these healthy donors displayed HLA-DR4, whereas 71% of donors displayed HLA-DP4 (Brentville et al., 2019). By assessing the T cell repertoire to citrullinated peptides in ovarian cancer patients and healthy donors, it was demonstrated that PBMC from 58% of patients proliferated in response to at least one of the PTM peptides and only 12% to both citrullinated Vim and ENO1 peptides (Brentville, Metheringham, et al.,

2020). Analyzing the type of HLA revealed that most responders were HLA-DR4 or HLA-DP4, but not all. With predictive methodologies, it was found that some even expressed HLA-DQ6, HLA-DR13, and HLA-DP18 (Brentville, Metheringham, et al., 2020). These data suggest that more HLA loci present citrullinated peptides, and those citrullinated peptides from TAA represent good candidates for vaccine approaches (Brentville, Metheringham, et al., 2020; Brentville, Vankemmelbeke, et al., 2020).

The binding of citrullinated Vim and ENO1 peptides to HLA-DP4 was tested by comparing it to that of HLA-DP4-known binding peptides like those from the Hepatitis B virus.–Unmodified Vim aa415-423 and aa28-49 peptides showed low binding to HLA-DP4 compared to the citrullinated Vim peptides, which showed stronger binding. Similarly, citrullinated ENO1 peptide had a higher binding capacity to HLA-DP4 compared to unmodified ENO1 peptide (Cook et al., 2018). In HLA-DP4 transgenic mice, the combination of a vaccine composed of citrullinated Vim and ENO1 peptides with granulocyte–macrophage colony-stimulating factor (GM-CSF) and TLR agonists (especially with TLR1/2 agonist) reduced by 10- to 100-fold the dose of the vaccine without losing the anti-tumor activity (Brentville, Metheringham, et al., 2020).

A combination of citrullinated peptides (Vim and ENO1) could also elicit similar immune responses in HLA-DR4 or HLA-DP4 transgenic mice (Brentville, Metheringham, et al., 2020; Brentville, Vankemmelbeke, et al., 2020). These combination peptide vaccines could elicit anti-tumor therapy against multiple tumor models in mice (Brentville, Metheringham, et al., 2020; Brentville, Vankemmelbeke, et al., 2020).

In a B16 melanoma mouse model, citrullinated peptides-induce IL10 release but also higher secretion of IFNγ than non-citrullinated peptides (Symonds et al., 2021). New citrullinated peptides could be extracted by peptide elution and mass spectrometry (Symonds et al., 2021). Another important confirmation of the relevance of modified

and specifically citrullinated peptides as targets to elicit an anti-tumor response is the presence of elevated levels of IgG-bound citrullinated peptides in the sera of newly diagnosed breast cancer patients (0-0.8 years) (Katayama et al., 2021). This suggests that citrullinated peptides-Ig complexes could be explored as biomarkers for early detection, just like they are used in the early identification of RA.

2.4 Phosphorylation

Phosphorylation is a reversible PTM catalyzed by phosphotransferase, which adds a phosphate group on the hydroxyl group of amino acid residues (Ser/Thr/Tyr) from the ATP molecule (Figure 2-4) (Cohen, 2002). It is one of the most widely studied PTMs (Ramazi & Zahiri, 2021). One of the hallmarks of tumor growth is, in fact, dysregulated phosphorylation, which contributes directly to oncogenic signaling cascades involved in cell growth, differentiation, and survival (Blume-Jensen & Hunter, 2001; Ehsanian et al., 2010; Y. Liu et al., 2021; T. Yang et al., 2019). This renders phosphorylation an interesting potential therapeutic tool, as the presence of PTMs increases the variety of naturally occurring peptide epitopes (Anderton, 2004; Engelhard et al., 2006; Petersen, Purcell, et al., 2009).

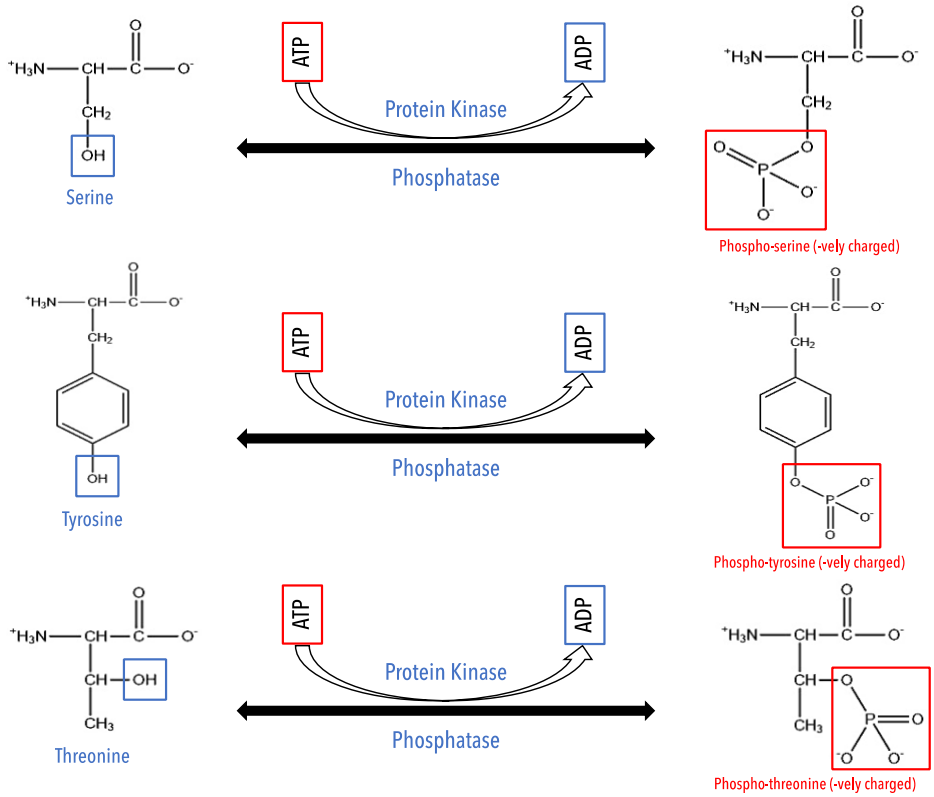

Figure 2-4: Schematic representation of phosphorylation on multiple amino acids

Phosphorylated peptides can be presented by HLA-II molecules. Structural analysis showed a 2.1 Å resolution of phosphorylated tumor-associated antigen MART-1 peptide (pMART-$1_{100-114}$) bound with HLA-DR1 (Y. Li et al., 2010). Specific $CD4^+$ T cell clones secreted GM-CSF in response to phosphorylated MART-1 peptide-pulsed onto HLA-DR1-expressing antigen-presenting cells (APCs) but not to the non-phosphorylated peptide. This demonstrates that the phosphate group is indeed a critical determinant for T Cell Receptor recognition (Y. Li et al., 2010).

A study validated the hypothesis that phosphopeptides can be immunotherapeutic targets by analyzing mixtures of more than 10,000 peptides presented by HLA-A*201 on the surface of human melanoma, ovarian cancer, and B cell lymphoma cell lines (Zarling et al., 2006). They were isolated, extracted, and phosphopeptides were enriched; 36 phosphopeptides presented by HLA-A*201 on one or more of the four cell lines were identified, sequenced, and employed to immunize transgenic mice expressing HLA-A*201. Isolated phosphopeptide-specific CD8 T cells secreted IFNγ when exposed to synthetic phosphopeptide epitopes but not to non-phosphorylated peptides (Zarling et al., 2006). Fresh PBMC from melanoma patients HLA-DRB1*01 and HLA-DQB1*0501 were stimulated *in vitro* with phosphorylated MART1 (pMART1) peptides for several rounds of simulation. Phosphorylated specific CD4+ T cells secreted IFNγ and GM-CSF in response to pMART1 but not in response to non-phosphorylated MART1 (Depontieu et al., 2009).

A phosphorylation site (Ser419) was also identified in the more acidic isoforms of the glycolytic enzyme ENO1 in PDAC and normal pancreatic ductal cells (Zhou et al., 2010). Autoantibodies against phosphorylated ENO1 were found in a greater percentage of PDAC patients and only in a small percentage of healthy individuals (Tomaino et al., 2011). Antibodies present in the sera of PDA patients recognized six different isoforms of ENO1 (ENO_1,2,3,4,5,6), while those in healthy individuals only had four isoforms. Notably, the presence of anti-ENO_1,2

autoantibodies improved the diagnostic performance of CA19.9 in pancreatic cancer patients with low levels of CA19.9 and correlated with a better prognosis and overall survival (OS) (Tomaino et al., 2011). This suggests that phosphorylation plays a role in breaking tolerance in cancer patients in an attempt to fight tumor growth. The association between the presence of the HLA-DRB1*08 allele and the production of autoantibodies against a phosphorylated epitope of ENO1 was also demonstrated (Capello et al., 2015). In effect, the HLA-DRB1*08 allele was more frequent in PDAC patients with autoantibodies against $pENO1_{413-422}$ (phosphate group at Ser419) than healthy controls or patients without these autoantibodies. Interestingly, PDAC patients with autoantibodies against pENO1 also displayed T cells that proliferated and secreted IFNγ in response to phosphopeptides but, to a lesser extent, in response to unmodified peptides (Capello et al., 2015).

Phosphopeptides from IRS2, CDC25b, p53, Vim, and TRAP1 also could elicit specific immune responses in different cancer models in terms of T cells secreting IFNγ (M.-H. Lin et al., 2019; K. Ohara et al., 2018; M. Ohara et al., 2020; Zarling et al., 2006). Phosphopeptides of IRS-2, CDC25b, and TRAP1 specifically elicited CD8 T cells, whereas the CD4 specific T cell response was elicited in response to phosphopeptides of p53 and Vim (M.-H. Lin et al., 2019; K. Ohara et al., 2018; M. Ohara et al., 2020; Zarling et al., 2006).

An open-label, pilot, proof-of-concept clinical trial study to assess the phosphopeptide vaccine safety and immunogenicity was performed on patients with resected stage II-IV melanoma (Craig L Slingluff, 2016; Engelhard et al., 2020). pIRS2 and phosphorylated BCAR3 were used as vaccines. Patients were divided into three groups: the first group (3 patients) was administered with pBCAR3, the second group (3 patients) with pIRS2, and the third group (9 patients) received both phosphopeptides. Vaccines were administered along with tetanus toxoid peptide in a water-in-oil emulsion with an equal volume of incomplete Freud's adjuvant. Immediately after vaccination, poly-L-lysine and carboxymethyl cellulose were injected into patients to stimulate the immune system (Engelhard et al., 2020). A total of 17% of patients administered with pBCAR3 showed a CD8+ T cell response,

whereas 42% of patients elicited a CD8+ T cell response when administered with the pIRS2 vaccine, with a greater increase in IFNγ production (Craig L Slingluff, 2016; Engelhard et al., 2020). None of the patients reported severe adverse effects after vaccination, showing that the phosphopeptide vaccine is safe and should be tried in larger clinical trials (Craig L Slingluff, 2016; Engelhard et al., 2020).

Overall, all these studies support the hypothesis that phosphorylated epitopes are recognized by the adaptive immune system and, therefore, imply that the anti-tumor response that can fight tumor growth.

2.5 Glycosylation

Protein glycosylation is a PTM where a carbohydrate molecule is attached to nitrogen or hydroxyl, or other functional groups of amino acids through enzymatic reactions. The glycosyltransferase enzyme catalyzes these reactions (Eichler, 2019). Protein glycosylation is classified into two major categories: N-Linked glycosylation, where glycans are attached to the nitrogen of an Asparagine (Asn) or Arg residues, and O-Linked glycosylation, where glycans are attached to the hydroxyl group of Ser or Thr residues (Eichler, 2019). Protein glycosylation plays a significant role in protein folding, activity, stability, and conformation. Almost half of the human proteins are glycosylated, and most cancer biomarkers, which the FDA has approved, consist of glycoprotein or carbohydrate antigens (Kirwan et al., 2015; Mereiter et al., 2019; Munkley, 2019; Silsirivanit, 2019).

Many glycoproteins are associated with cancer progression (Apostolopoulos et al., 1997; Itzkowitz SH, Yuan M, Montgomery CK, Kjeldsen T, Takahashi HK, Bigbee WL, 1989). One common glycoprotein whose role is well-established in tumors is MUC. The first identified membrane MUC protein in many solid tumors and hematopoietic cancers was MUC1 (Boland et al., 1982; Hasegawa et al., 2011; Nath & Mukherjee, 2014). MUC1 is often upregulated and aberrantly glycosylated, making it a potential therapeutic target for cancer immunotherapy. In some malignant transformations, MUC1 becomes hypo-glycosylated, carrying truncated

carbohydrates known as Tn antigens. MUC2 is another MUC protein commonly found in the intestinal lining and expressed in goblet cells of the small bowel and colon (S.-K. Chang et al., 1994). In mucinous carcinoma of the pancreas, prostate, breast, ovary, and colon, there is an overexpression of MUC2 (Hanski et al., 1997; O'Connell et al., 2002).

The potential of MUC1 as a vaccine was evaluated in preclinical models in different tumors (Apostolopoulos et al., 1997; Barratt-Boyes et al., 1999; Beatty et al., 2010; Fung et al., 1990; MacLean GD, Bowen-Yacyshyn MB, Samuel J, Meikle A, Stuart G, Nation J, Poppema S, Jerry M, Koganty R, Wong T, 1992; Reichenbach & Finn, 2013; M. M. Soares et al., 2001). Successful results in mice led to many phases I clinical trials showing that MUC1 is safe and well tolerated in patients (Goydos et al., 1996; Gulley et al., 2008; Karanikas et al., 1997; Lepisto et al., 2008; Loveland et al., 2006; MacLean et al., 1996; Ramanathan et al., 2005; Scholl et al., 2000; Wierecky et al., 2006). These exciting results began a new era of immunotherapy clinical trials over the following two decades. Many phase II and phase III clinical trials targeting glycosylation demonstrated an effective anti-tumor response but limited success in extending the survival of cancer patients. (Apostolopoulos et al., 2006; Arlen et al., 2006; Butts et al., 2014; Heery et al., 2015; Limacher & Quoix, 2012; MercK, n.d.; Miles et al., 2011; Mitchell et al., 2014; Posey et al., 2016; Quoix et al., 2016; Wu et al., 2011) Phase III clinical trials targeting MUC1 are shown in Table 2-2.

The majority of clinical trials proposed the use of a viral vector expressing MUC1 alone or in combination with other TAA in different solid cancers (Akbulut et al., 2010; Arlen et al., 2006; Gabitzsch et al., 2015; Gulley et al., 2008; Heery et al., 2015; Limacher & Quoix, 2012; Mohebtash et al., 2011; Quoix et al., 2016; Scholl et al., 2000; Tosch et al., 2017). Many phase III trials got terminated either prematurely or suspended because of lack of funding; hence, there are no related publications and limited information on the Clinicaltrials.gov website. One of the major phase III clinical trials used TG4010, a modified vaccinia Ankara vector expressing MUC1 and Interleukin 2, in combination with chemotherapeutic drugs or

placebo in 222 non-small cell lung cancer (NSCLC) patients (Quoix et al., 2016). Patients were subdivided into two arms: those receiving chemotherapeutic drugs with TG4010 and those receiving chemotherapeutic medication with a placebo. Patients treated with TG4010 combined with chemotherapeutic drugs had a longer significant PFS compared to that of patients treated with a placebo plus chemotherapeutic drugs (Quoix et al., 2016).

The most extensive phase III clinical trial of MUC1 was conducted by enrolling 1513 patients of NSCLC treated with Tecemotide, a lipopeptide derived from MUC1 (Butts et al., 2014). No significant differences in OS of the patients treated with Tecemotide or with a placebo were reported. However, 10.2 months improvement in median survival for patients who received Tecemotide after chemoradiotherapy was observed. This suggests that Tecemotide may have a potential role in the efficacy of maintenance therapy after initial concurrent chemoradiotherapy in NSCLC patients (Butts et al., 2014). Other phase III clinical trials targeting MUC1 are detailed in Table 2, with outcomes from the studies.

Many small Phase I/II trials evaluated the potential of autologous DC presenting MUC1 (Gray et al., 2016; Lepisto et al., 2008; Loveland et al., 2006; Mitchell et al., 2014; Scheid et al., 2016; Wierecky et al., 2006). The vaccine (CVac) was made with a recombinant fusion protein (FP) conjugated to oxidized mannan (M) and loaded into autologous DC (Loveland et al., 2006). The FP consisted of a variable number of tandem repeats (VNTR) region of the MUC1 protein and glutathione-S-transferase (Loveland et al., 2006). The benefit of mannan conjugated antigen is to induce DC activation and maturation by targeting the complex of mannose receptors of DC (Loveland et al., 2006). Oxidized mannan increases the efficiency of HLA I presentation of MUC1 recombinant protein (Apostolopoulos et al., 2000). Ovarian cancer patients in complete remission (CR) were treated with CVac to evaluate its efficacy and safety (Gray et al., 2016). Patients from the first CR (CR1) or second CR (CR2) were randomized to standard of care (SOC) observation to CVac treatment. Patients were given ten doses of CVac over 56 weeks and followed up for another 48 weeks at the end of the study to measure PFS. When both groups were

challenged with MUC1 antigen, SOC patients had few or no T cells, whereas CVac-treated patients had both CD8 and CD4 T cell responses (Gray et al., 2016). In addition, CVac-treated patients displayed a higher CD8 cytotoxic T-cell response compared to that elicited in CD4 T cells, for which it was not possible to evaluate the release of cytokines. In the CR1 group of patients, no significant change was observed in progression-free survival (PFS) and OS between the SOC and CVac arms. However, in CR2 patients CVac treated patients had higher PFS than the SOC control group (Gray et al., 2016). Another phase I/II clinical trial assessed the safety of a vaccine composed of Tn-MUC1 loaded onto autologous DC in 17 patients with non-metastatic castrate-resistant prostate cancer (nmCRPC) (Scheid et al., 2016). The Tn-MUC1 DC vaccine was found to be safe and elicited a robust CD4 T cell response by increasing the secretion of cytokines such as TNFα, IL2, IFNγ, and a more robust CD8 T cell response, although nmCRPC patients did not achieve the desired Prostate Specific Antigen (PSA) values (Scheid et al., 2016). However, the strong immune response monitored in the patients suggested that a larger trial in combination with chemotherapeutic drugs could improve both PFS and OS.

Many clinical trials also employed direct vaccination with MUC1 peptides in different tumors and even as a prevention strategy (Fiedler et al., 2016; Goydos et al., 1996; Karanikas et al., 1997; Kimura et al., 2013; Ramanathan et al., 2005). Patients with advanced colon adenoma were vaccinated with MUC1 to assess the ability of this vaccine to induce an anti-MUC1 immune response and long-term memory without toxicity (Kimura et al., 2013). The authors found that 44% of patients could produce high levels of anti-MUC1 immunoglobulin G (IgG) and long-lasting immune memory without toxicity (Kimura et al., 2013). The remaining 56% of patients, who did not show elevated levels of anti-MUC1 IgG, displayed higher levels of myeloid-derived suppressor cells (MDSC) already before the vaccination (Kimura et al., 2013). Another interesting phase I study used a humanized glycol-optimized monoclonal antibody against the MUC1 epitope (PankoMab-GEX) in different cancers (Fiedler et al., 2016). PankoMab-GEX was well tolerated in patients with advanced disease and was strong enough to elicit an anti-tumor activity. The best result in this trial was observed in ovarian and lung cancer patients: in the

former cohort, one patient had a complete response, and 32% of patients displayed disease stabilization (Fiedler et al., 2016).

MUC2 is commonly used as a biomarker for many cancers as well as other diseases (García-Labastida et al., 2014; Lakshmanan et al., 2015; C. Li et al., 2018; McIntire et al., 2011): a study with fifty patients with goblet cell metaplasia found that MUC2 expression in non-goblet epithelium may represent a specific biomarker. The authors concluded that in the esophagus, MUC2 expression represents a late event in converting mucinous columnar cells to goblet cells (McIntire et al., 2011). MUC2 overexpression was correlated with a lower tumor grade and a lower rate of lymphatic invasion in a large meta-analysis of 2,363 patients with gastric carcinoma (L. N. Hu et al., 2021). However, there was no statistically significant correlation between the expression of MUC2 and lymph node metastasis, gender, and five-year survival rate (L. N. Hu et al., 2021).

Clinical studies assessed the role of MUC2 as a potential therapeutic immune target in cancers (Astashchanka et al., 2019; Lesuffleur et al., 1993; Ragupathi et al., 1999; Rakha et al., 2005; S. F. Slovin et al., 1999). MUC2, conjugated to the immunologic carrier protein, keyhole limpet hemocyanin (KLH), and given with the saponin adjuvant, *Quillaja saponin* (QS-21), was safe and induced high IgM and IgG titer specific for the immunogen (Ragupathi et al., 1999; S. Slovin et al., 2002; S. F. Slovin et al., 1999; S. F. Slovin & Scher, 1999). Another interesting tumor-associated carbohydrate antigen is Globo H, which is expressed on the outer membrane of cancer cells but not in normal tissue cells. Indeed, antibodies against Globo H mediated complement lysis or ADCC (S. Slovin et al., 2002). In phase I clinical trial, a bivalent vaccine consisting of Globo H and MUC2 conjugated to the carrier, KLH, and mixed with adjuvant QS21 was administered to 43 patients with relapsed prostate cancer (S. F. Slovin et al., 2005). The vaccine was found to be safe and generated high titer of IgG and IgM antibodies to MUC2, but only IgM antibodies to Globo-H.(S. F. Slovin et al., 2005). The promising result from the Phase I clinical trial led to other Phase II clinical trials involving MUC2 and/or Globo H as vaccine targets with conjugates, which have no posted results yet (NCT00004929, 2013; NCT00016146, 2013; NCT00036933, 2013).

Even after some failed trials, an interest in finding a vaccine for cancer using glycosylated antigens as a target has not decreased. The number of new registrations for clinical trials has increased in the past two decades. This clearly shows the faith of corporate in investing a lot in immunotherapy.

Table 2-2:List of Phase III clinical trials using MUC1 antigen (retrieved from clinicaltrial.gov website)

Vaccine	Number of Patients	Treatment	Outcome	References
Oxidized mannan MUC1 peptide	31 doubly blind breast cancer stage II	Administered subcutaneous injections of either placebo or oxidized mannan-MUC1	5.5 years since the final patient began treatment (8.5 years from the start of treatment of the first patient), the recurrence rate in patients receiving the placebo was 27% (4/15; the expected rate of recurrence in stage II breast cancer); those receiving immunotherapy had no recurrences (0/16), and this finding was statistically significant (P = 0.0292).	(Apostolopoulos et al., 2006)
PANVAC-VF viral a vector expressing CEA. and MUC1 plus B7.1,	255 patients, advanced pancreatic cancer patients	PANVAC-VF versus palliative chemotherapy	No significant difference in OS of patients receiving PANVAC-VF versus palliative chemotherapy or best supportive care	(Madan et al., 2007)
Silayl Tn-KLH	1028 breast cancer patients	Silayl Tn-KLH versus KLH	No significant difference in OS in patients receiving Silayl Tn-KLH versus KLH alone	(Miles et al., 2011)

Tecemotide (L-BLP25) lyophilized 25mer MUC1	1513 NSCLC patients	Tecemotide (L-BLP25) versus placebo after chemoradiotherapy	No significant OS difference within the whole cohort	(Butts et al., 2014)
TG4010 (a modified vaccinia Ankara expressing MUC1) and interleukin 2	222 stage IV NSCLC patients (phase 2b/3)		TG4010 plus chemotherapy seems to improve progression-free survival compared to placebo plus chemotherapy	(Quoix et al., 2016)
Tecemotide (L-BLP25) lyophilized 25mer MUC1	285 Stage IV NSCLC patients		The study was prematurely terminated	(MercK, n.d.; Wu et al., 2011)

OS: Overall Survival
PANVAC-VF: Cancer vaccine targeting MUC1 and carcinoembryonic antigen delivered via two viral vectors, Vaccina (V) and Flowpox (F)
KLH: Keyhole Limpet Hemocyanin

2.6 Conclusion

It is becoming more apparent that immunotherapy represents a real promise for treating cancer in all its forms. Passive immunization or immune checkpoint blockade was the first approach to take hold, although an increase in clinical trials using the adoptive transfer of CAR T cells or TRC-engineered T cells has been reported since 2015 (Yu et al., 2019). Vaccines are still less successful than previous approaches due to the complex relationship between tumors, stroma, and immune cells, rendering the microenvironment extremely demanding and exhausting. However, the crucial point in active immunotherapy remains the choice of antigen to be targeted by the vaccine. TAA with PTMs represents an exciting option for cancer immunotherapy. New insights regarding the ability of acetylated and citrullinated peptides to elicit tumor-specific responses represent promising results which, nevertheless, need further investigation, especially in pilot-phase clinical trials. The promising results from combinational citrullinated ENO1 and Vim peptides also should be further explored with chemotherapeutic targets in pilot clinical trials for their immunogenicity and safety in cancer patients to open new possibilities for designing immunotherapeutic strategies. Efforts should first be focused on identifying and then exploiting aberrantly phosphorylated, acetylated,

citrullinated, and glycosylated proteins variably expressed from multiple cancers to develop vaccines for large-scale immunotherapy. The focus should be to translate these preclinical studies into clinical trials.

A pilot clinical trial into vaccines against phosphorylated peptides of pIRS2 and PBCAR3 along with the vaccine against MUC1, MUC2 showed promising results for those many vaccines which have yet to undergo clinical trials. Future vaccine strategies could involve many PTM antigens, which could enhance the magnitude of the immune response.

It is very important to deeply understand the meaning of PTM in cancer in parallel to their immunogenicity characterization. If the PTM becomes a general and key process acquired during carcinogenesis, it is expected that it will be maintained in all tumor cells and not only in certain clones, which happens for the so-called neoantigens. This will allow the extreme heterogeneity that has been well described in tumor cells to be overcome. Are PTM modulated by treatments and if yes, in which way and with which drugs? The answer to this open question will also allow designing the best vaccine for each patient based on which treatment they are receiving.

Chapter 3: Role of Adiponectin Receptors Expression in Cancerogenesis

This chapter reports the results of a bio-informatic study finalized to elucidate the role of Adiponectin and its receptors in cancer development. I got interested in the role of AdipoQ, and its receptors as the association of AdipoQ levels in serum with PDAC risk is controversial (positive, negative, or no association), and PDAC patients with worse survival after treatment had a higher concentration of AdipoQ in the immune-complexome. In this work, whose manuscript is in preparation, I performed an integrative analysis of the AdipoQ receptor genes in 33 types of cancers from The Cancer Genome Atlas (TCGA). I analyzed the transcriptome of patients using hierarchical clustering analysis. I identified two groups of patients for each tumor type, one with high levels of AdipoR1/AdipoR2 and one with low levels of AdipoR1/AdipoR2. I examined the expression profiles of approximately 10,000 cancer patients representing 33 cancer types from TCGA to identify the role of AdipoR1 and AdipoR2. I found out that lung adenocarcinoma patients with higher expression of AdipoR2 are predisposed to have more aggressive tumors, which could lead to poorer survival outcomes. To the best of my knowledge, the current study is the first to find an association between the expression of AdipoR1 in thymoma. Moreover, I found that patients with lower mRNA expression of AdipoR1 have better progression-free and overall survival outcomes. Overall, this study suggests that lower expression of AdipoR1 and AdipoR2 in multiple cancers is better for the survival of patients.

Role of Adiponectin Receptors Expression in the Pan-Cancer Study

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TCGA Study Abbreviations used in this work

Study Abbreviations	Study Name
ACC	Adrenocortical carcinoma
BLCA	Bladder urothelial carcinoma
BRCA	Breast invasive carcinoma
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
CHOL	Cholangiocarcinoma
COAD	Colon adenocarcinoma
DLBC	Diffuse large B-cell lymphoma
ESCA	Esophageal carcinoma
GBM	Glioblastoma
HNSC	Head and neck squamous carcinoma
KICH	Kidney chromophobe
KIRC	Kidney renal clear cell carcinoma
KIRP	Kidney renal papillary cell carcinoma
LAML	Acute myeloid leukemia
LGG	Low-grade gliomas
LIHC	Liver hepatocellular carcinoma
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MESO	Mesothelioma
OV	Ovarian serous cystadenocarcinoma
PCPG	Pheochromocytoma and Paraganglioma
PAAD	Pancreatic adenocarcinoma
PRAD	Prostate adenocarcinoma

SARC	Sarcoma
SKCM	Skin cutaneous melanoma
STAD	Stomach adenocarcinoma
TGCT	Testicular germ cell tumors
THYM	Thymoma
THCA	Thyroid carcinoma
UCS	Uterine Carcinosarcoma
UCEC	Uterine Corpus Endometrial Carcinoma
UVM	Uveal Melanoma

Abstract

Obesity and type-2 diabetes (T2D) are two of the three modifiable risk factors for PDAC. AdipoQ is one of the most abundant adipocytokines secreted by adipose tissue. AdipoQ is emerging as a link between obesity, T2D, and obesity/T2D-related tumors. AdipoQ and its receptors (AdipoR1 and AdipoR2) role in PDAC progression is debatable. We performed an integrative analysis of the AdipoQ receptor genes in 33 types of cancers from The Cancer Genome Atlas (TCGA). The transcriptome of patients was analyzed using hierarchical clustering analysis. Statistical analyses of clinical data were performed using R and GraphPad Prism 9. The clustering analysis of transcriptome data in each tumor type identified two groups of patients: one with high levels of AdipoR1/AdipoR2 receptors and one with low levels of AdipoR1/AdipoR2. Statistical analysis revealed that tumors with high AdipoR1/AdipoR2 are more aggressive compared to the low AdipoR1/AdipoR2 patients in terms of overall cancer grading, tumor stage grading, and in some cases, the aggressive subtypes. Additionally, it was observed that cancer patients with low AdipoR1/AdipoR2 expression display a better survival rate despite cancer subtypes than those with tumors highly expressing AdipoR1/AdipoR2. Our analysis confirms the role of AdipoR1/AdipoR2 expression in cancer, pointing out that targeting AdipoQ signaling could represent a novel therapeutic strategy for PDAC and other obesity/T2D-related tumors.

Keywords: AdipoR1, AdipoR2, TCGA, Pan-Cancer, Survival

3.1 Methods

3.1.1 Data Acquisition

RNASeq data of 33 tumor types of TCGA Pan-Cancer study and clinic-pathological data were downloaded from cbioportal (https://www.cbioportal.org/). The clinic-pathological parameters included age, cancer subtypes, TNM staging, neoplasm staging, and sex of the 10,967 patients. mRNA expression of each patient was in Fragments Per Kilobase of transcript per Million (FPKM)-Z-score transformed format.

3.1.2 Clustering and Heatmaps

All the analyses were performed by R version 3.2.1. Adiponectin and its receptors in each cancer dataset were filtered with *filter* function in the *dplyr (0.7.8)* package from the mRNA FPKM Z-score transformed data files. Hierarchical clustering was performed using *Heatmap.2* function in *gplots (3.1.3)* package with the Euclidean distance and the Ward.D2 clustering method on patients in each cancer type based on the expression level of AdipoQ, AdipoR1, and AdipoR2. Heatmaps were made using the *gplots (3.1.3)* and *RColorBrewer (1.1.3)* packages in R.

3.1.3 Survival analysis

We performed Kaplan-Meier survival analysis with higher and lower expressions of AdipoR1 and AdipoR2 to find an association between their expression and patient survival. The analysis was used to plot survival curves. We employed the log-rank test to compare the survival rates between the high- and low-expression groups. We performed these analyses using GraphPad Prism 9.1.

3.1.4 Spearman correlation

Spearman correlation analysis was performed between mRNA expressions level of ADIPOR1 and ADIPOR2 in all 33 types of cancer using GraphPad Prism 9.1.

3.2 Results

3.2.1 Correlation analysis finds that two adiponectin receptors expression are correlated in DLBC

To find whether the AdipoR1 expression values are correlated with the expression values of AdipoR2 in 33 types of cancer, we performed the Spearman correlation analysis between the mRNA expression levels of AdipoR1 and AdipoR2 (Table 3-1). We retrieved from cbioportal (43) the mRNA expression FPKM Z-Score transformed data of the TCGA Pan-Cancer study Atlas (40) along with their clinical information. We observed that there was little to no correlation between the expression levels of AdipoR1 and AdipoR2. Only diffused large B cell lymphoma (DLBC) expression data was moderately correlated between AdipoR1 and AdipoR2 with a spearman r coefficient of 0.5579 (p-value <0.0001) (Figure 3-1).

Table 3-1: Spearman correlation analysis between ADIPOR1 and ADIPOR2 expressions

Type of Cancers	Spearman R coefficient	Confidence Interval	p-value
ACC	-0.05838	-0.2835 to 0.1728	0.6116
BLCA	0.1646	0.06563 to 0.2604	0.0009
BRCA	0.1852	0.1252 to 0.2438	<0.0001
CHOL	0.1021	-0.2438 to 0.4249	0.5536
GBM	0.1232	-0.03723 to 0.2774	0.1207
LGG	0.1958	0.1088 to 0.2799	<0.0001
PAAD	0.1783	0.02724 to 0.3214	0.0176
CESC	0.2172	0.1021 to 0.3266	0.0002
ESCA	0.08692	-0.06402 to 0.2340	0.2446
HNSC	0.1210	0.03236 to 0.2077	0.0060
KIRC	0.05044	-0.03911 to 0.1392	0.255
LIHC	0.08092	-0.02481 to 0.1849	0.1222

Ovary	0.02574	-0.01419 to 0.09109	0.6570
PRAD	0.2882	0.2026 to 0.3694	<0.0001
STAD	0.1485	0.04975 to 0.2443	0.0025
KICH	-0.2036	-0.4324 to 0.04972	0.1037
KIRP	0.1953	0.07711 to 0.3081	0.0010
LAML	-0.6944	-0.02206 to -0.08501	0.3640
LUAD	-0.01908	-0.01083 to 0.07042	0.6672
LUSC	0.1025	0.01081 to 0.1924	0.0242
COAD	0.03317	-0.04992 to 0.1158	0.4205
TGCT	0.1269	-0.03940 to 0.2864	0.1230
THCA	-0.07961	--0.1688 to 0.01092	0.0759
THYM	0.1289	-0.05769 to 0.3068	0.1624
UCEC	0.06724	-0.02081 to 0.1543	0.1231
UCS	0.1004	-0.1721 to 0.3587	0.4574
DLBC	0.5579	0.3175 to 0.7308	<0.0001
MESO	-0.02008	-0.1975 to 0.2357	0.8535
PCPG	-0.04923	-0.1029 to 0.1991	0.5141
SARC	0.03004	-0.09727 to 0.1564	0.6344
SKCM	0.2079	0.1142 to 0.2978	<0.0001
UVM	-0.03393	-0.2579 to 0.1935	0.7651

Yellow highlighted part in the table means statistically significant correlation value.

3.2.2 Clustering analysis classifies cancer patients into lower and higher expression group of adiponectin receptors

We used hierarchical clustering analysis to divide the patients based on their mRNA expression levels of AdipoR1 and AdipoR2. Clustering analysis of AdipoR1 and

AdipoR2, mRNA expression data, identifies two main groups of patients corresponding to High and Low AdipoR1/AdipoR2. Almost all the cancer types had a significant difference in the mRNA expression level of AdipoR1 and AdipoR2, as represented by a scatter plot in Figure 3-2 A and B, respectively.

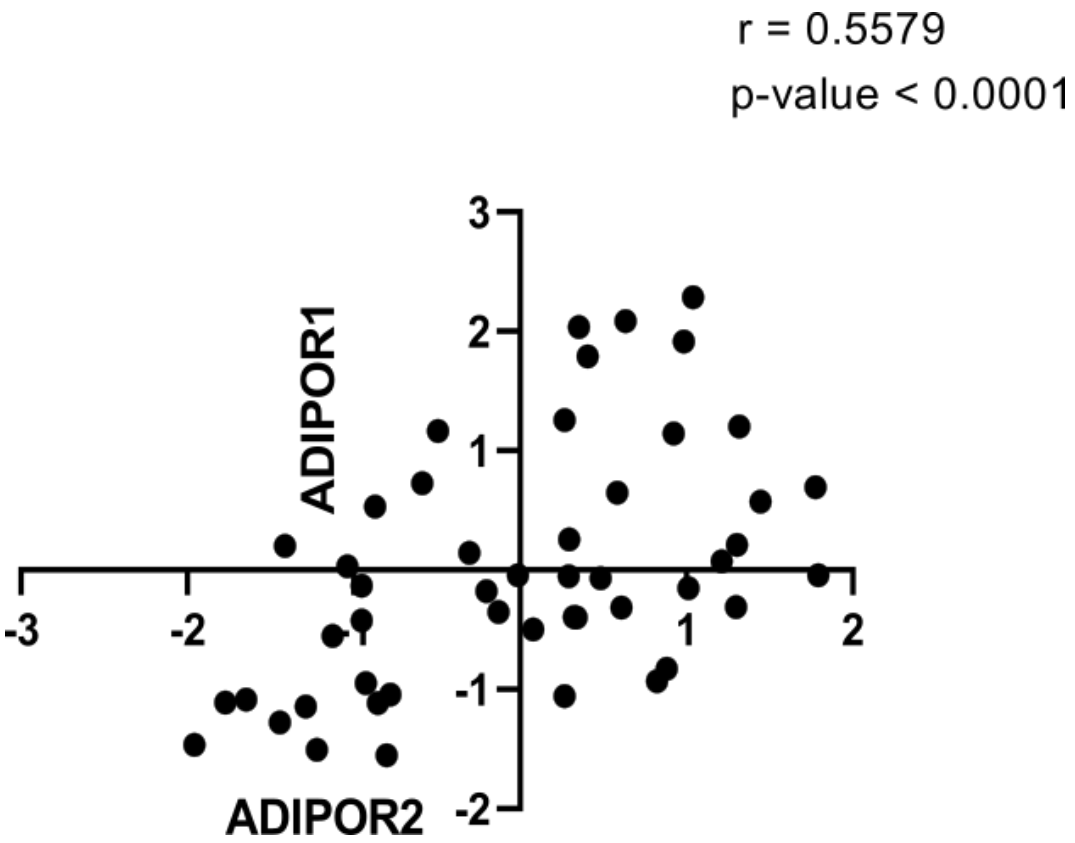

Figure 3-1: Scatter plot representing the correlation between AdipoR1 and AdipoR2 mRNA expression levels in diffused large B-cell lymphoma

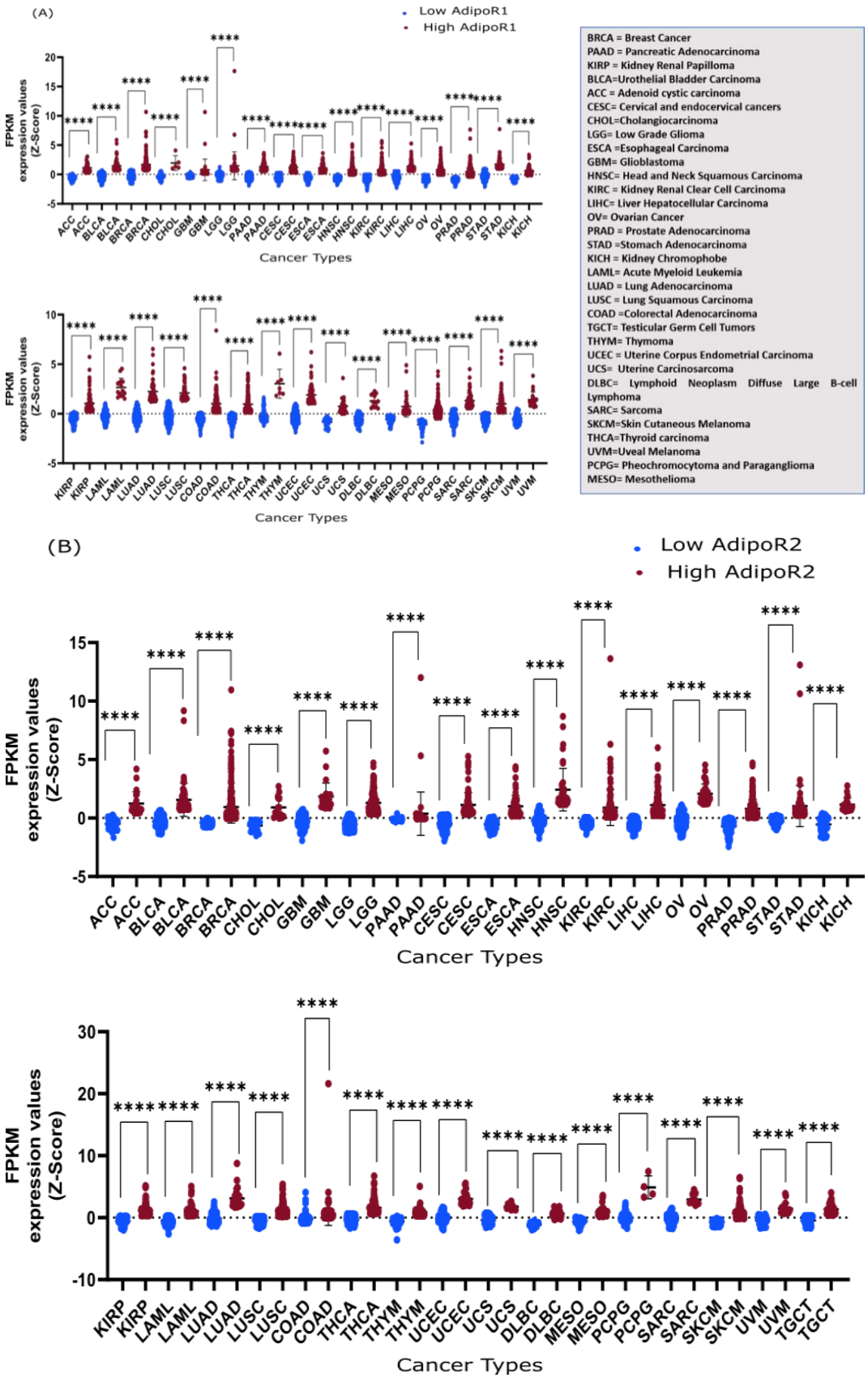

Figure 3-2: Scatter dot plot representing the hierarchical clustering analysis:
A) Hierarchical clustering analysis of AdipoR1 gene expression in 33 types of tumors; B). Hierarchical clustering analysis of AdipoR2 gene expression in 33 types of tumors Clustering analysis

significantly divided patients into high (red dots) and low (blue dots) AdipoR1/AdipoR2 expression. P-values from Wilcoxon test, ****=p<0.0001.

3.2.3 Adiponectin receptors expression profiles characterize cancer patients with their diagnosis age

We performed clinicopathological association of patients in pan-cancer studies with mRNA expression profiles of AdipoR1 and AdipoR2. Based on the hierarchical clustering of AdipoR1 and AdipoR2, the patients in each cancer group were divided into lower mRNA expression and higher mRNA expression groups of AdipoR1 and AdipoR2. As the age of diagnosis followed a normal distribution, we used the mean age of the patients for each group of mRNA expression for AdpioR1 and AdipoR2.

The mean age at diagnosis for the breast cancer (BRCA) patients belonging to the high AdipoR1 and AdipoR2 group (Figure 3-3A) is 61.15 (SD 13.98, n=158) and 58.53 (SD 13.43, n=333), respectively, whereas for the patients belonging to the low AdipoR1 and AdipoR2 groups are 57.93 (SD 12.99, n=924) and 58.34 (SD 13.08, n= 749) respectively. We found a significant difference in the mean age at diagnosis for AdipoR1 (p-value =0.007924, from the Wilcoxon test). In the case of AdipoR2, no such difference is observed (p-value =0.2838, from the Wilcoxon test).

The mean age at diagnosis for the esophagus cancer (ESCA) patients belonging to the high AdipoR1 and AdipoR2 group (Figure 3-3B) is 58.93 (SD 10.42, n=65) and 62.42 (SD 11.89, n=66), respectively, whereas for the patients belonging to the low AdipoR1 and AdipoR2 groups are 64.12 (SD 12.19, n=116) and 62.17 (SD 11.82, n= 115) respectively. We found a significant difference in the mean age at diagnosis for AdipoR1 (p-value =0.0099, from the Wilcoxon test). In the case of AdipoR2, no such difference is observed (p-value =0.90, from the Wilcoxon test).

The mean age at diagnosis for colorectal cancer (COAD) patients belonging to the high AdipoR1 and AdipoR2 group (Figure 3-3C) is 67.81 (SD 10.42, n=216) and 66.73 (SD 12.53, n=270), respectively, whereas for the patients belonging to the low AdipoR1 and AdipoR2 groups are 65.2 (SD 13.02, n=374) and 65.6 (SD 13.06, n= 320) respectively. In the colorectal cancer patients, we found a significant difference in the mean age at diagnosis for AdipoR1 (p-value =0.028, from the Wilcoxon test).

In the case of AdipoR2, no such difference is observed (p-value =0.29, from the Wilcoxon test).

The mean age at diagnosis for the head and neck cancer (HNSC) patients belonging to the high AdipoR1 and AdipoR2 group (Figure 3-3D) are 61.71 (SD 12.53, n=221) and 64.04 (SD 11.41, n=41), respectively, whereas for the patients belonging to the low AdipoR1 and AdipoR2 groups are 60.1 (SD 11.01, n=293) and 60.5 (SD 11.7, n= 473) respectively. For head and neck cancer patients, a significant difference was observed in the mean age at diagnosis for AdipoR1 (p-value =0.02, from Wilcoxon test) in the case of AdipoR2; no such distinction is observed (p-value =0.101, from Wilcoxon test).

The mean age at diagnosis (Figure 3-3E) for the low-grade-glioma (LGG) patients belonging to the high AdipoR1 and AdipoR2 groups is 47.07 (SD 16.18, n=56) years and 40.96 (SD 11.65, n=144) years respectively, whereas for the patients belonging to the low AdipoR1 and AdipoR2 groups are 42.38 (SD 12.87, n=458) years and 43.67 (SD 14.07, n= 370) years respectively. No significant difference was observed in the mean age in LGG patients at diagnosis for AdipoR2 (p-value =0.0966, from Wilcoxon test). Still, a significant difference was observed in the case of AdipoR1 (p-value =0.0343, from the Wilcoxon test).

The mean age at diagnosis (Figure 3-3F) for testis cancer (TGCT) patients belonging to the high AdipoR1 and AdipoR2 groups are 28.8 (SD 8.2, n=22) years and 34.08 (SD 11.73, n=36) years, respectively, whereas for the patients belonging to the low AdipoR1 and AdipoR2 groups are 32.58 (SD 9.2, n=111) years and 31.08 (SD 8.07, n= 96) years respectively. We found a significant difference in the mean age at diagnosis for AdipoR1 (p-value =0.049, from the Wilcoxon test), but no such difference was observed in the case of AdipoR2 (p-value =0.466, from the Wilcoxon test).

These results show that patients with lower mRNA expression of AdipoR1 in BRCA, LGG, COAD, and HNSC are diagnosed at an early age, whereas patients with lower mRNA expression of AdipoR1 in ESCA and TGCT are diagnosed at a later age.

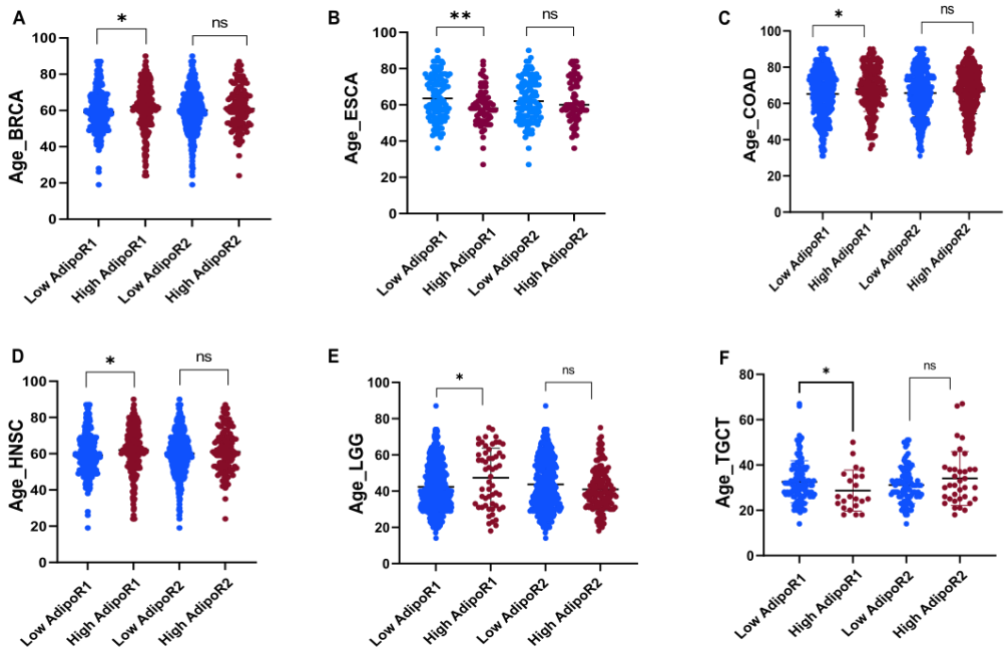

Figure 3-3: Diagnosis Age of patients in various cancers

A) BRCA patients' diagnosis age to low and high mRNA expression of AdipoR1 and AdipoR2 B) ESCA patients' diagnosis age to low and high mRNA expression of AdipoR1 and AdipoR2 C) COAD patients diagnosis age with respect low and high mRNA expression of AdipoR1 and AdipoR2 D) HNSC patients diagnosis age with respect low and high mRNA expression of AdipoR1 and AdipoR2 E) LGG patients diagnosis age with respect low and high mRNA expression of AdipoR1 and AdipoR2 F) TGCT patients diagnosis age with respect low and high mRNA expression of AdipoR1 and AdipoR2

3.2.4 Classification of cancer subtypes with expression profiles of adiponectin receptors

Knowing the subtype of cancer is essential to planning treatment and determining prognosis. Cancer subtypes describe the smaller groups that a type of cancer can be divided into based on specific characteristics of the cancer cells. These characteristics include how cancer cells look under a microscope. To understand how the mRNA expression of AdipoR1/R2 is distributed among the patients of various cancer subtypes, we performed a chi-square test on patients with higher and lower mRNA expression of AdipoR1 and AdipoR2.

We observed that patients with lower mRNA expression of AdipoR1 or AdipoR2 had a higher distribution of cancer subtypes which are worse in prognosis and survival. In BRCA patients, triple negative subtype or basal (BRCA_Basal) was significantly more in patients with lower mRNA expression of AdipoR1 (17.42% of patients vs. 6% of patients with higher expression of AdipoR1) or AdipoR2 (19% of patients vs. 7.8% of patients with higher expression of AdipoR2) (Figure 3-4A).

Similarly, in ESCA patients with chromosomal instability (ESAC_CIN), significantly more in lower mRNA expression profiles of AdipoR1 (54% of patients vs. 17% of patients with higher expression of AdipoR1) (Figure 3-4B).

In the TCGA, sarcoma cancer (SARC) patients are divided according to their complex karyotypes: (1) dedifferentiated liposarcoma (DDLPS), an undifferentiated sarcoma usually arising in association with well-differentiated liposarcoma and characterized by 12q13~15 amplification; (2) Leiomyosarcoma (LMS), showing smooth muscle differentiation, arising in both gynecologic (ULMS) and soft tissue (STLMS) sites; (3) undifferentiated pleomorphic sarcoma (UPS), lacking any defined line of differentiation and myxofibrosarcoma (MFS), showing fibroblastic differentiation with myxoid stroma; (4) and the rest kind of alterations are combined in Other sections which include malignant peripheral nerve sheath tumor (MPNST), arising in peripheral nerves and simple-karyotype sarcoma, synovial sarcoma (SS), defined by the translocation t(X;18)(p11;q11) (Abeshouse et al., 2017). Our analysis of the expression profile of AdipoR1 and AdipoR2 mRNA revealed that SARC patients with higher mRNA expression of AdipoR2 had a significantly higher fraction of MFS/UPS subtypes. Whereas, in the case of AdipoR1 expression, patients had a substantially higher fraction of the LMS subtype with their higher expression (Figure 3-4C).

LGG is divided into subtypes based on whether or not there is a mutation in isocitrate dehydrogenase (IDH) (Aliotta et al., 2019). We observed that patients with lower mRNA expression of AdipoR1 had a higher percentage of patients (84% vs. 60% of patients with higher expression of AdipoR1) with IDH mutation subtype. In contrast, patients with higher mRNA expression of AdipoR2 had a significantly large proportion of IDH mutations (92% vs. 75% of patients with lower expression of AdipoR2) (Figure 3-4D).

The TCGA classified stomach cancer (STAD) into six subtypes using genomic and molecular platforms, including genome/exome/methylome DNA sequencing, RNA sequencing, protein arrays, and sophisticated statistical and informatics analyses of data from 295 tumors (Zhang, 2014). The subtypes were named Epstein-Barr virus (EBV)-positive tumors, microsatellite instable (MSI) tumors, genomically stable (GS) tumors, and tumors with chromosomal instability (CIN) (Zhang, 2014). We

observed that STAD patients with CIN subtypes had a significantly higher percentage of higher mRNA expression of AdipoR1 (63% vs. 52% for patients with lower mRNA expression of AdipoR1) or AdipoR2 (72% vs. 49% for patients with lower mRNA expression of AdipoR2).In the case of a more aggressive subtype of STAD, GS subtypes were found with lower mRNA expression of AdipoR1 (13% vs. 5% for patients with higher mRNA expression of AdipoR1) or AdipoR2 (14% vs. 5.5% for patients with higher mRNA expression of AdipoR2) (Figure 3-4E).

Based on genomic characteristics of endometrial cancer (UCEC), TCGA similarly classified it into four subtypes, namely POLE hyper-mutation, high-mutation microsatellite instability (MSI), and high-copy number type (such as p53 gene mutation), and low-copy number type (Y. Hu et al., 2021). With our analysis, we noted that higher mRNA expression of AdipoR2 had a significantly more fraction of UCEC patients (48%) with high copy numbers compared to a group with lower mRNA expression of AdipoR2 (19%). Whereas in the AdipoR1 expression profile, the order was reversed for copy number high subtypes, we found that lower mRNA expression of AdipoR1 (33.5%) had a significantly higher fraction of patients compared to higher mRNA expression of AdipoR1 (15%) (Figure 3-4F).

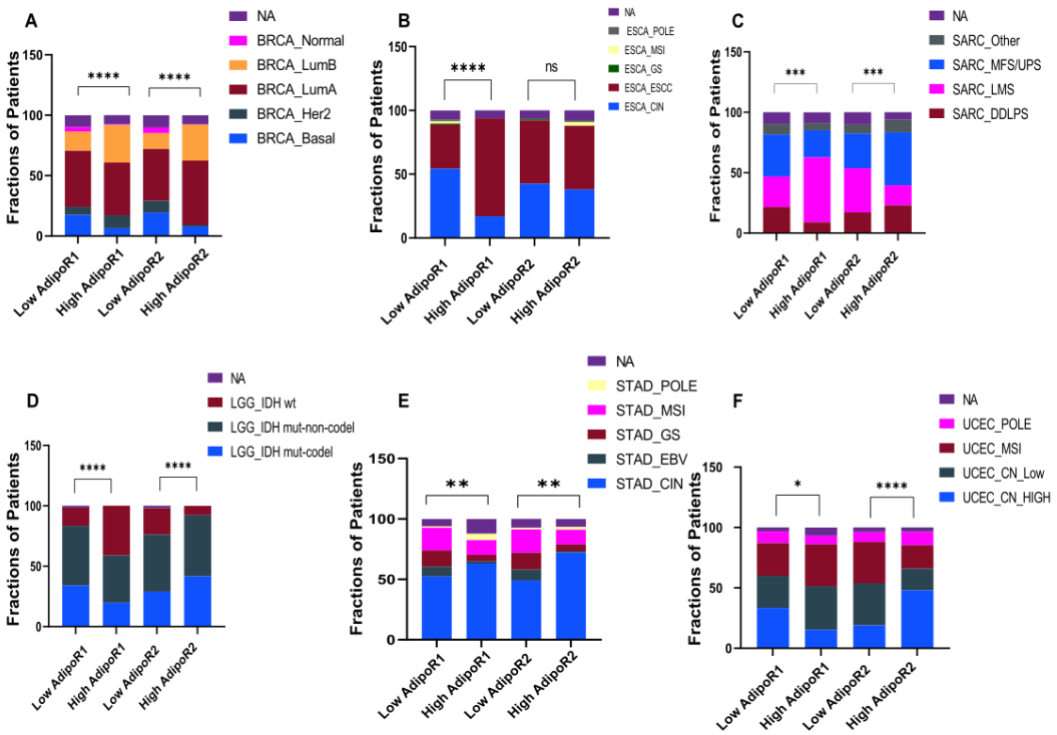

Figure 3-4 Subtypes of cancers

A)BRCA subtypes in patients with low and high mRNA expression of AdipoR1 and AdipoR2 B) ESCA subtypes in patients with low and high mRNA expression of AdipoR1 and AdipoR2 C) SARC subtypes in patients with respect low and high mRNA expression of AdipoR1 and AdipoR2 D) LGG cancer subtypes in patients with respect low and high mRNA expression of AdipoR1 and AdipoR2 E) STAD subtypes in patients with respect low and high mRNA expression of AdipoR1 and AdipoR2 F) UCEC subtypes in patients with respect low and high mRNA expression of AdipoR1 and AdipoR2

3.2.5 Lower expression of adiponectin receptors is better for the least aggressive tumor

To better understand the clinical behavior of cancer malignancies, a staging system is used to classify every tumor. American Joint Committee on Cancer developed a standard staging system (AJCC) called TNM staging. In the TNM staging system, cancer is classified based on the dimensions of the primary tumor, the presence and extent of regional lymph node metastases, and the presence and absence of distant metastases (Hortobagyi et al., 2018).

The TNM system provides clinical significance by establishing the anatomic extent of the disease. Combining all three factors can help to describe the overall stage of the tumor with stages from I-IV, with stage I being the least aggressive stage, whereas stage IV is the most severe stage (Rosen & Sapra, 2021). The detail of each stage is described in Table 3.

Table 3-2: Description of each stage derived from TNM staging (Adapted from (Rosen & Sapra, 2021))

Stage	Description
Stage 0	Indicates carcinoma in situ. Tis, N0, M0
Stage I	Localized cancer. T1-T2, N0, M0
Stage II	Locally advanced cancer, early stages. T2-T4, N0, M0
Stage III	Locally advanced cancer, late stages. T1-T4, N1-N3, M0
Stage IV	Metastatic cancer. T1-T4, N1-N3, M1

To get an in-depth understanding of how the mRNA expression profile of AdipoR1 and AdipoR2 is distributed among patient tumor staging. We observed that patients with lower mRNA expression of AdipoR1 have a significantly higher percentage of smaller tumor size (less than 5 cm, more T1 and T2) in kidney renal papilloma (KIRP) (85% of patients vs. 61% of patients with higher mRNA expression of

AdipoR1) (Figure 3-5A). In STAD, we observed that patients with higher mRNA expression of AdipoR1 had a significantly lower fraction of patients with more than 7cm (T4) size of tumor (12% of patients vs. 30% of patients with lower mRNA expression of AdipoR1) (Figure 3-5B). In COAD, we observed that patients with tumor size more than 7cm (T4) were significantly less in a group with lower mRNA expression of AdipoR1 (8% vs. 16% of patients with higher mRNA expression of AdipoR1) (Figure 3-5C). In lung adenocarcinoma (LUAD), the distribution of patients with more than 5 cm tumor (T3 and T4) was found to be significantly more in a group with higher mRNA expression of AdipoR1 (24% vs. 11.5% of patients with lower mRNA expression of AdipoR1). A similar observation was made in patients with higher expression of AdipoR2 (16% vs. 10% of patients with lower mRNA expression of AdipoR2) (Figure 3-5D).

We observed similar results in clinical neoplasm staging. Patients with lower mRNA expression of AdipoR1 have a significantly higher percentage of less aggressive tumors (Stage I and Stage II) in KIRP (75% of patients vs. 53% of patients with higher mRNA expression of AdipoR1) (Figure 3-6A). We observed that patients with higher mRNA expression of AdipoR1 significantly lower fraction of STAD patients with more aggressive tumors (stage III and Stage IV) (35% of patients vs. 55% of patients with lower mRNA expression of AdipoR1) (Figure 3-6B). Our analysis found that COAD patients with the most severe stage of the tumor (Stage IV) were significantly less in a group with lower mRNA expression of AdipoR1 (10% vs. 20% of patients with higher mRNA expression of AdipoR1) (Figure 3-6C). In LUAD, the distribution of patients with more severe stages of tumor (Stage III and Stage IV) was found to be significantly more in a group with higher mRNA expression of AdipoR2 (26% vs. 18% of patients with lower mRNA expression of AdipoR2) (Figure 3-6D).

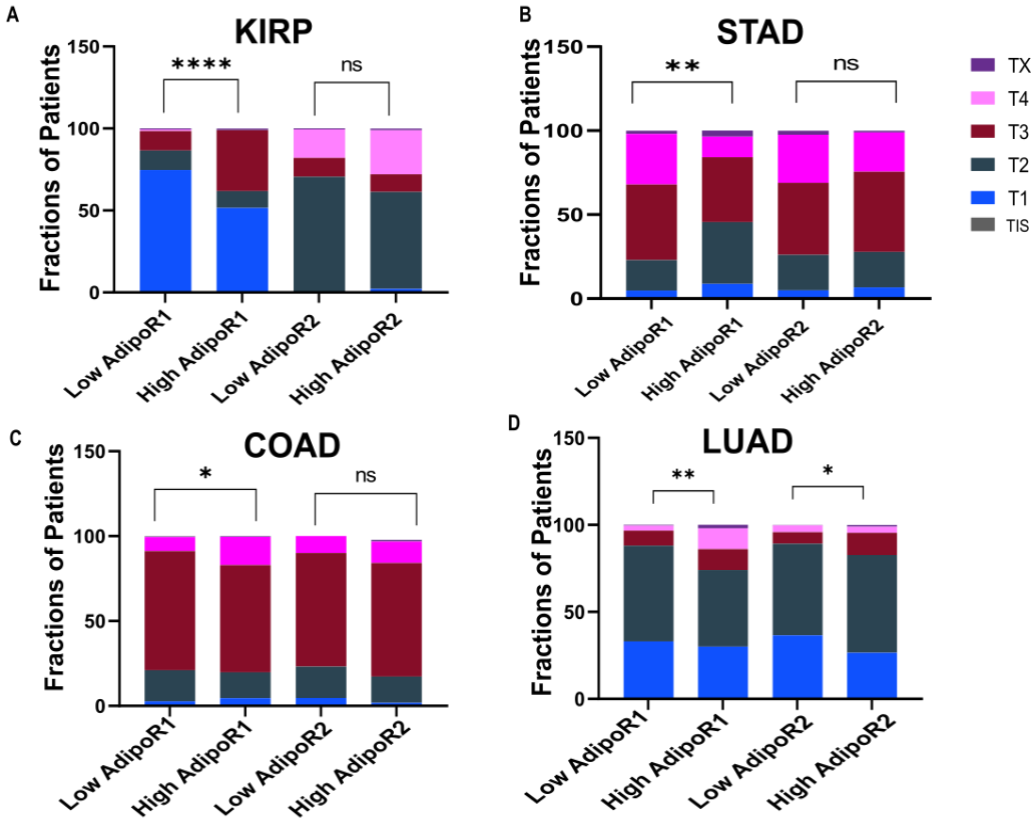

Figure 3-5: Tumor staging of cancers

A) Proportional bar plots showing tumor staging of KIRP patients with respect to mRNA expression of AdipoR1 and AdipoR2 group B) Proportional bar plots showing tumor staging of STAD patients with respect to mRNA expression of AdipoR1 and AdipoR2 group C) Proportional bar plots showing tumor staging of COAD patients with respect mRNA expression of AdipoR1 and AdipoR2 group D) Proportional bar plots showing tumor staging of LUAD patients with respect mRNA expression of AdipoR1 and AdipoR2 group

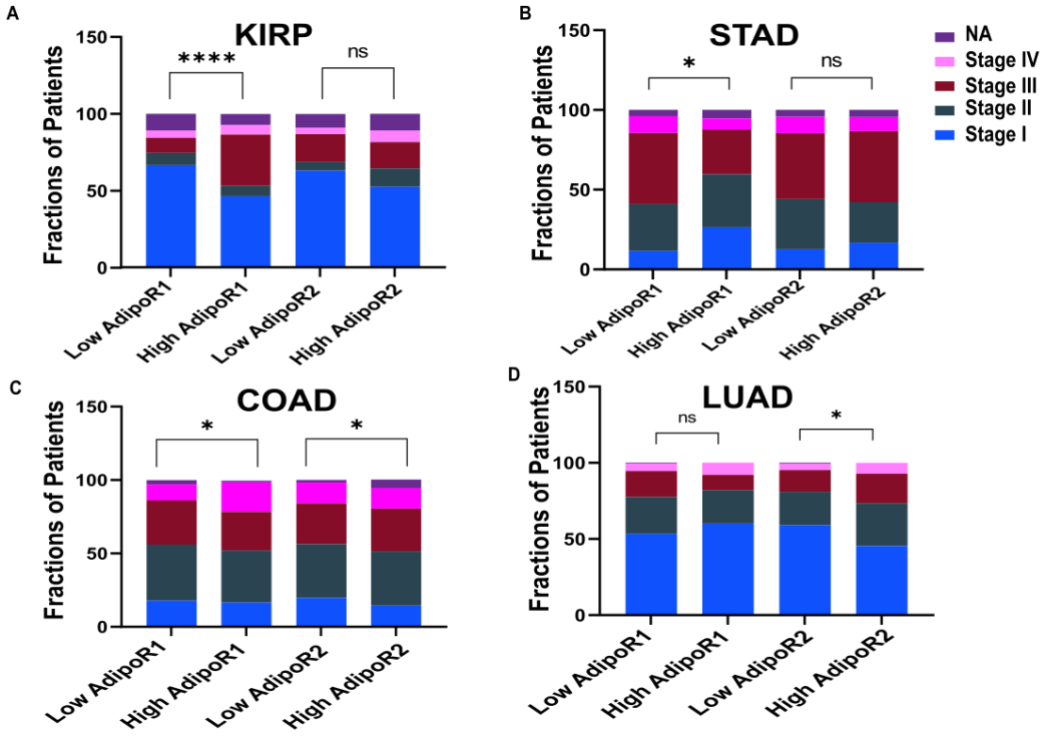

Figure 3-6: Neoplasm staging in cancer

A) Proportional bar plots showing neoplasm staging of KIRP patients with respect to mRNA expression of AdipoR1 and AdipoR2 group B) Proportional bar plots showing neoplasm staging of STAD patients with respect to mRNA expression of AdipoR1 and AdipoR2 group C) Proportional bar plots showing neoplasm staging of COAD patients with respect mRNA expression of AdipoR1 and AdipoR2 group D) Proportional bar plots showing neoplasm staging of LUAD patients with respect mRNA expression of AdipoR1 and AdipoR2 group

3.2.6 Lower expression of adiponectin receptors are better for survival in four cancer types

The overall objective of the current study was to find whether the expression of AdipoR1 and AdipoR2 had any role in the patient's survival. Our survival analysis revealed that mRNA expression of AdipoR1 or AdipoR2 played a significant role in progression-free (PFS) and overall survival (OS) of four cancer types.

A lower expression profile of AdipoR1 was associated with better survival (PFS and OS) in patients with KIRP, LGG, and thymoma (THYM). In the case of KIRP and THYM patients, the median survival time for PFS and OS was statistically undefined, as more than 50% of patients were alive at the end of the study. The median survival months with higher mRNA expression of AdipoR1 in KIRP patients for PFS and OS were 106 and 96 months, respectively (Figure 3-7A and Figure

3-8A). Our analysis revealed that the median survival months of LGG patients with lower mRNA expression of AdipoR1 in PFS and OS were 45 and 93 months, respectively. At the same time, the median survival time for patients with lower expression for PFS and OS was 19 and 63 months, respectively (Figure 3-7B and Figure 3-8B). Additionally, the median survival months for THYM patients with higher mRNA expression of AdipoR1 for OS was 114 months, whereas for PFS, it was statistically undefined (Figure 3-7C and Figure 3-8C).

We observed that lower expression of AdipoR2 was significantly correlated with better survival in LUAD patients. The median survival months of LUAD patients with lower mRNA expression of AdipoR2 in PFS and OS were 45 and 59 months, respectively. In comparison, the median survival time for patients with higher mRNA expression of AdipoR2 for PFS and OS was 25 and 34 months, respectively (Figure 3-7D and Figure 3-8D).

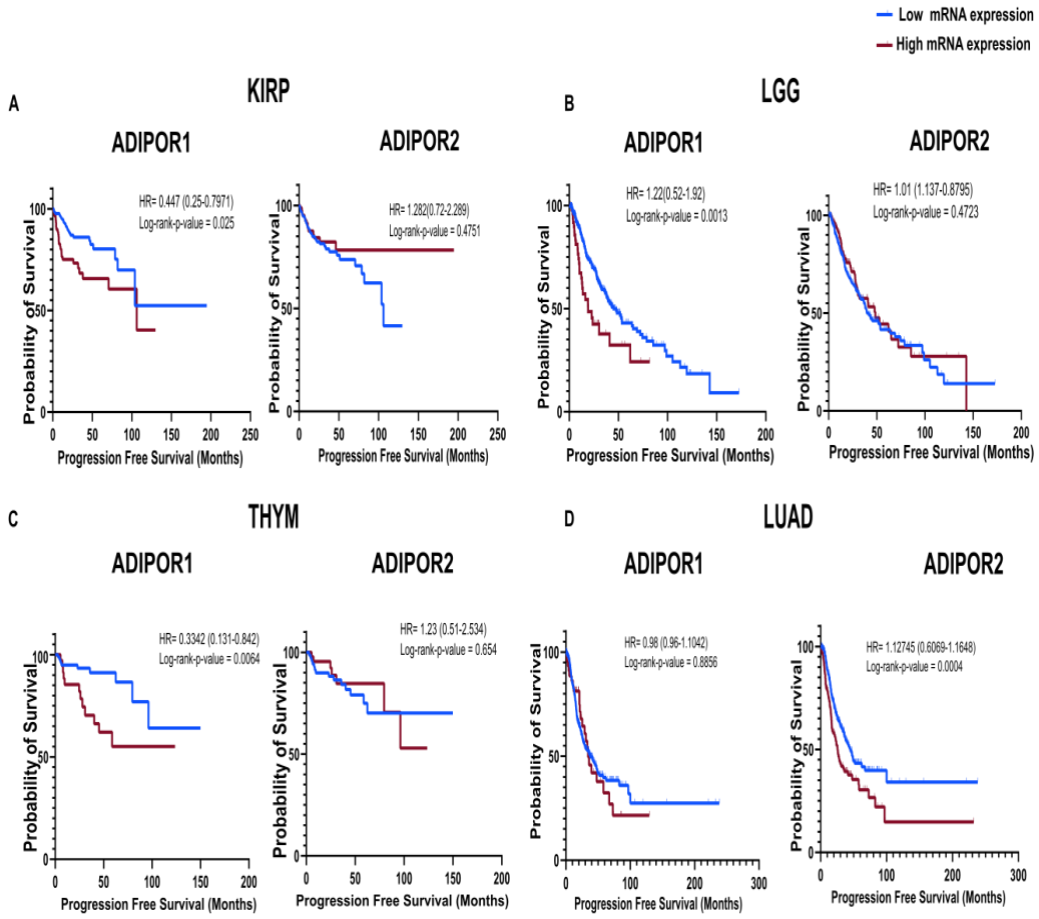

Figure 3-7: Progression-Free Survival in cancer patients

A) Survival curve showing KIRP progression-free survival months for Low and High AdipoR1/AdipoR2 B) Survival curve showing LGG progression-free survival months for Low and High AdipoR1/AdipoR2 C) Survival curve showing THYM progression-free survival months for Low and High AdipoR1/AdipoR2 D) Survival curve showing LUAD progression-free survival months for Low and High AdipoR1/AdipoR2

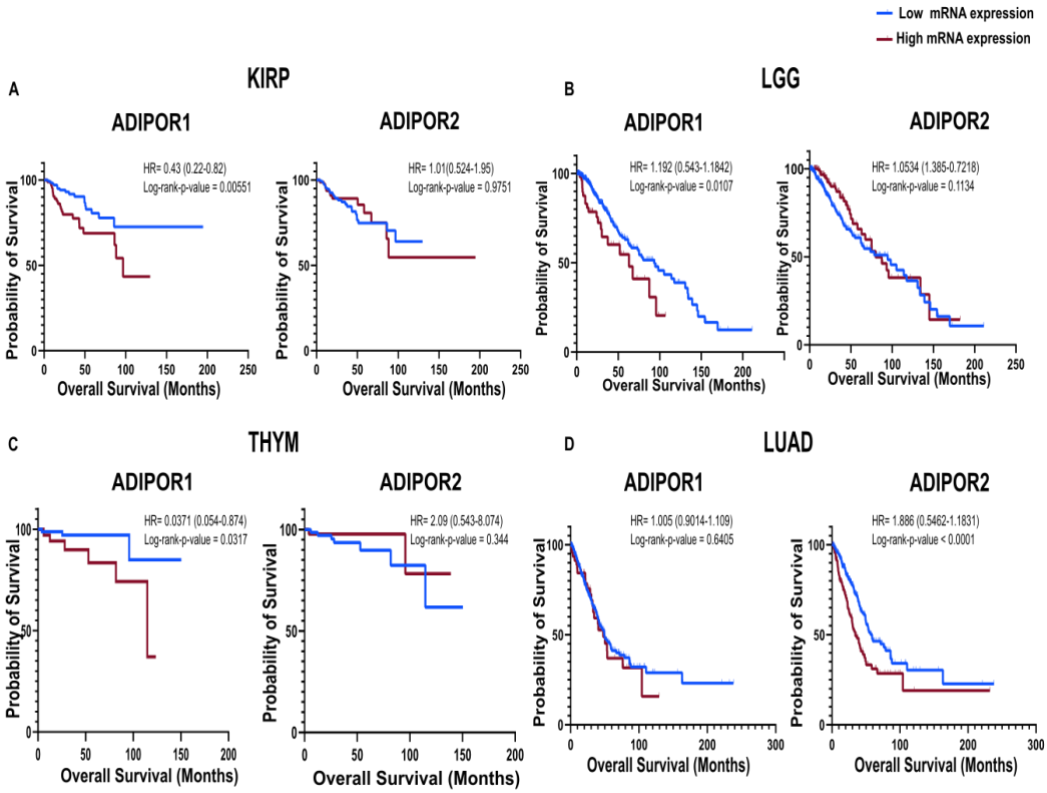

Figure 3-8: Overall survival of patients

A) Survival curve showing KIRP overall survival months for Low and High AdipoR1/AdipoR2 B) Survival curve showing LGG overall survival months for Low and High AdipoR1/AdipoR2 C) Survival curve showing THYM overall survival months for Low and High AdipoR1/AdipoR2 D) Survival curve showing LUAD overall survival months for Low and High AdipoR1/AdipoR2

3.3 Discussion

In the present study, we used more than 10,000 cancer patients' expression profiles in 33 types from TCGA pan-cancer analysis to understand the role of AdipoR1 and AdipoR2. We performed statistical analysis on clinical parameters in 33 types of cancers. We used hierarchical clustering to classify the mRNA expression of AdipoR1 and AdipoR2 in patients as higher and lower expression groups.

Lung cancer has been associated with obesity, and a report found higher expression of AdipoR1 in serum levels of patients with lung cancer compared to regular healthy

patients (Nigro et al., 2020). Our analysis shows the presence of more aggressive tumors for lung adenocarcinoma patients expressing higher AdipoR2. In simpler words, it could lead to lesser survival, and in the analysis, we find the same, that patients with higher mRNA expression of AdipoR2 had worse progression-free and overall survival.

Even though there are no studies on the role of AdipoR1 and AdipoR2 in thymoma, our analysis shows that patients with lower mRNA expression of AdipoR1 are better for progression-free and overall survival. The new findings could be used to establish the role of AdipoQ and its receptors in thymoma.

Kidney cancer has been associated with obesity (C. J. Lin et al., 2020). Lower AdipoQ concentration was reported in renal cell carcinoma patients compared to those without disease in a meta-analysis of ten published studies and could be used as biomarkers (X. Liu et al., 2018). There is no study investigating kidney renal papilloma. Our analysis found that patients with lower mRNA expression of AdipoR1 had a significantly higher fraction of less aggressive and smaller tumor sizes. The lesser aggressive tumor meant that patients had better survival; in both progression and overall survival studies, more than 50% of patients were alive at the end of the investigations.

Even though obesity is not correlated as a causative factor in LGG, expression of AdipoQ, AdipopR1, and AdipoR2 were reported in all the cell lines of gliomas at the mRNA and protein level (Porcile et al., 2014). It was also observed that AdipoQ expression was acting as a negative regulator for the gliomas (Porcile et al., 2014). Our analysis also shows that lower expression of AdipoR1 is better for the overall and progression-free survival of low-grade glioma. Further investigation is required to establish the role of AdipoQ and its receptor in LGG.

Overall, our pan-cancer analysis of AdipoR1 and AdipoR2 shows that lower mRNA expression is better for patients. These preliminary data need to be further investigated to understand the role of AdipoR1 and AdipoR2 in obesity-associated cancer.

Chapter 4: Transcriptional landscape of adiponectin and its receptors in pancreatic cancers

Taking advantage of the data shown in Chapter 3 on the role of AdipoR1 and AdipoR2 in the survival of patients with multiple cancer. In collaboration with the group of Prof. Rudolf Fehrmann at the Department of Medical Oncology, University Medical Center Groningen, Groningen, the Netherlands, where I spent more than six months of work. The stay at Fehrmann Lab allowed me to dive deep into understanding the role of AdipoQ and its receptors in PC. 184 transcriptomic data of PC patients were downloaded from TCGA, and the gene expression profiles of PC were disentangled with c-ICA. The study found ten transcriptional components where AdipoQ or its receptors were outliers after applying c-ICA. I performed further analysis on these AdipoQ-TCs with gene set enrichment analysis and co-functionality network analysis. With these biological process analyses, I observed that AdipoQ-TCs were active in innate immune pathways, DNA repair, lipid metabolism, and organ development. I employed random forest survival analysis and found that five of the AdipoQ-TCs played an active role in the overall survival of the patients. This analysis, which represents the basis of a manuscript that is under preparation for submission, revealed that a potential linkage between AdipoR1 and kallikrein (KLK) enzyme family expression exists. As one of the AdipoQ-TCs with AdipoR1 as an outlier, four KLKs genes upregulated. Further analysis is warranted to understand this mechanism.

Transcriptional landscape of adiponectin and its receptors in pancreatic cancers

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Abstract

Adiponectin is produced by adipose tissue that has been found to play a role in the development and progression of pancreatic cancer. So far research on the role of adiponectin in pancreatic cancer remains controversial. The objective of this chapter is to elucidate the role of adiponectin and its receptor in pancreatic cancer using consensus-independent component analysis (c-ICA). This work included 184 transcriptome profiles of pancreatic cancer patients downloaded from the TCGA. c-ICA disentangled transcriptome profiles into statistically independent transcriptional components. We found ten TCs where adiponectin or its receptors were overexpressed. Our random forest survival analysis resulted in five TCs found to be significant classifiers for the overall survival of the patients.

4.1 Methods

4.1.1 Data Acquisition

We downloaded the pre-processed and RNAseq with Expectation Maximization (RSEM) normalized gene expression profiles of PAncreatic ADenocarcinoma (PAAD) patients from TCGA using the Broad GDAC Firehose portal (https://gdac.broadinstitute.org/)). We standardized gene expression profiles to a mean of zero and a variance of one on the gene level across all the patients with PAAD. This transformed gene expression dataset is referred to as the PAAD-TCGA data set throughout the manuscript. Clinicopathological information of the patients from the PAAD-TCGA dataset (Hoadley et al., 2018) was downloaded using the cbioportal website (https://www.cbioportal.org/study/clinicalData?id=paad_tcga_pan_can_atlas_2018) (cbioportal, n.d.; Cerami et al., 2017; Gao et al., 2013). Clinicopathological information of the patients included neoplasm staging, tumor staging in TNM (Tumor size, degree of spread to regional lymph Nodes, presence of Metastasis), age at the time of diagnosis, tumor subtypes, number of mutations counts, and tumor subtype the clinicopathological dataset is referred to as the PAAD-clinical dataset.

4.1.2 Consensus Independent Component Analysis

We used consensus-independent component analysis (c-ICA) to segregate the average gene expression profiles of complex biopsies into statistically independent

transcriptomic footprints. The PAAD-TCGA dataset containing standardized gene expression profile of *n* genes and *s* samples was pre-processed using whitening transformation, making all profiles uncorrelated and giving them a variance of one. We performed ICA on the whitened dataset using the in-house *AnalyzerTool* developed by the Fehrmaan group version 6, based on the FastICA algorithm. c-ICA resulted in the extraction of *i* independent components (hereafter estimated sources (ES)), a matrix of *n* i* dimension, and a mixing matrix of *i*s* dimension. The number of principal components capturing 100% explained variance in the whitened dataset were chosen as the number of ESs to extract. Each ES contains separate weights of all genes. This weight represents the direction and magnitude of the influence of an underlying transcriptional regulatory process on the expression level of that gene. The mixing matrix contains the coefficients of ES in each sample, representing the activity of an ES in the corresponding sample.

In the ICA, an initial random weight vector with a variance of 1 must be chosen to obtain statistically independent ES. Hence, varying initial random weight vectors could result in different sets of Ess. To obtain a global solution, we performed 250 ICA runs with different randomly initialized weight vectors. ESs extracted from different runs were clustered together if the absolute value of the Pearson correlation between them was > 0.9. Representative ESs were selected based on distance correlation with all other cluster members which are maximally correlated to each other. To further remove ES's with dependency on any other ESs, "xi" correlation was performed between the representative ES's (S. Chatterjee, 2021). Representative ESs with an xi-correlation coefficient value less than 0.1 were selected as statistically independent ES, and representative ESs with an xi-correlation coefficient between 0.1 and 0.5 were removed from the analysis. For representative ESs with an xi-correlation coefficient value of more than 0.5, the ESs with maximum correlation with other representative ESs was selected. Next, we calculated a credibility index (CI) for each representative ES by determining the proportion of ICA runs the ESs appeared as a solution. We used a cutoff of CI more than 10% of ICA runs. Consensus ESs were selected when representative ESs appeared in more than cutoff value of run. Consensus ESs will be referred as Transcriptional Components (TC) in the manuscript. These TCs were used for further analysis. After applying the consensus algorithm, we obtained a TC matrix of the j*n dimension with j

transcriptional components and the n number of genes. TCs, in combination with the original input expression profiles, were used to obtain the consensus mixing matrix (MM).

4.1.3 Identification of transcriptional components with high absolute weights of AdipoQ and its receptors

To better understand the role played by AdipoQ and its receptor in PAAD, we identified the TCs with high absolute weights of AdipoQ and its receptors. We used an absolute cutoff weight of 3 to determine the TC where AdipoQ or its receptors are outliers. We called this new matrix AdipoQ-TCs.

4.1.4 Gene Set Enrichment Analysis

AdipoQ plays a vital role in metabolism via various signaling pathways like AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptors (PPARs) signaling pathways (Yamauchi et al., 2014). We performed gene set enrichment analysis (GSEA) to investigate the enrichment of different biological processes in AdipoQ-TCs. We used gene sets from the MsigDB for GSEA analysis (Carbon et al., 2021; De Preter et al., 2008; Gillespie et al., 2022; Kanehisa et al., 2021; Köhler et al., 2021; Liberzon et al., 2011, 2015; Martens et al., 2021; Schaefer et al., 2009; Segal et al., 2003; Subramanian et al., 2005; X. Wang, 2008; Wong & Wang, 2015). Gene sets containing less than ten genes or more than 500 genes (after filtering out genes that were not present in our data sets) were excluded from further analysis. Enrichment of each gene set against TC was tested using the Fisher exact test. The Fisher exact test checks whether the two variables are independent. We defined above 3 as high weight, below -3 as low weight, and values between 3 and -3 as the intermediate weight for Fisher's exact test. The enrichment score was calculated as a Z-score obtained by transforming the p-value of Fisher's exact test. As we performed multiple Fisher exact tests, it would have introduced multiple-testing errors. To correct the multiple-testing error, we performed a Bonferroni correction. Bonferroni corrected p-value (α) was calculated by dividing the p-value of 0.05 by the number of tests performed. The α was then converted to a Z-score value. We showed the enriched gene sets in each AdipoQ-TCs by heatmaps. We generated heatmaps using the *ComplexHeatmap package* (v2.10.0) in R with

hierarchical clustering on the enriched gene sets with *spearman correlation* distance method and *hclust* method.

4.1.5 Identifying biology of AdipoQ-TCs outliers

We identified the biological processes associated with the AdipoQ-TCs using multiple methods. Firstly, we performed GSEA on each TC of AdipoQ-TCs separately using MsigDB gene set collections (explained in detail above). Secondly, we constructed separate co-functionality networks using GenetICA network on top 250 genes with positive values and bottom genes with negative values from each of the AdipoQ-TCs (available at this site) (Urzúa-Traslaviña et al., 2021). The enrichment of predicted functionality was calculated for clusters containing ≥ five genes and subsequently used to identify biological processes associated with the TC under investigation.

4.1.6 Survival Analysis

To determine the association between the AdipoQ-TC activity and OS, we performed a univariate Cox proportional hazard regression analysis using the patients with clinicopathological information (n= 184, Table 1). We performed the Cox proportional hazard regression model using *survminer* (v0.4.9) and *survival (*v3.3.1) packages in R.

4.1.6.1 ***Survival Tree Analysis***

Survival tree analysis was performed for PAAD patients using activities of TCs from AdipoQ-TCs to identify significant classifiers. We used PAAD-Survival-TCs as an input matrix for survival tree analysis.

We conducted a recursive process, samples were divided into two groups with a threshold of activities of TC, and a log-rank test was applied to each TC activities with survival information. The log-rank test would assign a p-value for each patient's activity score of a TC column. This step will be repeated for each TC column. A tree would be formed based on the p-values of TC column. TC whose activity score would be lowest among other will come on the top of the tree. For each resulting subset, this recursive step was repeated. This process stopped if the number of patients in the two subsets dropped below 50, one subset dropped below 17, or the

number of events for both subsets was < 25. We obtained survival probabilities for both subsets.

We used *MST (v2.2)* package in R to create a multi-survival tree. For splitting the tree with correlation, we used the "independence model" method. The independence model ignores dependence and uses log-rank statistics as the splitting rule. The classifier in our analysis is a numeric value, and we obtained a cutoff for each activity score of classifiers. To assess the goodness of fit survival curve, we created a new variable survival cohort to store the terminal node number in the survival tree of each sample using *partykit* (*v 1.2.15*) package in R.

4.2 Results

4.2.1 Data set containing 184 pancreatic cancer tissue samples

We collected gene expression profile for 184 patient samples from TCGA-Broad-GDAC-Firehose. Of these PAAD, major tumor types included pancreatic ductal adenocarcinoma (PDAC, n=153), mucinous non-cystic PAAD (n=4), and 25 representing other subtypes of PAAD. Patient median diagnosis age was 65 years (range 57-73). 83%of patients were diagnosed with clinical stage II. We retrieved follow-up and survival data for all patients (Table 4-1).

Table 4-1: Patient Characteristics

Clinico-pathological features	**Patients (184)**
Median Age at diagnosis	65 (range 57-73)
Male	101 (55%)
Female	83 (45%)
Stage I	22 (12%)
Stage II	152 (83%)
Stage III	6 (3%)

Stage IV	6 (3%)
PDAC	153 (83.15%)
PAAD, other subtypes	31 (16.85%)
Alive	85 (46%)
Dead	99 (54%)

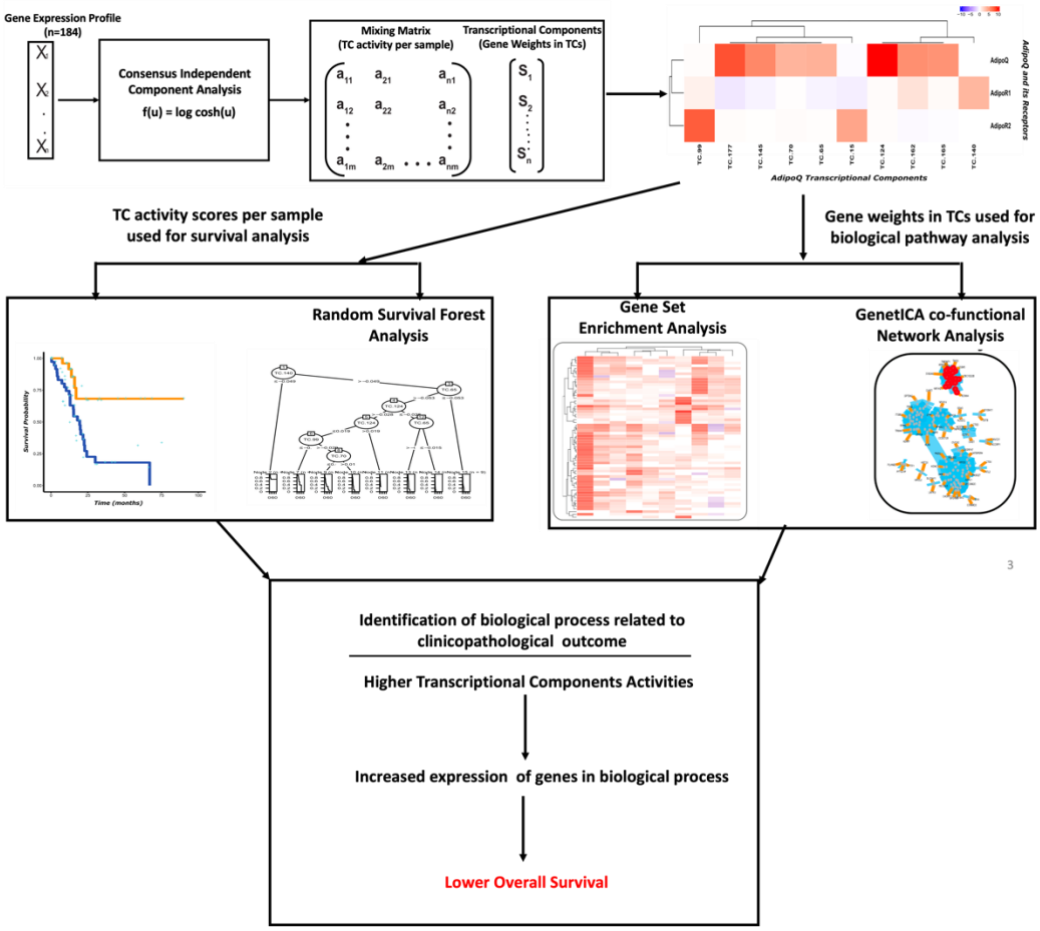

Figure 4-1: Workflow indicating the data acquisition and relations between the methods

4.2.2 Consensus independent component analysis identifies 182 transcriptional components

c-ICA on the 184 gene expression profiles resulted in 182 statistically independent TCs. Even though we kept credibility index (CI) as 0.1 while performing c-ICA, 90.1% (164/182) of TC had CI of 1 and remaining had CI value more than 0.75 (Supplementary Table 1, Figure 4-1). High CI value showed the robustness of our

method for generating TCs. We found AdipoQ or its receptors as outliers in 10 TCs (AdipoQ-TC) and those TCs were used for further analysis (Figure 4-2 and Supplementary Table 2).

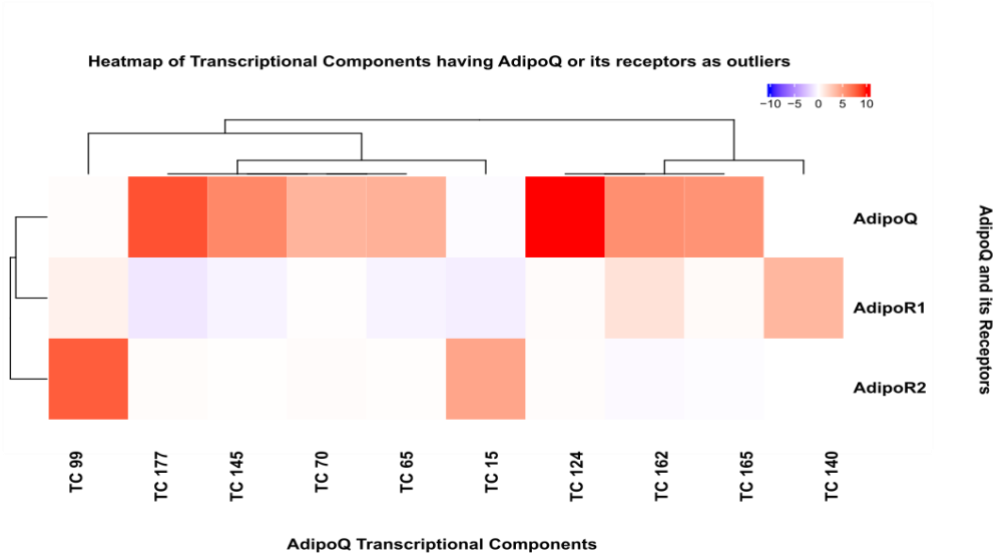

Figure 4-2: Heatmap of transcriptional components with AdipoQ or its receptors as an outliers (AdipoQ-TCs)

4.2.3 Distinct biological processes show enrichment in the AdipoQ-TCs

4.2.3.1 Geneset enrichment analysis shows enrichment of AdipoQ-TCs in 274 genesets from 18 databases

GSEA with 32,880 gene sets that defines biological processes was performed to identify TCs in AdipoQ-TCs enriched for biological processes. The gene sets were selected from all the gene set collections within the Molecular Signatures DataBase (MSigDB, v7.5.1; for the systematic selection strategy, see the "Methods" section). We used a separate transformed z-score from Bonferroni corrected p-value cutoff of 0.05 for identifying enriched gene sets corresponding to each database. Gene set with a higher enrichment score than the threshold was considered enriched gene sets in an individual TC. GSEA showed that each TC from AdipoQ-TCs was significantly enriched for at least one gene set in one of the 18 available gene sets database (significance was defined as Bonferroni corrected Z score for p-value <0.05). For example, the number of enriched gene sets from Gene Ontology Biological Process (GOBP) gene set collection in an individual AdipoQ-TC ranged from zero to 32

enriched gene sets (Figure 4-3). The median top Z score was 2.56 (range 0.00017 - 7.026, interquartile range 1.90 - 5.02) for each GOBP gene set. Similarly, the number of enriched gene sets from Reactome gene set collection in an individual TC of AdipoQ-TC ranged from zero to six enriched gene sets. The median top Z score was 3.39 (range 1.33 -7.44, interquartile range 2.35 - 4.77) for each Reactome gene set.

4.2.3.2 GenetICA co-functionality network shows AdipoQ-TCs involved in DNA repair, innate immunity, and organ development

GenetICA network analysis (Supplementary Table 3) of the top 250 genes in TC 145, and TC 140 were involved in innate immunity, DNA repair, and organ development. TC 140 was also involved in apoptosis and skin development, whereas TC 145 was additionally involved with RNA splicing. Co-functionality network revealed that the top genes from TC 124 were involved in neuronal signals , DNA repair and innate immunity. The top genes of TC 65 showed their involvement in DNA repair and innate immunity. Co-functionality network prediction showed that top genes of TC 70 were enriched in DNA repair RNA splicing, and chromosomal segregation. The top genes of TC 162, TC 165, and TC 177 showed their involvement in DNA repair, organ development and innate immunity. The top genes of TC 15 and TC 99 showed their involvement in DNA repair, innate immunity, and organ development. Co-functionality network revealed that one of the clusters of TC 99 were enriched in digestion process . We observed that AdipoQ was present in cluster showing DNA repair for components with AdipoQ as an outlier.

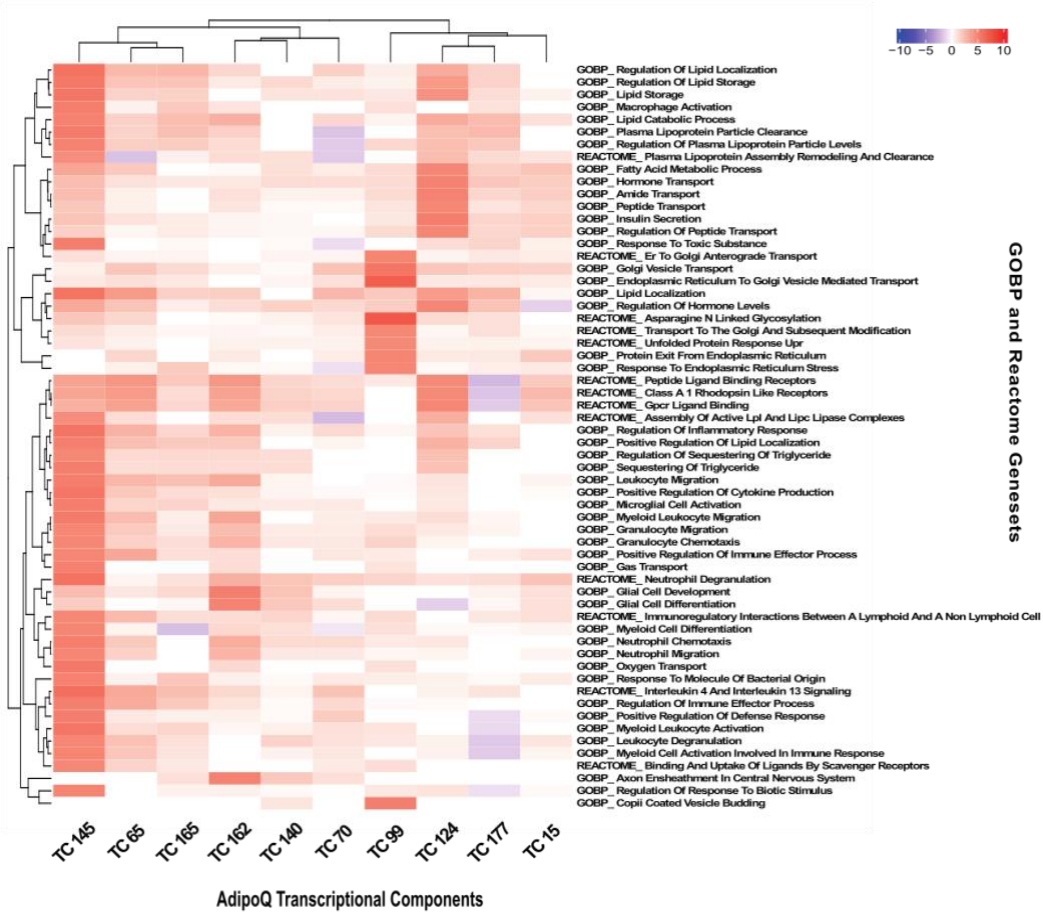

Figure 4-3:Enrichment heatmap of GOBP and Reactome gene sets in each TC of AdipoQ-TCs:

GSEA results of all TC from AdipoQ-TC are present. GOBP and Reactome gene sets were included if the enrichment for at one TC passed the Bonferroni threshold for multiple testing correction. Gene sets were clustered using Pearson correlation and Ward D2. To facilitate interpretation, heatmap colours were based on Z-scores and truncated at a value of ten.

4.2.4 The activity of three TC from AdipoQ-TC is associated with OS

Univariate Cox regression revealed that the activities of TC 15, TC 124, and TC 140 from AdipoQ-TC were significantly associated with OS after multiple testing corrections (significance based on Bonferroni adjusted p-value less than 0.05, Supplementary Table 4).

4.2.5 Random survival forest identifies five TCs as significant classifiers from AdipoQ-TCs in OS

Random survival forest analysis revealed five TCs namely TC 99, TC 124, TC 140, TC 145, and TC 162 from AdipoQ-TCs as a significant classifier for OS (significance based on p-value less than 0.05 and importance score more than or equal to 0.5, Table 4-2). Eight robust cohorts of patients were observed using random survival forest analysis. All the patients belonging to cohort 6 had the best OS, whereas those belonging to cohort 3 had worse OS (Figure 4-4).

Table 4-2: Random Survival Forest Reveals significant classifiers for OS

TCs	Input tree	Number of. significant classifiers	Importance. Score	P value
TC 99	**126**	**126**	**1**	**8.39E-29**
TC 162	**126**	**120**	**0.80**	**7.74E-24**
TC 140	**126**	**116**	**0.64**	**8.43E-21**
TC 145	**126**	**125**	**0.64**	**6.10E-28**
TC 124	**126**	**102**	**0.51**	**6.90E-12**
TC 65	126	113	0.48	1.15E-18
TC 15	126	63	0.25	1
TC 165	126	86	0.24	6.10E-05
TC 70	126	48	0.15	0.00978
TC 177	126	55	0.13	0.181449

Input Tree: Number of trees in the random forest

Number of Significant classifiers: Number of times a TC appeared in the tree

Importance score: Number of times a TC appeared at the top of the tree (TC with importance score above or equal to 0.5 and with p-value less than 0.05 were considered as significant classifier for the survival analysis)

Table 4-3 Summary of biological process of AdipoQ-TCs obtained from GSEA and GenetICA

TCs	Biology captured by AdipoQ-TCs from GSEA and GenetICA
TC 15	Innate immunity, DNA repair and organ development
TC 65	Innate immunity, and DNA repair
TC 70	DNA repair, RNA splicing, and chromosomal segregation
TC 99	ER biology, DNA repair, innate immunity
TC 124	Lipid metabolism, neuronal development, transport
TC 140	Innate immunity, DNA repair, apoptosis, organ development
TC 145	Innate immunity, Lipid metabolism, and DNA repair
TC 162	Neuronal development, DNA repair, and innate immunity
TC 165	Organ development, DNA repair, and innate immunity
TC 177	Organ development, DNA repair, and innate immunity

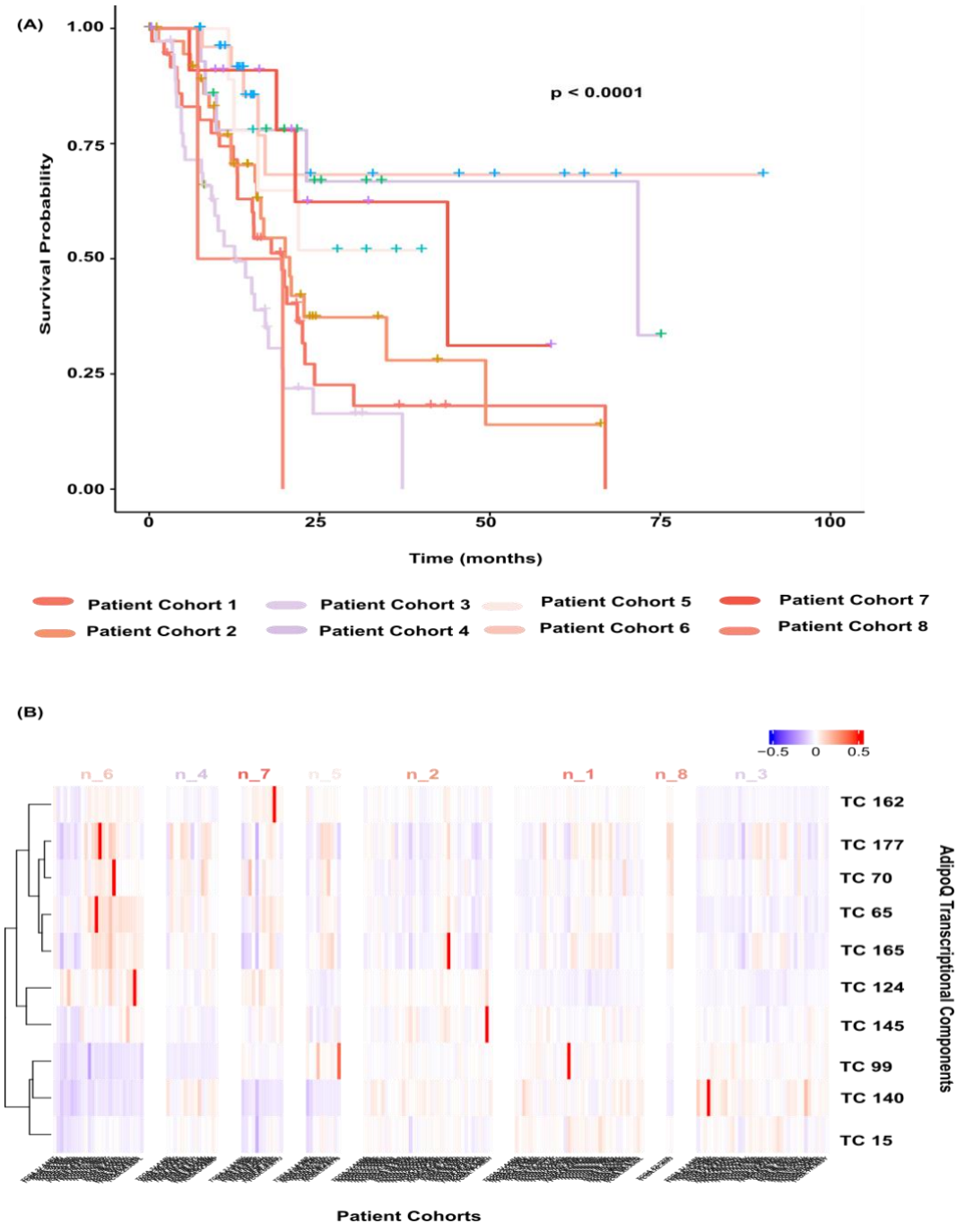

Figure 4-4: Random-forest survival analysis of patients defines survival cohorts:

A) Survival curve for eight cohort's patients in OS (B) Heatmap showing the activity of TCs from best to worst survival cohorts of patients (right to left) in OS

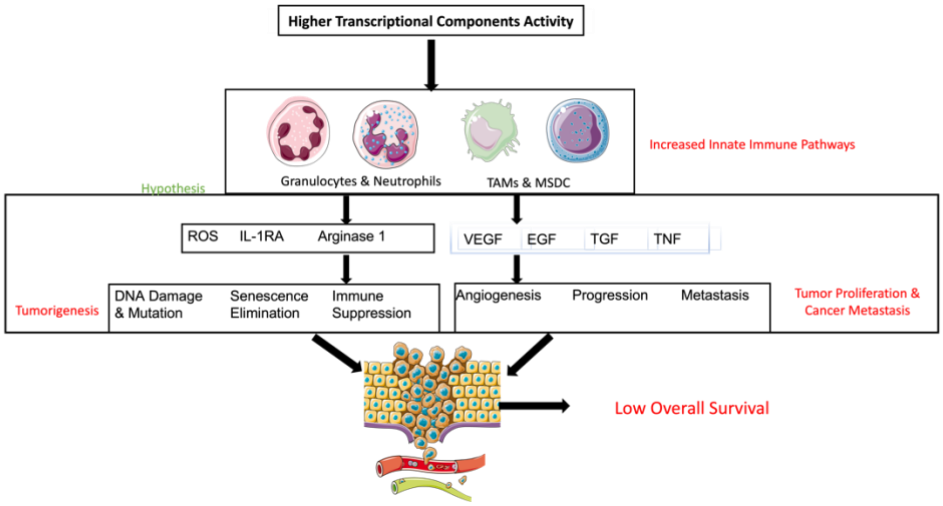

Figure 4-5: Hypothetical model of biological process associated with the activities of TCs and its clinical outcomes

4.3 Discussion

The current research utilized 184 gene expression profiles in conjunction with c-ICA to uncover the landscape of AdipoQ-TC in PAAD patients. We described each of the 10 AdipoQ-TC with GSEA and genetICA network analysis. The composition of all TCs, gene set enrichment results and the activity of the TCs in each sample are publicly available via http:// opendatainscience.net/. This portal provides a new tool to gain further insight into the adiponectin biology that impacts the patient's survival in pancreatic cancer.

Our analysis revealed that patients with worse OS were characterized by higher activity of TC 140 and TC 145. These TCs were enriched for gene sets related to innate immune pathways, such as neutrophil degranulation, interleukin 4 (IL-4) and interleukin 13 (IL-13) signaling, activation of myeloid-derived suppressor cells (Briukhovetska et al., 2021; Masucci et al., 2019; Pergamo & Miller, 2017; Shi et al., 2021; Thyagarajan et al., 2019; Toedebusch et al., 2021; Xiong et al., 2021). Significantly higher level of IL-4 and IL-13 was reported in blood plasma level of pancreatic ductal adenocarcinoma (PDAC) patients compared to the control participant (Chen et al., 2009; Gabitass et al., 2011; Yako et al., 2017). IL-4 and IL-13 were reported to be a strong predictor of disease-free survival (DFS) in patients with resectable PDAC (Formentini et al., 2009; Piro et al., 2017).Furthermore, higher levels of IL-13 and IL-4R are associated with increased lymph node metastasis (Formentini et al., 2009). Additionally, the close connection between IL-4 and

tumor-associated macrophages (TAMs) suggests that IL-4 can activate the cathepsin protease in TAMs, leading to PDAC tumor growth and angiogenesis (Shi et al., 2021). IL-4 treatment of TAMs promoted the malignancy of PC cells by inducing epithelial-mesenchymal transition (EMT), resulting in increased cell growth and movement (C.-Y. Liu et al., 2013). These observations from studies suggest that increased IL-4 and IL-13 signaling are detrimental to PC patients. As we observed that higher activity of TC 140 and TC 145 are associated with worse survival of the patients, we can hypothesize that increased signalling of IL-4 and IL-13 could be one of the factors.

Studies have found that neutrophils and granulocytes are involved at every stage of tumorigenesis, from cancer initiation growth and metastasis (Masucci et al., 2019; Xiong et al., 2021). Neutrophils promote carcinogenesis by inducing DNA damage and mutation through reactive oxygen species. They also play a role in the immunosuppression by the release of arginase-1, which inhibits CD3-mediated T cell activation and proliferation. This results in the creation of an acidic environment which inhibits the anticancer activity of T cells and NK cells. The cancer progression is promoted by the neutrophils and granulocyte through IL-1RA which eliminates senescence (Masucci et al., 2019; Xiong et al., 2021). A study discovered that increased neutrophil infiltration was at the center of metastasis of PDAC in the liver, lung, and stomach (Jiang et al., 2022). Studies have established that neutrophils mediate tumor metastasis by vascular epidermal growth factors (VEGF), transforming growth factor beta (TGFβ), and tumor necrosis factor (TNF) (Jiang et al., 2022; Masucci et al., 2019; Patel et al., 2018; Y. Wang et al., 2018; Xiong et al., 2021). With our in-silico analysis we hypothesize that higher activities of TCs impact those innate immune pathways which are pro-tumorigenesis and that could have an impact on the OS of patients (Figure 4-5).

We also observed that TC 65, TC124, TC 145, TC 165, and TC 177 had higher expression of leptin gene (Supplementary Table 5). The result is concurrent with in-vitro studies of obesity, where it was shown that leptin induces the adiponectin expression (E. Hu et al., 1996; Makimura et al., 2002). A randomized clinical trial on weight gain in 44 patients showed a positive correlation between increase in adiponectin expression to leptin expression (Singh et al., 2016). However, a study in

breast cancer found that leptin expression is not associated with expression of adiponectin (Jardé et al., 2009).

Furthermore, our study from GSEA and genetICA analysis revealed that TCs of AdipoQ-TCs were majorly involved in lipid metabolism, innate immunity, DNA repair, and organ development (Table 4-3). AdipoQ is known to be an active player in lipid metabolism, and innate immunity (Ruan & Dong, 2016; Yamauchi et al., 2014). TC 145 captured the biology of innate immunity and lipid metabolism with multiple gene sets enriched in GOBP and Reactome, belonging to innate immunity as well as lipid metabolism (Supplementary Table 6 and Figure 4-3).

Random survival forest analysis showed that TC 99, TC 124, TC 140, TC 145, and TC 162 are significant classifiers. We observed that lower activity of TC 140, TC 99, TC 145, and TC 162 were associated with better OS of patients. Whereas higher activity of TC 124 was associated with better OS of patients. TC 140, TC 145, TC 99, and TC 162 showed biological function of innate immunity, and DNA repair pathways. DNA damage repair deficiency has already been shown to be associated with significantly better survival than patients with proficient DNA damage repair pathways independent of cancer subtypes (Zimmermann et al., 2021). Our result also shows that a lower or deficiency of DNA damage repair pathway could be better for the PAAD patient's OS. One of the innate immune pathways shown to be enriched in TC 145 is IL-4 and IL-13 signalling pathway (Supplementary Table 6). IL-4 is a known player in the tumor progression of PDAC. A prospective study of 287 PDAC patients found that IL-4 was significantly associated with worse disease-free survival (Piro et al., 2017). Our analysis also supports that lower the innate immunity pathways is better for PAAD patients OS.

We also checked for the top genes in these components and what is known about them in the literature. We found that in TC 140, four of the kallikrein (KLK) family of enzymes were upregulated. KLK 6, KLK7, KLK8, and KLK10 were upregulated (Supplementary Table 5). KLKs are a subgroup of serine proteases, enzymes capable of cleaving peptide bonds in proteins. KLKs encode a family of fifteen closely related serine proteases. KLKs are upregulated in pancreatic cancer and aberrant KLK levels are associated with poor prognosis (Iakovlev et al., 2012). KLK7-positive staining is associated with almost a three-fold increase in mortality rate of

patients with unresected PAAD (Iakovlev et al., 2012). Another study observed that higher expression of KLK6, KLK 7, and KLK 10 in PAAD patients were associated with poor OS and disease-free survival (Rückert et al., 2008). Aberrant upregulation of KLK10 promotes metastasis and was associated with poor prognosis, survival and tumor progression of PAAD patients (X.-Y. Cao et al., 2018). KLK7 was overexpressed in PAAD patients by 30 folds and was involved in tumor proliferation and migration (Du et al., 2018). Positive KLK8 staining was associated with the progression and poor survival of PAAD (Hua et al., 2021). KLKs have been shown to be associated with poor survival in oral cancer (H. Zhao et al., 2011), colorectal cancer (Alexopoulou et al., 2013; Inoue et al., 2010; Talieri et al., 2009), breast cancer (Talieri et al., 2004) , and ovarian cancer (Kyriakopoulou et al., 2003). TC 140 had KLK6, KLK7, and KLK8 highly upregulated with their presence in the top 20 genes. Due to the aberrant upregulation of KLKs, there could be a plausible explanation for the higher activity of TC 140 associated with poor survival in OS Further investigation is needed to establish the role of KLKs and AdipoQ in pancreatic cancer.

Chapter 5: Conclusions

The thesis elucidated in Chapter 2: the role of PTMs in TAAs as a platform for novel immune-oncology therapy. The future perspective of that chapter would focus on translating the success of many preclinical studies in citrullinated peptide as a vaccine in clinical settings. The start of the open-label pilot phase clinical trial of phosphorylated peptides in melanoma opens the arena for more clinical trials. These promising results suggest that in the future, we could be able to target cold tumors with vaccines having PTMs in TAAs.

I showed in Chapter 3: that lower expressions of AdipoR1 and AdipoR2 are better for survival and least aggressive tumors in multiple cancers. Further studies should be carried out to find the physiological and biological role of AdipoR1 and AdipoR2 in Thymoma, low-grade glioma, and Kidney renal papilloma. As there is limited literature on the presence of AdipoR1 and AdipoR2 in these tumors. A detailed investigation could open up a new arena of therapeutic targets in these cancers.

The final chapter of the thesis (Chapter 4:) focused on the transcriptomic landscape of AdipoQ and its receptors in pancreatic cancer. One of the key findings from this study is the presence of KLKs enzyme overexpression in TCs with AdipoR1 as an outlier. We should investigate in tumor model mice what happens when AdipoR1 is overexpressed, whether we are able to replicate the overexpression of KLKs in these mice models, and how the immune system behaves as well as over survival of the mice should be investigated. Our future studies should focus on analyzing the role of the top 20 genes in each AdipoQ-TCs found as significant classifiers of survival.

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Acknowledgment

I started my life in research a decade ago, and the end point of my dream was to have a doctorate. I am extremely excited to finally arrive at the destination. It was a long arduous journey and wouldn't have been possible without the support of many people. I would like to first thank my mentor and supervisor, **Prof. Francesco Novelli.** Franco has been there to support me through thick and thin. He always encouraged me to bring new ideas. He supported me in venturing into bioinformatics and meta-analysis. He was there to help me during the process of the international research exchange program in The Netherlands. It was his guidance that I was able to fruitfully complete this thesis. I will always remember Franco's words of wisdom during our numerous meetings. Franco was there for me as a guardian during the COVID. His sharp and critical comments on our published work as well as during my thesis helped me a lot to learn.

My journey would have been incomplete without **Prof. Paola Cappello**. She is not just my co-PI but my biggest support in the Ph.D. journey. I will always cherish the moment we had constructive discussions in lab meetings and your input on improving my project. You taught me many skills in the lab as well as improved my personality. While I was working in The Netherlands, you always took extra steps to understand the complex bioinformatic project, despite not being your area of interest. I will never forget your constant motivation during the drafting of the review article. The constructive criticism you provided helped a lot to improve the quality of my thesis. Paola became my pillar of strength at the time of the second COVID wave when I was suffering personal losses. I have learned from you how to be a perfectionist and humbler while working.

I would like to thank my thesis reviewer **Prof. Surva Chatterjee**, and **Prof. George Priya Doss** for accepting the invite to review in a short time. Your comments have helped to improve the quality of the thesis.

My first bioinformatic project wouldn't be a possibility if I did not get the support and guidance of **Prof. Francesca Cordero**, **Prof. Marco Beccuti**, and **Dr. Laura Follia**. Marco took an extra effort to teach me the basics of R. I would always cherish my after-class interaction with Marco. Francesca provided me the constant support

and feedback on the project's progress as well as on the chapter's draft. I will always be indebted to Laura for introducing me to the world of bioinformatics. I will never forget our constant chatter on any random topic during the lunch break and your strong recommendation of gelato at La Romana.

I wanted to do an international exchange as part of our Ph.D. program, but due to COVID, no lab was willing to have an exchange student. I have to say that **Dr. Rudolf Fehrmann** was an exception and allowed me to join and work in his lab. I learned so much while working in Rudolf's group due to the openness created by Rudolf himself. I had never expected a PI to be so involved in a visiting student project. Even though it was a highly complex mathematical model, you were always there to explain it to me multiple times. I will never forget your stories of transitioning from computer scientist to medical doctor. You were always there to support me in every aspect of my stay in Groningen. I will never forget the happiness you got when I first received an interview call from a company. You gave me everything to succeed and learn. It was the best experience of working abroad in my long research career. I will always be thankful to you for your encouragement, motivation, and support. I never felt that I was working with a supervisor but had a feeling of being with a friend.

The thesis and my progress would be incomplete without **Dr. Arkajyoti Bhattacharya**. I loved the way you taught me by always saying, "*You will learn only by doing mistakes*." Every time I came to you with some doubts, you challenged me to solve it by myself. You helped me understand the mathematical concept behind ICA. There are so many things that I learned from you in research that even though I am a storyteller, I am at a loss for words to explain your magnanimity. Apart from research, I will always miss those small TED talks that you gave during office hours to motivate everyone. Your determination to run can inspire so many and make a lot of people jealous. One of the best parties of my life was your defense party and the way we danced in the bar. I will never forget the delicious "Kulfi" you made at the dinner party at your place. I would also like to thank **Bashundhara** for those delicious meals and for hosting me at parties.

My journey to Ph.D. wouldn't be complete without the support I received from my collegues at Novelli Lab. I would especially like to thank **Dr. Giorgia Mandilli** for

teaching me the proteomic analysis of pancreatic cancer. I would take this opportunity to thank **Dr. Gianluca Mucciolo** for being my first friend in a foreign land. Gianluca introduced me to the various part of this beautiful city and helped me in shifting home. I would also like to thank other lab members of Novelli Lab namely **Dr. Sara Bulfamante**, **Dr. Cludia Curcio, Dr. Cecilia Roux, Silvia Brugiapaglia, Alessandro Scagliott, and Laura Barbasio.** I would also like to especially thank **Giorgia Guadagnin** for her support during my project on adiponectin. Giorgia helped me a lot during the review and especially with making the figure. Your suggestion on some recent literature helped me improve the quality of the thesis as well as the review.

My life at Groningen would have become a boring research job had I not found some amazing people at Rudolf's Lab. I would like to thank **Stefan** for being a work buddy, which I have always missed during my research career. I will always cherish the rides we had to go back to our home from the office. The fun we had during the open day while exploring historical buildings in Groningen. Your introvertedness and how you always needed "me time" after spending energy at parties amused me always. I will never forget how you helped me plan the trip to Zaans Schans during last year's Easter holiday. You are my constant cheerleader in the writing journey.

I never knew how to start the job search journey; it was difficult and challenging. But things become simpler if you have a friend and guide like **Setareh.** She helped me overhaul my LinkedIn profile and assisted me with the cover letter drafting. Your inspiring journey kept me motivated during my low phases, I always used to say when Setareh can make it big in her career despite so much adversity, I can also do it. I will always be indebted to you, Setareh for transforming me. I will take this opportunity to thank **Hamid** also for being such a gentle friend and joining us for all the parties.

When I went to Rudolf's lab, it was intimidating to be surrounded by all the bioinformaticians and statisticians. It was a sigh of relief to meet **Manasa,** with the same background as mine. I will always be grateful for the support you showed in finding accommodation during my first visit to Groningen. I will cherish working with you on the project of the projection of my data. The way you took extra effort to explain to me the intricacies of the projection will always stay with me. How

would I ever forget the memorable bike ride we (I, Stefan and You) took after the defense party of Arko.

I would also like to thank **Dr. Carlos** for always motivating me during my job search phase. I really enjoyed your defense party. I will also thank **Shanlin** for his inquisitive questions during many lab meetings.

My arduous journey would have been incomplete had I not gotten the support of my wonderful friends. I would like to thank **Dr. Tushar Tomar**, for being a constant support and guiding me in all difficult phases like an elder brother. Your advice has changed my life. I would like to thank my cousin and friend, **Dhwani Srivastava.** You have always been there to support me, especially during our virtual joint food cooking session during COVID. You were always there with me during my ups and downs. I wish you could have come here for the defense and lit up the ceremony as you did during my master's graduation. It is time to say thanks to one of my dearest friends **Dr. Siddharth Omar**. Sid, along with Dhwani, played an important role in keeping me motivated during the low phases of my Ph.D. Sid was an awesome host in the Netherlands whenever I had to go there to visit him. I wish you could have joined me at this occasion. I would like to thank Dr. **Raj and Dr. Sweta** for always being there with me whenever I needed support.

I would also thank **Dr. Shubham** for being my soundboard. Our Ph.D. journeys were similar in many ways, and we fed each other with positivity during this journey. You helped me in building the website and supported me throughout. You have been such a great emotional support throughout my master and Ph.D. journey. It is so great that you were not only part of those journey, but you made sure to be present even at the final destination. Thank you for your continued support. I would also like to thank **Bhanupriya.** Bhanu zeal to achieve new heights motivates me to push further. I am so much impressed with your progress in the last year. You were a constant support during my Ph.D. and current job search phase.

I would also thank my friend from my master's degree program, **Jonathan** and **Dr. Marijke.** We started our WhatsApp group during COVID, and it has been a great mental support for me. Jonathan, your journey from molecular biologist to sales manager motivates me. You always pushed me and appreciated my efforts. Marijke, despite your tumultuous Ph.D. journey, your love for research remains unhindered.

The way you supported me during my journey is amazing. I congratulate you for making the transition to industry and hope that I will make it soon.

I will also like to thank my friends from bachelor and childhood who were always there for me as a soundboard. **Dr. Devesh Singh** and **Dr. Chandrashekar Misra** have been with me in the entire journey. We always wanted to make a trip together, and finally, it happened in Dec 2018, when we all went to roam around Rome, Venice, Padua, and Turin. We were three musketeers, two got the doctorate, and finally, I am finishing the race. It would have been great if you would have come to my defense personally. A special thanks to Devesh for making sure to attend my sister's wedding in December. My other friends who have supported me throughout are **Dr. Sujeet, Pratyush, Dr. Anirudh Gupta, Abhay Singh, Debobrat Sabat, Suraj Pandey, Vikash Paswan, Abhishek Kumar, Dr. Ritu Singh, Manish Kumar, Dr. Pooja, Sansriti, Manish Kumar, Dr. Asutosh Tripathi, Dr. Pallav Gupta, Dr. Ashish Kumar, Dr. Neh Nupur, Dr. Sumedha Ojha, and Dr. Stephanie**. A special thanks to my childhood friend **Sushmita.** You have played a pivotal role in my educational journey. The moment we have cherished together are some of the best moments of my life. I will always cherish our last meeting in Kolkata. You have been a constant support and a soundboard during my Ph.D. journey.

I met many people via online platforms who are helping me in my career with various roles. I would like to thank **Dr. Adam, Dr. Surya, and Murali Karthik** for their constant support.

The journey would not be a possibility if I did not find some amazing landladies during my stay in Turin and Groningen. I would like to thank **Emanuele, Shweta, and Anna** for being such lovely hosts. A special word to put in for Anna, I got in your house at the last phase of my Ph.D., but you became the most important person I have ever met in Turin. You made me feel like I am at home. I see my grandmother in you and the way you feed me food is incredible and bear me. Thank you for all the love and kindness.

During my doctoral studies, I also guided students to higher education abroad. These guiding sessions have provided me with gems of people who became close friends of mine. I would like to thank **Sucheta Patnaik,** one of my first students who

introduced me to the group of new people at VIT Vellore. I will always remember our meeting during my visit in 2017 before starting the position in Italy. I am thrilled with your career progress and wish to meet you soon. Another student who went on to become my friend is **Murali Mishra.** You brought some extraordinary people into my life and have been a constant support during my journey toward Ph.D. Now moving to some other close people, **Shivohum, Harshita, Hansika, and Aakash**. Shiv, you have been an inspiration for me for your humbleness and attention to detail-attitude. I could never express my gratitude in words as you have been my biggest cheerleader for the completion of my Ph.D. your effort to come to my defense is a testimony of that. Thank you for being my friend and supporter. Harshita, you have been one of the sweetest people I have ever met in my life. Even though we speak occasionally but the enthusiasm you spread is unmatched. I would never forget our Netherlands trip before COVID. Hansika, even though you live in a different continent, you make sure to keep me updated on all your progress. You supported me emotionally when I was down during the COVID lockdown. It was a great pleasure to meet you during my Amsterdam trip. Aakash, you helped me prove the hypothesis that I can guide students irrespective of their background. I will never forget your unrelentless faith in me. I was more than happy to help you during your master's thesis, and I look forward to meeting you in the future.

A special mention to **Prachi;** you are not only my student but one of my closest friends I have. I saw you grow from your bachelor studies to your life in Italy and now working woman in Denmark. You are an example of breaking the myth that you don’t need the language to get a job in Europe. I will always cherish my trip to Trento and the neighboring part during your master defense. I enjoyed being hosted by you and **Edordo.** I wish you and Edordo could have come for my defense but given your hectic schedule. We will meet soon.

Other students who played an influential role in shaping me as a person are **Pujitha, Ramya, Lohitesh, Bibhudatta, Ranjan, Rohan, Rishika, Rajan, Rutuja, Chelsea, Bhumika, Ruhi, Dhanbala, Indraja, Meera, Aswathy, Aishwarya, Kavya, Brinda, Chitra, Sara, Naveen, Anuvind, Lakshmi,** and many others.

A special thanks to **Vasundhara,** your grit to keep moving always motivates me. I would like to thank you for lighting up Sangeet at my sister's wedding. I wish you

all the success in your career. You are one of the first person to appreciate my writing in full form. A word of thanks to **Ben,** you have taught me that following passion is never a difficult choice. Your Ph.D. search journey is a testament of never giving up your dream. It is a pleasure that you will be present at my defense.

I have always been blessed to be under the shadow of some great mentors. I would like to thank **Prof. Anil Kumar Gopinathan**, my godfather in research. You baptized me with the virus of research, and it was your dream to see me complete my doctoral studies. I am thankful to **S.K. Jha Sir**, my high school biology teacher, who made the subject so interesting that even today, I am unable to come out of this. I will always be indebted to Prof. Amitava Mukherjee; you have been a motivator and friend in my journey. Whenever I hit rock bottom, you were there to bring me out of that. I take this opportunity to thank **Prof. T.Subromaniom**, my mentor at the summer research fellowship. Our relationship goes beyond research; thank you for making me a part of your family life. I always looked up to you for taking inspiration. Your recent autobiography is a must-read for every researcher to learn how to overcome adversity and be happy. I met **Prof. Peter Stamp** at one of the conferences at VIT University during my undergraduate studies. You became my cheerleader and gave me the name "*Little Crab Philosopher*." You have always supported me through your motivational word over emails; your words of wisdom kept me going. I would like to thank **Prof. Jan Kok**, my mentor in the master's degree program and constant motivator, and it was great meeting you during my last stint in the Netherlands.

My extended family has always been appreciative of my work, I would like to thank them for their support. **Bua, Badi Mami, Choti mami, Chote Mama, Ravi Mama, Beena Mami, Manu Mama, Anita Mami, Shatru Mama, Meena Mami. My cousins have also supported me throughout this journey, Pankaj Bhaiya, Shweta Bhabhi, Prasoon Bhaiya, Kirti Bhabhi, Gaurav Bhaiya, Garima didi, Amit Jijaji, Puja di, Arvind Jijaji, Vaibhav, Priyanaka, Sankalp, Toshi, Tushar, Shlok, Tanvi Di, and Garima Di.**

My journey was going in a downward spiral when I met **Azra Thaseen**. Azra is my confidant I never had. You appreciated my writing as no one else did, and your appreciation pushed me to chart a career in storytelling. If you were not there, I don't

think my review would have seen the light. When my laptop stopped working, I remembered how I finished the draft at Amsterdam Airport on your laptop. I will always cherish those Pizzas, Thai Food, and Gelato we had together and how to forget our best dining place ISKCON temple of Turin. Your constructive criticism has helped me improve as a person. Throughout my thesis writing, you have pushed me hard to never ever give up. The special moment we have shared while watching "Crime Patrol", can never be replaced by anything. I get the positivity whenever we visit open market with you every weekend. There are so many things I can write but how to construct those sentences which explain my feelings in the truest sense. I hope to cherish every moment with you. Thank you for being the positivity in my life.

Last but not least, I would like to thank my parents for always pushing me to pursue higher education. You have been an inspiration to me. **Maa and Papa**, this whole journey is worthless without your effort. This thesis is for you, and I am happy to tell you that in our generation, I will be the first one to achieve a doctorate degree. I would also like to thank my elder brother **Abhishek, sister Shubham, and brother-in-law Rupesh Jijaji** for their constant support.

As I stated that the journey is incomplete without support from enormous people, and I might have missed out on many on the list. I apologize for not mentioning their name. I am and will always be thankful to everyone for their support.

Personal Profile

Education

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Publication

Srivastava Anurag Kumar, Arkojyoti Bhattacharya, Giorgia Guadagnin, Paola Cappello, Francesco Novelli, and Rudolf SN Fehermann, (Manuscript under prep) "Role of adiponectin and its receptor in pancreatic cancer."

Srivastava, Anurag Kumar, Laura Follia, Marco Beccuti, Francesca Cordero, Paola Cappello, and Francesco Novelli. (Manuscript under prep) "Adiponectin receptors expression in the pan-cancer study."

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Patel, Vikash, Abhishek Dutt Tripathi, Kundan Singh Adhikari, and Anurag Srivastava. "Screening of physicochemical and functional attributes of fermented beverage (wine) produced from local mango (Mangifera indica) varieties of Uttar Pradesh using novel saccharomyces strain." Journal of food science and technology 58, no. 6 (2021): 2206-2215. https://doi.org/10.1007/s13197-020-04731-9

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Srivastava, A.K, Halder, K., Kok J., Neumann H., Dutt S., (2016) Investigation of Protein dynamics using genetically encoded unnatural amino acid (Abstract), European Journal of Cancer Supplements, 54, S70 (Conference Paper).

Honors and Awards

Visiting researcher fellowship of €10,000 awarded by the University of Turin, Italy, to pursue research at UMCG, The Netherlands (Jan 2022)

Research grant of €80,000 on research proposal by University of Turin, Italy: Carried out Ph.D. research work (Oct 2018)