

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**MACHINE LEARNING-BASED SUBTYPING OF RENAL
CANCER VIA NON-APOPTOTIC CELL DEATH PATHWAYS**

HAKAN KAHRAMAN

A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOENGINEERING
BIOINFORMATICS AND SYSTEMS BIOLOGY PROGRAM

ADVISOR: ASSOC. PROF. PINAR PIR

JULY 2025

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**MACHINE LEARNING-BASED SUBTYPING OF RENAL
CANCER VIA NON-APOPTOTIC CELL DEATH
PATHWAYS**

HAKAN KAHRAMAN

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOENGINEERING
BIOINFORMATICS AND SYSTEMS BIOLOGY
PROGRAM**

ADVISOR: ASSOC. PROF. PINAR PİR

JULY 2025

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

APOPTOTİK OLMAYAN HÜCRE ÖLÜM YOLLARI
ÜZERİNDEN BÖBREK KANSERİNİN ALT TIPLERİNE
MAKİNE ÖĞRENMESİ TABANLI AYRILMASI

HAKAN KAHRAMAN

YÜKSEK LİSANS TEZİ
BİYOMÜHENDİSLİK ANABİLİM DALI
BİYOİNFORMATİK VE SİSTEM BİYOLOJİSİ
PROGRAMI

DANIŞMAN: DOÇ. DR. PINAR PİR

TEMMUZ 2025

MASTER of SCIENCE JURY APPROVAL FORM

A thesis submitted by Hakan Kahraman, defended on 10/07/2025 before the jury formed with the 08/07/2025 date and 2025/35 numbered decision of the GTU Graduate Administration Board, has been accepted as a MASTER of SCIENCE thesis in the Department of Bioengineering, Bioinformatics and Systems Biology Program.

JURY

MEMBER

(THESIS ADVISOR) : Assoc. Prof. Dr. Pınar Pir

MEMBER

: Prof. Dr. Tunahan Çakır

MEMBER

: Assoc. Prof. Dr. Beste Turanlı

APPROVAL

Gebze Technical University Graduate Administration Board
...../...../..... date and/..... numbered decision.

SIGNATURE/SEAL



*To my
Father ...*

ABSTRACT

Non-apoptotic programmed cell death (PCD) has attracted growing interest due to the discovery of alternative mechanisms that function independently of traditional apoptosis. This study aims to elucidate the molecular foundations of recently characterized non-apoptotic PCD pathways—namely necroptosis, pyroptosis, ferroptosis, parthanatos, autophagic cell death, and cuproptosis—and to explore their specific roles across various cancer types. Emphasis is placed on understanding how these pathways contribute to cancer biology and their potential utility in distinguishing cancer types based on gene expression patterns.

Non-apoptotic PCD mechanisms have emerged as pivotal regulators of tumor progression, presenting novel avenues for biomarker discovery and cancer subtype classification. In this study, we evaluate the diagnostic relevance of gene expression signatures associated with these pathways across a diverse set of cancer types. Leveraging data from The Cancer Genome Atlas (TCGA), we identified specific cancers—particularly subtypes of kidney cancer—where the regulation of key activator and inhibitor genes is significantly altered.

By constructing biologically informed features such as pathway-specific means, principal components, and entropy-based metrics, we trained multi-class machine learning models that achieved 94% accuracy in classifying kidney cancer subtypes (KIRC, KIRP, KICH). Dimensionality reduction techniques and SHAP-based interpretability analyses revealed distinct molecular profiles for each subtype, with pyroptosis and autophagic cell death emerging as dominant discriminative pathways.

To facilitate real-time diagnostic application, we developed a Flask-based web platform (kidneycancerscope.com) capable of performing tissue classification and tumor subtype prediction. Overall, our findings highlight the potential of non-apoptotic cell death gene signatures as biomarkers for cancer classification and support the advancement of AI-powered diagnostic tools.

Keywords: Non-Apoptotic Programmed Cell Death, Gene Set Variation Analysis, The Cancer Genome Atlas, Transcriptomic Profiling, Machine Learning, Precision Oncology

ÖZET

Apoptoz dışı programlanmış hücre ölümü, geleneksel apoptoz mekanizmasının ötesine geçen yeni yolların ortaya çıkmasıyla birlikte giderek daha fazla ilgi görmektedir. Bu çalışma, yakın zamanda tanımlanan apoptoz dışı hücre ölümü mekanizmalarının—nekroptoz, piroptoz, ferroptoz, parthanatos, otofajik hücre ölümü ve cuproptosis—moleküler temelini ve bu yolların farklı kanser türlerindeki özgül rollerini ortaya koymayı amaçlamaktadır. Araştırma, bu mekanizmaların kanser biyolojisindeki önemini ve gen ekspresyon profilleri aracılığıyla kanser türlerini ayırt etme potansiyelini incelemektedir.

Apoptoz dışı programlanmış hücre ölümü (PCD) mekanizmaları, tümör davranışının kritik düzenleyicileri olarak öne çıkmış ve biyobelirteç keşfi ile kanser alt türlerinin sınıflandırılması için yeni fırsatlar sunmuştur. Bu çalışma, bu PCD yollarından türetilen gen ekspresyon imzalarının çeşitli kanser türlerinde tanısal değerini araştırmaktadır. The Cancer Genome Atlas (TCGA) veriseti kullanılarak özellikle böbrek kanserlerinde (KIRC, KIRP, KICH), aktivatör ve inhibitör genlerin düzenlenmesinin belirgin şekilde bozulduğu kanser türleri sistematik olarak belirlenmiştir.

Biyolojik olarak anlamlı özellikler (örneğin entropi, PCA, yolak ortalamaları) kullanılarak, böbrek kanseri alt türlerini %94 doğrulukla sınıflandırabilen çok sınıflı makine öğrenmesi modelleri geliştirilmiştir. Boyut indirgeme ve SHAP tabanlı yorumlanabilirlik analizleri, bu alt türlerin özellikle piroptoz ve otofajik hücre ölümü tarafından şekillenen belirgin moleküler desenler sergilediğini ortaya koymuştur. Doku sınıflandırması ve tümör tanımlaması için gerçek zamanlı tahminler sunan Flask tabanlı bir web platformu (kidneycancerscope.com) geliştirilmiştir.

Bu çalışma, apoptoz dışı hücre ölümü gen imzalarının kanser sınıflandırması için biyobelirteçler olarak kullanılabileceğini göstermekte ve yapay zeka destekli tanı platformlarının geliştirilmesini desteklemektedir.

Anahtar Kelimeler: Apoptoz Dışı Programlanmış Hücre Ölümü, Kanser Genom Atlası, Gen Seti Varyasyon Analizi, Makine Öğrenimi, Transkriptomik Profilleme, Hassas Onkoloji

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Assoc. Prof. Dr. Pınar Pir, for her invaluable guidance and dedicated support throughout the preparation of this thesis. Her profound knowledge and expertise have played a crucial role not only in scientific aspects but also in the development of my work discipline and critical thinking skills. Her patient, understanding, and encouraging approach has been instrumental in the successful completion of this study.

Throughout my life, my beloved father, Ali Kahraman, whom I lost in 2022, has been my source of inspiration, and I offer him my deepest gratitude and appreciation. His passion for science and learning, along with the support he gave me in choosing this field, forms one of the cornerstones of my work. His curiosity, determination, and unwavering love, which always pushed me forward, have been my greatest motivation in this journey.



TABLE OF CONTENTS

	<u>Page</u>
ABSTRACT	vi
ÖZET	vii
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS	ix
LIST OF SYMBOLS AND ABBREVIATIONS	x
LIST OF FIGURES	xi
LIST OF TABLES	xii
1. INTRODUCTION	1
2. NON APOPTOTIC PROGRAMMED CELL DEATH MECHANISMS	4
2.1. Necroptosis and Related Genes	5
2.2. Pyroptosis and Related Genes	9
2.3. Ferroptosis and Related Genes	12
2.4. Parthanatos and Related Genes	17
2.5. Autophagic Cell Death and Related Genes	20
2.6. Cuproptosis and Related Genes	23
3. OBJECTIVES AND METHODS	27
3.1. Data Collection and Preprocessing	29
3.2. GSVA of Non-Apoptotic Programmed Cell Death Mechanisms Across 15 Cancer Types with Paired Samples	32
3.3. Feature Engineering and Dimensionally Reduction	34
3.4. Visualization with UMAP and t-SNE	36
3.5. Multi-Class Machine Learning Models for Classifying Kidney Cancer Subtypes	37
3.6. Platform for Kidney Cancer Diagnosis Using Genes Linked to Non- Apoptotic Programmed Cell Death	39
4. RESULTS AND DISCUSSION	42
4.1. Regulation of Non-Apoptotic Cell Death Mechanisms in Cancer: Distinct Patterns Concentrated in Renal Malignancies	44
4.2. Machine Learning Clustering with Mechanism-Derived Features: Investigating Cancer Heterogeneity	52
4.3. Classification of Kidney Cancer Subtypes Using Non-Apoptotic Cell Death Mechanisms and XGBoost	55
4.4. Validation of a Web-Based XGBoost Model for Kidney Cancer Tissue Detection and Tumor/Normal Classification Using Non- Apoptotic Cell Death Mechanisms	63
4.5. Discussion and Future Directions	70
REFERENCES	73
BIOGRAPHY	81
PUBLICATIONS AND PRESENTATIONS FROM THE THESIS	82

LIST OF SYMBOLS AND ABBREVIATIONS

ACD	: Autophagic Cell Death
ACC	: Adrenocortical Carcinoma
BLCA	: Bladder Urothelial Carcinoma
BRCA	: Breast Invasive Carcinoma
CCRCC	: Clear Cell Renal Cell Carcinoma
CESC	: Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma
CHOL	: Cholangiocarcinoma
COAD	: Colon Adenocarcinoma
DLBC	: Lymphoid Neoplasm Diffuse Large B-cell Lymphoma
ESCA	: Esophageal Carcinoma
GBM	: Glioblastoma Multiforme
GSVA	: Gene Set Variation Analysis
HNSC	: Head and Neck Squamous Cell Carcinoma
KICH	: Kidney Chromophobe
KIRC	: Kidney Renal Clear Cell Carcinoma
KIRP	: Kidney Renal Papillary Cell Carcinoma
LAML	: Acute Myeloid Leukemia
LGG	: Brain Lower Grade Glioma
LIHC	: Liver Hepatocellular Carcinoma
LUAD	: Lung Adenocarcinoma
LUSC	: Lung Squamous Cell Carcinoma
MESO	: Mesothelioma
OV	: Ovarian Serous Cystadenocarcinoma
PAAD	: Pancreatic Adenocarcinoma
PCD	: Programmed Cell Death
PCPG	: Pheochromocytoma and Paraganglioma
PRAD	: Prostate Adenocarcinoma
READ	: Rectum Adenocarcinoma
SARC	: Sarcoma
SKCM	: Skin Cutaneous Melanoma
STAD	: Stomach Adenocarcinoma
TGCT	: Testicular Germ Cell Tumors
THCA	: Thyroid Carcinoma
THYM	: Thymoma
UCEC	: Uterine Corpus Endometrial Carcinoma
UCS	: Uterine Carcinosarcoma
UVM	: Uveal Melanoma
TCGA	: The Cancer Genome Atlas

LIST OF FIGURES

	<u>Page</u>
Figure 2.1: Necroptosis Mechanism with Pathway Genes	6
Figure 2.2: Functional Enrichment Analysis of Necroptosis-Related Genes	8
Figure 2.3: Pyroptosis with Pathway Genes	9
Figure 2.4: Functional Enrichment Analysis of Pyroptosis-Related Genes	12
Figure 2.5: Ferroptosis with Pathway Genes	14
Figure 2.6: Functional Enrichment Analysis of Ferroptosis-Related Genes	16
Figure 2.7: Parthanatos with Pathway Genes	17
Figure 2.8: Functional Enrichment Analysis of Parthanatos-Related Genes	19
Figure 2.9: Autophagic Cell Death with Pathway Genes	21
Figure 2.10: Functional Enrichment Analysis of ACD-Related Genes	22
Figure 2.11: Cuproptosis with Pathway Genes	24
Figure 2.12: Functional Enrichment Analysis of Cuproptosis Genes	26
Figure 3.1: Overall Workflow of the Study	28
Figure 3.2: Distribution of Paired Tumor and Normal Samples Across 15 Cancer Types	30
Figure 3.3: Distribution of Tumor and Normal Samples Across 33 Cancer Types	31
Figure 4.1: GSVA Score Differences and Statistical Significance Analysis of Necroptosis-Related Gene Sets Between Tumor and Normal Tissues	45
Figure 4.2: GSVA Score Differences and Statistical Significance Analysis of Pyroptosis-Related Gene Sets Between Tumor and Normal Tissues	47
Figure 4.3: GSVA Score Differences and Statistical Significance Analysis of Ferroptosis-Related Gene Sets Between Tumor and Normal Tissues	48
Figure 4.4: GSVA Score Differences and Statistical Significance Analysis of Parthanatos-Related Gene Sets Between Tumor and Normal Tissues	49
Figure 4.5: GSVA Score Differences and Statistical Significance Analysis of ACD-Related Gene Sets Between Tumor and Normal Tissues	50
Figure 4.6: GSVA Score Differences and Statistical Significance Analysis of Cuproptosis-Related Gene Sets Between Tumor and Normal Tissues	51
Figure 4.7: Normal Tissue-Specific Clustering of Normal Samples Across Cancer Types Using t-Sne and UMAP Dimensionality Reduction	53
Figure 4.8: Tumor Tissue-Specific Clustering of Normal Samples Across Cancer Types Using T-SNE and UMAP Dimensionality Reduction	54
Figure 4.9: Performance Comparison Between XGBoost, Random Forest, SVM, Gradient Boosting and KNN	55

Figure 4.10: Confusion Matrix of Multi-Class XGBoost Model for Kidney Cancer Subtype Classification	57
Figure 4.11: Confusion Matrix of Multi-Class XGBoost Model for Kidney Cancer Subtype Classification with Random Genes	58
Figure 4.12: Feature Importance Ranking Determined by XGBoost Feature Importance	60
Figure 4.13: Mechanism Importance Ranking of The XGBoost Model	60
Figure 4.14: Feature Importance Ranking Determined by SHAP	61
Figure 4.15: Mechanism Importance Ranking Determined by SHAP	61
Figure 4.16: Confusion Matrix for Multi-Class Tissue Detection - Kidney-Focused Analysis	64
Figure 4.17: Confusion Matrix for Kidney Normal vs. Tumor Classification	65
Figure 4.18: Web Application for Kidney Tissue and Tumor Prediction Using an XGBoost Model with Features Extracted from Non-Apoptotic Programmed Cell Death Mechanisms	67
Figure 4.19: The Analysis Result of a Dataset Other Than Kidney Cancer in the Web Application	69

LIST OF TABLES

	<u>Page</u>
Table 2.1: Necroptosis Pathway Genes	7
Table 2.2: Pyroptosis Pathway Genes	10
Table 2.3: Ferroptosis Pathway Genes	14
Table 2.4: Parthanatos Pathway Genes	18
Table 2.5: ACD Pathway Genes	21
Table 2.6: Cuproptosis Pathway Genes	25
Table 4.1: The Genes Associated with Necroptosis, Pyroptosis, Ferroptosis, Parthanatos, ACD and Cuproptosis	42
Table 4.2: Classification Report of Multi-Class XGBoost Model for Kidney Cancer Subtype Classification	57
Table 4.3: Classification Report of Multi-Class XGBoost Model for Kidney Cancer Subtype Classification with Random Genes	59
Table 4.4: Accuracy Rates of the 5 Runs Conducted with Random Genes for Kidney Cancer Subtype Classification	59
Table 4.5: Classification Report of Multi-Class XGBoost Model for Kidney Cancer Normal/Tumor Classification using all Data in TCGA	65
Table 4.6: Classification Report of Multi-Class XGBoost Model for Kidney Cancer Normal/Tumor Classification using Paired Data in TCGA	66

1. INTRODUCTION

Cell death in multicellular organisms is an essential biological process required for maintaining tissue homeostasis, regulating developmental processes, and eliminating damaged or infected cells **(Tang et al., 2019)**. Beyond its physiological roles, cell death plays a critical role in the onset and progression of various pathologies, including cancer, neurodegenerative diseases, and inflammatory disorders **(Galluzzi et al., 2018)**. Recent research has revealed the existence of numerous regulated cell death pathways beyond classical apoptosis, highlighting the complexity of this process.

The activation of cell death pathways is widely acknowledged as an effective approach in cancer therapy. Apoptosis, a thoroughly investigated mechanism of cell death, is induced by many anticancer agents to achieve their therapeutic outcomes. Nevertheless, resistance to apoptosis is a defining characteristic of cancer, enabling cancer cells to withstand chemotherapy. As a result, non-apoptotic modes of programmed cell death mechanisms (Necroptosis, Pyroptosis, Ferroptosis, Parthanatos, Autophagic Cell Death, Cuproptosis) have gained growing prominence in cancer biology and therapeutic development in recent years **(Chen et al., 2023)**. Mechanisms of programmed non-apoptotic cell death are sensitive to the balance between inhibitors and activators. Various inhibitors and activators of these pathways have been evaluated as targetable mechanisms in disease models **(Hadian & Stockwell, 2023)**.

This study addresses necroptosis, pyroptosis, ferroptosis, parthanatos, autophagic cell death, and the newly discovered cuproptosis. Each of these mechanisms plays varying roles in different tumor types, either inhibiting or supporting tumor progression. Particularly in cancer, targeting these alternative death pathways may enable the development of new treatment strategies. The objective of this study is to identify the diagnostic gene signatures associated with novel non-apoptotic cell death mechanisms using bioinformatics and machine learning methodologies.

The hypothesis proposed in this study posits that the balance of activators and inhibitors associated with non-apoptotic cell death, which is maintained in normal tissues, may be disrupted in tumor tissues. Consequently, if significant changes in the

expression levels of activator and inhibitor genes associated with non-apoptotic cell death mechanisms are observed in tumor tissues compared to normal tissues, this could indicate the malignant nature of the tumor and suggest that the regulation of cell death mechanisms has become disrupted in these tissues. Within this framework, using the TCGA pan-cancer dataset, cancer types exhibiting disrupted expression balance of activator and inhibitor genes associated with non-apoptotic cell death have been systematically identified.

Cancer is a highly heterogeneous group of diseases in terms of its biological mechanisms and clinical progression. Accurate identification of cancer types and subtypes is critically important for the development of effective treatment strategies. However, reliably classifying cancer subtypes with current methods remains a major challenge, particularly in the early stages of the disease. Therefore, machine learning approaches based on gene expression profiles have emerged as powerful tools for more precise and reliable identification of cancer subtypes. Gene expression data can reveal the molecular signatures of cancer, offering the potential to improve diagnostic processes and support personalized treatment approaches.

One of the primary objectives of this study is to develop machine learning models trained on biologically meaningful features to accurately distinguish between kidney cancer subtypes (KIRC, KIRP, KICH). Through the application of feature engineering, dimensionality reduction, and interpretable artificial intelligence approaches, the study reveals distinct molecular patterns specific to these subtypes.

Another objective of this study is to develop a web-based diagnostic platform that utilizes machine learning methodologies to detect kidney tissue and classify whether the tissue is tumorous, based on gene sets associated with non-apoptotic cell death mechanisms. The developed platform serves as a significant example of the integration of systems biology and artificial intelligence into clinical diagnostics. For this purpose, various classification algorithms—including XGBoost, Random Forest, Support Vector Machines (SVM), and K-Nearest Neighbours (KNN)—were compared, and XGBoost was selected based on its superior cross-validation performance. The model's performance was evaluated using metrics such as accuracy, precision, and F1-score, and its interpretability was enhanced through SHAP (SHapley Additive exPlanations)

analysis. This enabled an in-depth examination of the gene sets and mechanisms that contributed most significantly to the classification process.

In summary, this study consists of three parts investigating the roles of non-apoptotic programmed cell death (PCD) mechanisms in cancer biology.

In the first part, the transcriptomic regulation of activator and inhibitor genes of alternative cell death mechanisms such as necroptosis, pyroptosis, ferroptosis, parthanatos, autophagic cell death, and cuproptosis was analyzed at a pan-cancer level. Differences in the activity of these pathways between tumor and normal tissues were identified using GSVA and statistical analyses. This analysis aims to determine whether these cell death mechanisms involve tissue-specific or tumor-specific regulatory disruptions in cancer development.

In the second part, the focus was on kidney cancer subtypes (KIRC, KIRP, KICH). Multi-class machine learning models were developed, trained with biologically significant features (e.g., mean expression, PCA components, and entropy) derived from the aforementioned pathways. These models classify kidney cancer subtypes with high accuracy and provide biological interpretability through SHAP analyses.

In the third and final part, a transcriptome-based diagnosis model was implemented via a Flask-based web platform, tested with external datasets, and a real-time decision support system capable of identifying kidney tissue and distinguishing between normal and tumor tissues was established. Thus, the theoretical aspects of the study were complemented by a concrete, clinically applicable software product. Through this three-pronged structure, the thesis offers a holistic approach encompassing biological discovery, classification performance, and software application dimensions.

2. NON APOPTOTIC PROGRAMMED CELL DEATH MECHANISMS

Although the concept of cell death has been recognized historically for a long time, its modern understanding was shaped by the work of Kerr, Wyllie, and Currie in 1972, which introduced the term “apoptosis” into the scientific literature **(Kerr et al., 1972)**. Apoptosis is characterized by distinct morphological features such as cell shrinkage, chromatin condensation, and the formation of apoptotic bodies, representing a genetically programmed, active process **(Galluzzi et al., 2018)**. This non-immunogenic mechanism is fundamental to development, tissue balance, and the removal of damaged cells. Today, it is recognized as just one of several regulated cell death pathways, each with its own unique molecular mechanisms and physiological effects.

Cell death can be generally divided into two principal categories: programmed cell death (PCD) and accidental (unprogrammed) cell death. PCD is a genetically controlled, energy-dependent process governed by specific signalling pathways and molecular regulators. In contrast, accidental cell death, such as necrosis, results from extreme physical, chemical, or mechanical stress and lacks the molecular regulation observed in PCD **(Galluzzi et al., 2018; Tang et al., 2019)**.

Programmed cell death can be further subdivided into apoptotic and non-apoptotic forms based on morphological and biochemical characteristics. While apoptosis is often regarded as the prototype of PCD, the programmed nature of non-apoptotic cell death pathways is frequently questioned. This confusion may arise because the term “programmed” is sometimes associated solely with apoptosis. However, non-apoptotic cell death pathways—such as necroptosis, pyroptosis, ferroptosis, or cuproptosis—are also programmed, as they are controlled by specific molecular mechanisms rather than occurring randomly, and they serve physiological purposes. For instance, necroptosis is regulated by the RIPK1/RIPK3 pathway, pyroptosis by gasdermin proteins, and cuproptosis by copper ion interactions with mitochondrial proteins. Thus, despite their often-inflammatory outcomes, these processes are active, genetically orchestrated events and fall under the umbrella of PCD **(Galluzzi et al., 2018)**.

Over the past decade, significant progress has been made in identifying and characterizing non-apoptotic cell death pathways that differ from classical apoptosis in mechanism and function. These pathways include necroptosis, pyroptosis, ferroptosis, parthanatos, autophagic cell death, and the recently identified cuproptosis (Tang et al., 2019; Tsvetkov et al., 2022; Nafe and Hattingen, 2024). While apoptosis dominates developmental processes, non-apoptotic pathways provide alternative elimination mechanisms, particularly in pathological conditions like cancer and infectious diseases where apoptosis may be insufficient (Galluzzi et al., 2018).

2.1. Necroptosis and Related Genes

Necroptosis is a form of programmed necrosis regulated by receptor-interacting protein kinases RIPK1 and RIPK3. Unlike apoptosis, necroptosis is immunogenic, resulting in membrane rupture and the release of intracellular contents, thereby triggering strong inflammatory responses. This mode of cell death is typically induced by the activation of death receptors, such as tumor necrosis factor (TNF) receptors, and serves as an alternative mechanism for cell elimination, especially under conditions where apoptosis is inhibited (Galluzzi et al., 2017; Tang et al., 2019).

The relationship between necroptosis and cancer is bidirectional; it can exert both tumor-suppressive and tumor-promoting effects. This duality depends on the cancer type, the tumor microenvironment, and the regulation of cell death pathways (Yan et al., 2022). Resistance to apoptosis represents a major obstacle in cancer therapy. Necroptosis provides an alternative cell death pathway by targeting apoptosis-resistant cancer cells. For instance, one study demonstrated that necroptosis effectively overcame apoptosis resistance and enhanced anti-tumor immunity (Gong et al., 2019).

By promoting the release of intracellular contents, necroptosis activates the immune system and may enhance the immunogenicity of tumor cells. However, chronic necroptosis may lead to the formation of a pro-inflammatory microenvironment, potentially supporting tumor progression (Yan et al., 2022). A review emphasized that while therapy-induced necroptosis can trigger anti-tumor immune responses, it must be carefully regulated (Yan et al., 2022).

Understanding necroptosis is of critical importance for the development of potential therapeutics targeting cancer and inflammatory diseases. RIPK3 plays significant roles

in cancer and potentially in cancer therapy by inducing cancer cell death, suppressing tumor growth, and/or enhancing effective anti-cancer immune responses (**Morgan and Kim, 2025**).

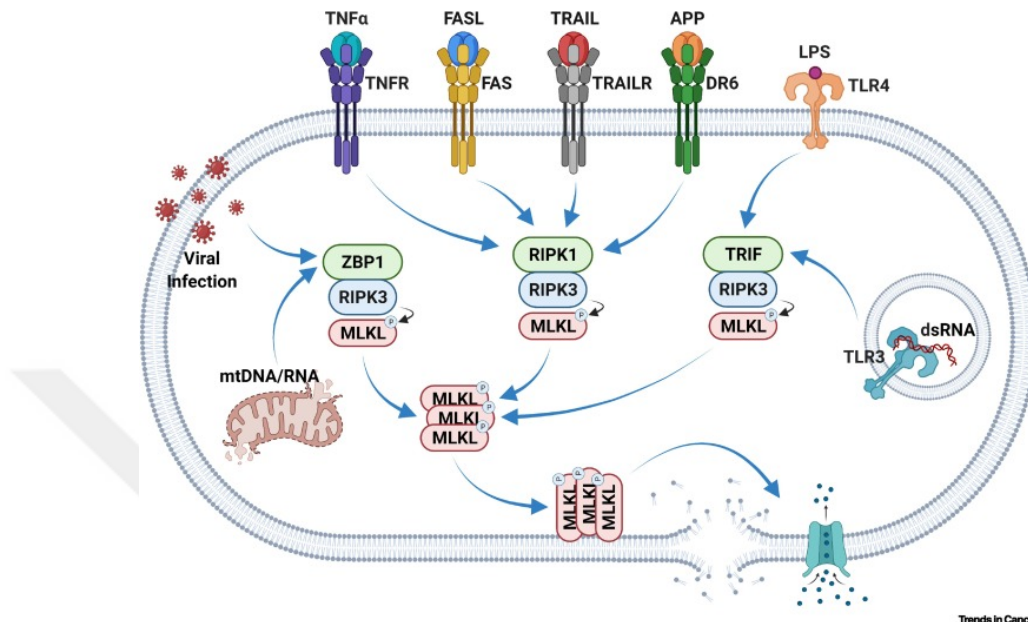


Figure 2.1: Necroptosis Mechanism with Pathway Genes (Yan et al., 2022)

The genes associated with necroptosis, including activators, inhibitors, and parallel effectors, along with their respective roles, were identified through a literature review and are presented in Table 2.1. Activators are genes that initiate or trigger cell death pathways. Inhibitors are genes that suppress this process, preventing cell death. Effector genes, on the other hand, are responsible for physically executing the death process after the pathway is initiated; in other words, they are the genes that carry out the final stage of the process by perforating the cell membrane, fragmenting DNA, or directly causing metabolic disruption.

Table 2.1: Necroptosis Pathway Genes

Gene	Role	Description
RIPK1	Activator	Forms a complex with RIPK3 after TNFR1 signalling to initiate necroptosis (Yao et al., 2025).
RIPK3	Activator	Interacts with RIPK1 and phosphorylates MLKL, transmitting the necroptotic signal (Yao et al., 2025).
ZBP1	Activator	Can activate RIPK3 independently of RIPK1 in response to viral DNA (Yao et al., 2025).
TNF	Activator	Triggers RIPK1 activation via TNFR1 (Yao et al., 2025).
CYLD	Activator	Deubiquitinates RIPK1, enhancing its pro-necroptotic activity; promotes necrosome formation (Yao et al., 2025; Ye et al., 2023).
TICAM1/TRIF	Activator	Activates RIPK3 through TLR3/4 (Morgan et al., 2022).
CASP8	Inhibitor	Cleaves RIPK1/RIPK3, preventing necroptosis and directing cells towards apoptosis (Li et al., 2022).
FADD	Inhibitor	Forms a complex with CASP8, thereby inhibiting necroptosis (Li et al., 2022).
cFLIP	Inhibitor	Modulates CASP8 activity, potentially halting necroptosis (Li et al., 2022).
MLKL	Effector	Phosphorylated by RIPK3, forming pores in the membrane (Li et al., 2022).

The genes associated with necroptosis, as identified in Table 2.1, are categorized as follows: activators include RIPK1, RIPK3, ZBP1, TNF, CYLD, and TICAM1; inhibitors consist of CASP8, FADD, and cFLIP; and the parallel effector is MLKL.

In this study, a functional enrichment analysis was conducted using the g:Profiler platform to evaluate whether the analyzed gene sets are biologically associated with the corresponding cell death mechanisms. As a result of this analysis, the selected genes were found to be significantly associated with various biological processes across different Gene Ontology (GO) categories.

Figure 2.2 presents the functional enrichment analysis of genes related to necroptosis, as obtained from the literature. Notably, the term “necroptotic process” yielded a highly significant FDR-adjusted p-value, strongly supporting the direct involvement of the analyzed gene set in the necroptosis mechanism. Similarly, other death-related signalling terms such as “death-inducing signalling complex” and “tumor necrosis factor receptor superfamily binding” also showed significant enrichment. These results are consistent with previous studies indicating that the genes derived from the literature play critical roles in the necroptosis pathway.

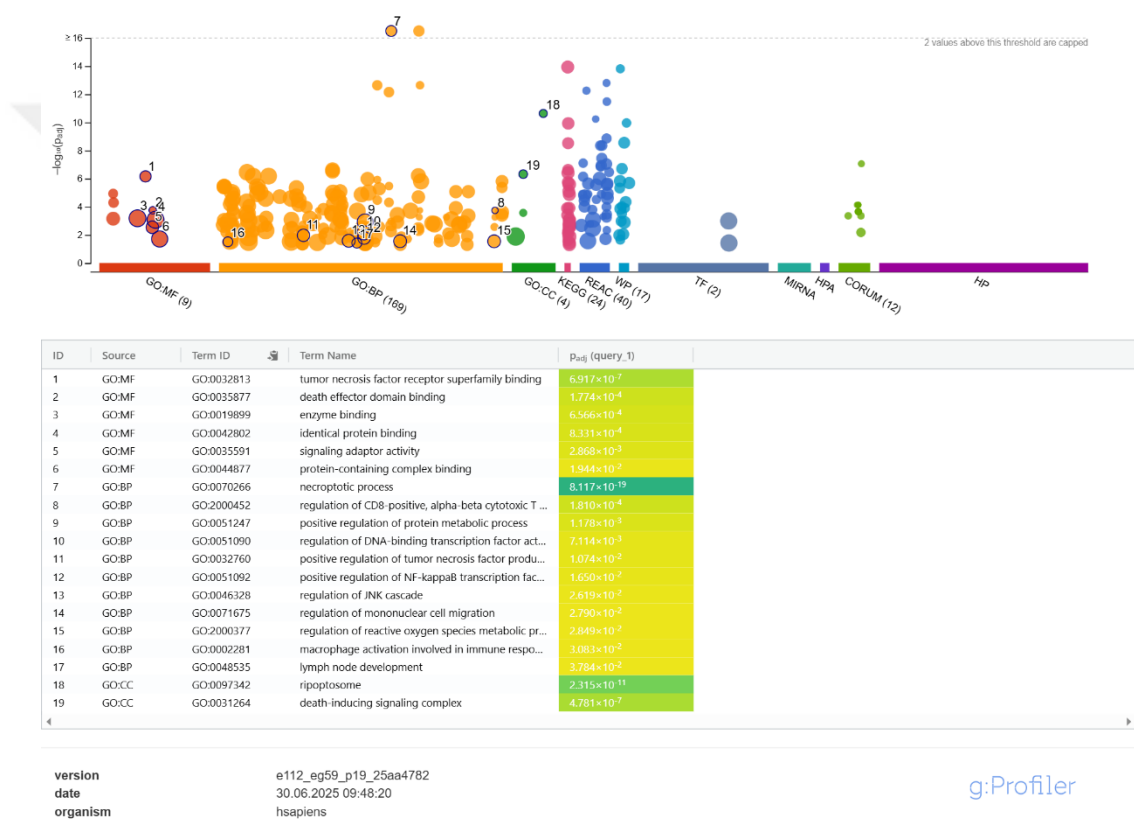


Figure 2.2: Functional Enrichment Analysis of Necroptosis-Related Genes

Additionally, the significant enrichment observed in processes such as signalling adaptor activity, enzyme binding, macrophage activation, and regulation of NF-κB transcription indicates that cell death mechanisms operate in coordination with immune responses and signalling networks. These findings demonstrate that the analyzed genes are biologically relevant to the associated cell death mechanisms not only at the genetic level but also at the functional level.

2.2 Pyroptosis and Related Genes

Pyroptosis is an inflammatory type of programmed cell death (PCD) that, unlike apoptosis, leads to the rupture of the cell membrane and the release of inflammatory cytokines (e.g., IL-1 β and IL-18). It was first described in 2001, but in recent years, research on its association with cancer has increased. Pyroptosis primarily plays a role as a defence mechanism against pathogens in immune cells (macrophages, dendritic cells). In the context of cancer, pyroptosis can exhibit both tumor-suppressive and tumor-promoting effects (Shi et al., 2017; Fang et al., 2020; Man & Kanneganti, 2015; Xia et al., 2019).

Pyroptosis is typically triggered by inflammatory caspases. The process begins with the activation of multiprotein complexes called inflammasomes, which detect pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Inflammasome activation leads to the cleavage of caspase-1, which then cleaves the gasdermin D (GSDMD) protein. The N-terminal fragment of GSDMD forms pores in the cell membrane, causing cell swelling and rupture, thereby releasing cytokines (Broz & Dixit, 2016; Zheng et al., 2020; Ding et al., 2016).

Recent studies have demonstrated that pyroptosis can be utilized as an anti-tumor immunity mechanism and holds clinical potential. Many researchers are exploring ways to combine pyroptosis with other tumor therapies, aiming to regulate the pyroptosis mechanism to suppress tumor cell proliferation, migration, and invasion, thereby treating cancers (Wei et al., 2022).

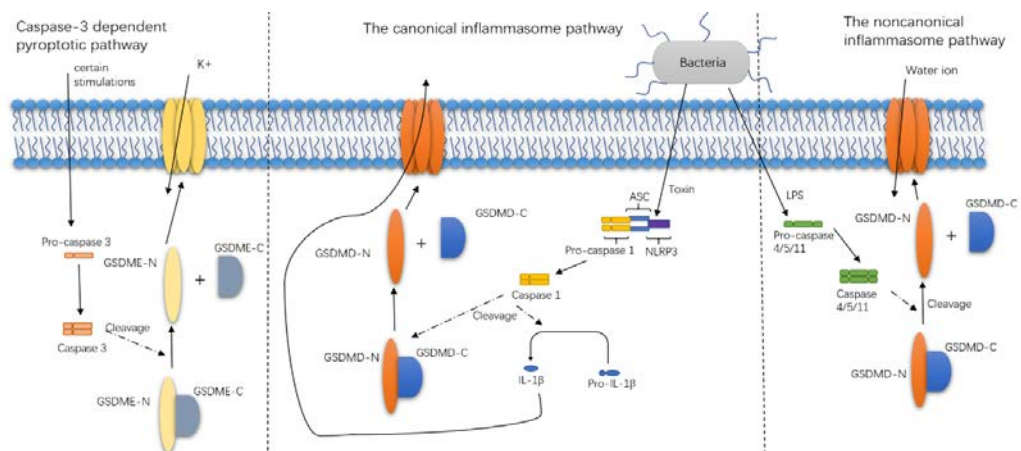


Figure 2.3: Pyroptosis with Pathway Genes (Fang et al., 2020)

The genes associated with pyroptosis, including activators, inhibitors, and parallel effectors, along with their respective roles, were identified through a literature review and are presented in Table 2.2.

Table 2.2: Pyroptosis Pathway Genes

Gene	Role	Description
CASP1	Activator	Activated by inflammasome; cleaves GSDMD, IL-1 β , IL-18 (Eckhart & Fischer, 2024).
CASP4	Activator	Binds LPS directly; initiates non-canonical pyroptosis, cleaves GSDMD (Eckhart & Fischer, 2024).
CASP5	Activator	Works with CASP4; cleaves GSDMD (Eckhart & Fischer, 2024).
NLRP3	Activator	Sensor protein; triggers CASP1 activation (Wei et al., 2022).
AIM2	Activator	Detects cytoplasmic DNA; forms inflammasome (Wei et al., 2022).
NLRC4	Activator	Detects bacterial components; triggers inflammasome (Wei et al., 2022).
GZMA	Activator	Cleaves GSDMB; initiates pyroptosis (Yu et al., 2021).
GZMB	Activator	Cleaves GSDME; induces pyroptosis (Yu et al., 2021).
PYRIN	Activator	Detects Rho GTPase changes; activates CASP1 (Yu et al., 2021).
ASC (PYCARD)	Activator	Adaptor linking inflammasome sensors to CASP1 (Yu et al., 2021).
NLRP1	Activator	Detects toxins; activates CASP1 (Yu et al., 2021).
CARD16 (COP)	Inhibitor	Suppresses CASP1 activation (Devi et al., 2020).
CARD18 (ICEBERG)	Inhibitor	Competes with CASP1; reduces inflammation (Devi et al., 2020).
NLRX1	Inhibitor	Negatively regulates NLRP3 activation (Liu et al., 2022).
POP1/POP2	Inhibitor	Blocks ASC binding; prevents inflammasome formation (Ratsimandresy et al., 2017).
CASP8	Inhibitor	Cleaves GSDMD; promotes apoptosis, inhibits pyroptosis (Hughes et al., 2023).

Table 2.2 (Continued): Pyroptosis Pathway Genes

XIAP	Inhibitor	Inhibits CASP3, CASP7; indirectly suppresses pyroptosis (Hughes et al., 2023) .
BCL2	Inhibitor	Prevents mitochondrial damage; inhibits NLRP3 (Shi et al., 2019) .
IL10	Inhibitor	Anti-inflammatory; reduces inflammasome activity (Wang et al., 2018) .
TNF	Effector	Activates inflammatory genes via NF- κ B; indirect pyroptosis effect (Jin et al., 2023) .
CASP3	Effector	Cleaves GSDME; converts apoptosis to pyroptosis (Jin et al., 2023) .
GSDMD	Effector	Main pyroptosis executor; forms pores (Jin et al., 2023) .
GSDME	Effector	Cleaved by CASP3, GZMB; forms pores (Jin et al., 2023) .
GSDMB	Effector	Cleaved by GZMA; forms pores (Jin et al., 2023) .
GSDMC	Effector	Induced by TNF- α ; triggers pyroptosis with CASP8 (Jin et al., 2023) .
IL1B	Effector	Processed by CASP1; enhances inflammation (Jin et al., 2023) .
IL18	Effector	Processed by CASP1; boosts immune response (Jin et al., 2023) .
P2X7R	Effector	ATP-dependent ion channel; activates NLRP3 (Kong et al., 2022) .
ESCRT-III	Effector	Repairs membrane; delays GSDMD pore formation (Li et al., 2022) .

The genes associated with pyroptosis, as identified in the Table 2.2, are categorized as follows: activators include CASP1, CASP4, CASP5, NLRP3, AIM2, NLRC4, GZMA, GZMB, PYRIN, ASC (PYCARD), and NLRP1; inhibitors consist of CARD16 (COP), CARD18 (ICEBERG), NLRX1, POP1/POP2, CASP8, XIAP, BCL2, and IL10; parallel effectors are TNF and CASP3, GSDMD, GSDME, GSDMB, GSDMC, IL1B, IL18, P2X7R, and ESCRT-III.

Figure 2.4 presents the functional enrichment analysis for the gene set identified as associated with pyroptosis, clearly revealing that the genes are strongly and significantly linked to this particular cell death mechanism at the biological level.

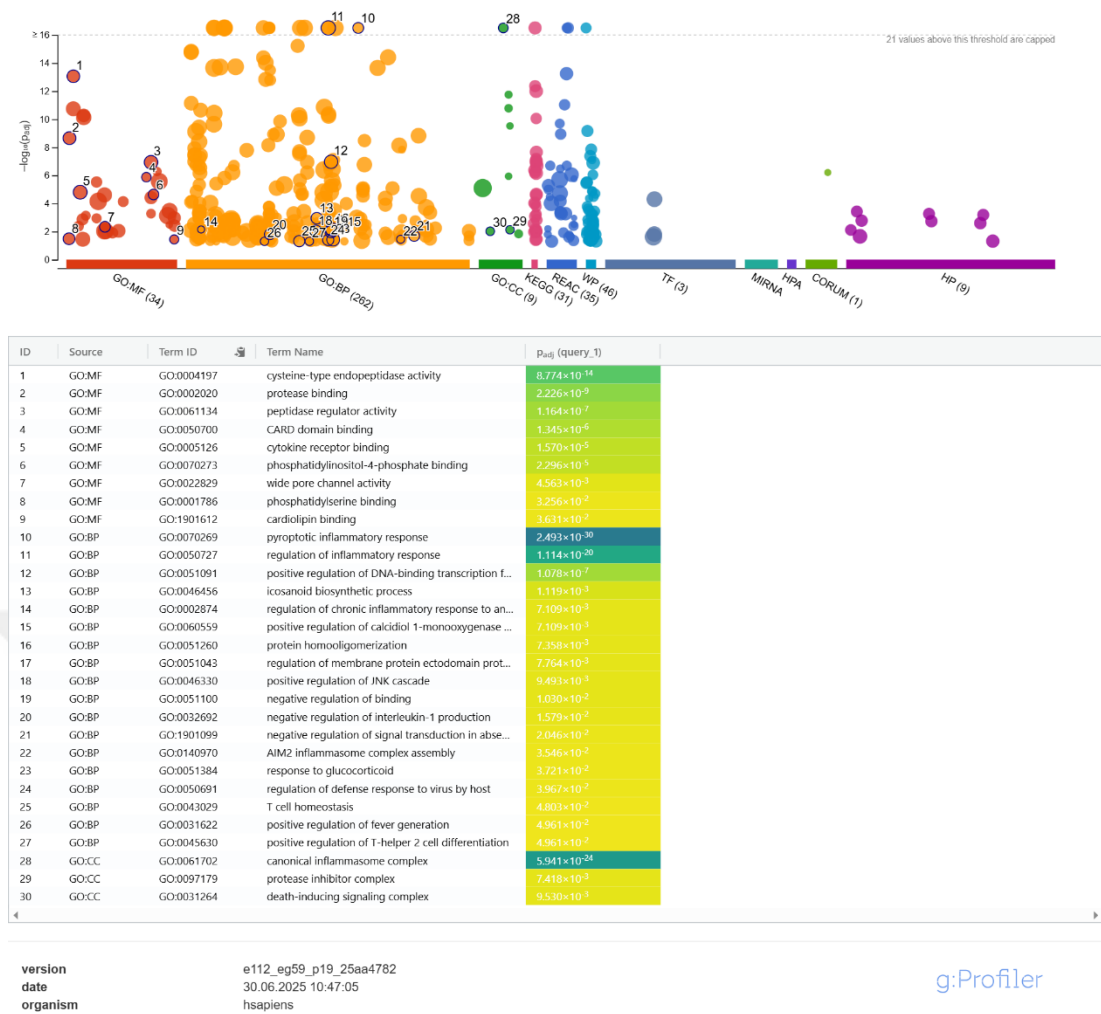


Figure 2.4: Functional Enrichment Analysis of Pyroptosis-Related Genes

In particular, the strong enrichment of terms such as "pyroptotic inflammatory response" indicates that the analyzed genes are actively involved in the stages of the pyroptosis process.

2.3 Ferroptosis and Related Genes

Ferroptosis is an iron-dependent form of programmed cell death characterized by the accumulation of lipid peroxidation (Yan et al., 2021). Identified in 2012, it differs biochemically, genetically, and morphologically from other cell death types like apoptosis and necrosis (Dixon et al., 2012). Its core mechanism involves iron accumulation and increased reactive oxygen species (ROS) in cells, leading to oxidative damage of lipid membranes and ultimately cell death (Stockwell et al., 2017).

Ferroptosis is triggered by elevated intracellular free iron (Fe^{2+}) levels; iron catalyzes ROS production via the Fenton reaction, initiating lipid peroxidation **(Yan et al., 2021)**. Ferroptosis involves several key mechanisms. Lipid peroxidation occurs when phospholipids containing polyunsaturated fatty acids (PUFAs) undergo oxidation, a process facilitated by enzymes like ACSL4 and LPCAT3, which integrate PUFAs into membranes. The collapse of antioxidant defences plays a critical role; glutathione peroxidase 4 (GPX4) inhibits ferroptosis by reducing lipid peroxides, but glutathione (GSH) deficiency or GPX4 inhibition triggers it **(Li et al., 2020)**. Additionally, blocking cysteine uptake through the system xc- (SLC7A11) transporter reduces GSH synthesis, accelerating ferroptosis **(Lee et al., 2022)**. Other pathways also regulate ferroptosis independently of GPX4, such as FSP1/CoQ10 and DHODH; for example, FSP1 reduces coenzyme Q10 (ubiquinone) to prevent lipid peroxidation **(Bersuker et al., 2019)**.

In ferroptotic cells, mitochondria shrink, membrane density increases, and cristae structures diminish or disappear; however, unlike apoptosis, there is no caspase activation, and unlike necrosis, there is no cell swelling **(Pedrera et al., 2025)**. Ferroptosis holds potential for tumor suppression, particularly in drug-resistant cancer cells (e.g., mesenchymal-state cells), which are more susceptible to ferroptosis **(Zhang et al., 2022)**.

Pharmacologically targeting ferroptosis offers significant promise for cancer therapy. However, realizing the clinical potential of ferroptosis drugs requires overcoming additional challenges in future research. First, the lack of well-developed animal models to assess ferroptosis in cancer in vivo poses a major obstacle. Second, the complex biological effects of ferroptosis in cancer present another challenge; in some cases, inducing ferroptosis may initially promote tumor formation while later causing tumor cell death and suppression. Third, the absence of effective drugs that can safely and specifically trigger ferroptosis in cancer cells is a serious issue. Fourth, inducing ferroptosis may negatively impact anti-tumor immunity, hindering complete tumor eradication. Finally, identifying the patient groups most likely to benefit from ferroptosis therapy is critical for successful clinical trials. Overcoming these challenges will fully unlock ferroptosis's potential in cancer treatment **(Zhou et al., 2024)**.

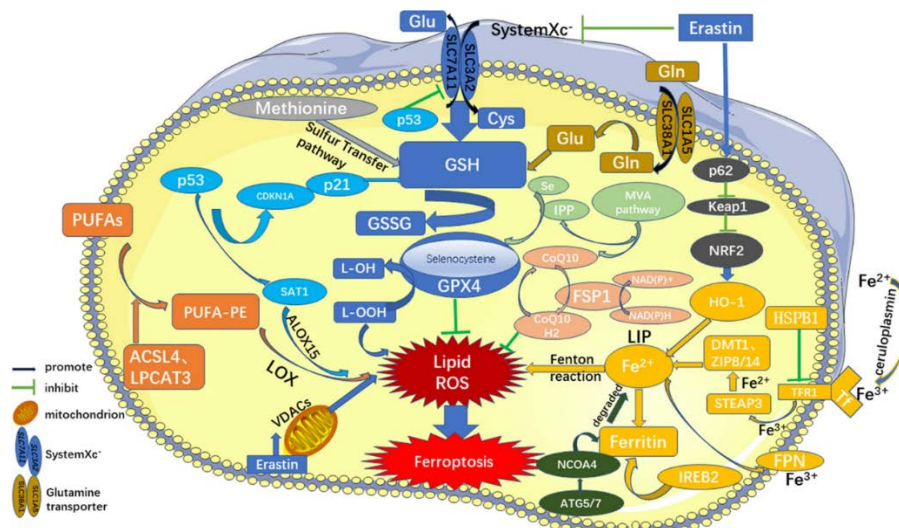


Figure 2.5: Ferroptosis with Pathway Genes (Li et al., 2020)

The genes associated with ferroptosis, including activators, inhibitors, and parallel effectors, along with their respective roles, were identified through a literature review and are presented in Table 2.3.

Table 2.3: Ferroptosis Pathway Genes

Gene	Role	Description
ALOX15	Activator	Initiates lipid peroxidation; triggers ferroptosis signalling (Tang et al., 2021).
ACSL4	Activator	Esterifies PUFA-PEs; targets lipids for peroxidation.
LPCAT3	Activator	Incorporates PUFAs into membranes, enhances lipid peroxidation with ACSL4 (Tang et al., 2021).
TFRC	Activator	Facilitates iron uptake; increases ROS production, triggers ferroptosis (Tang et al., 2021).
NOX1/NOX4	Activator	NADPH oxidases: produce ROS, enhance lipid peroxidation (Tang et al., 2021).
SAT1	Activator	P53 target gene; enhances lipid peroxidation and promotes ferroptosis (Tang et al., 2021).
P53	Activator/Inhibitor	Context-dependent; can enhance or inhibit ferroptosis via SLC7A11 and GPX4 (Tang et al., 2021).
GPX4	Inhibitor	Main ferroptosis suppressor; halts lipid peroxidation (Li et al., 2020).

Table 2.3 (Continued): Ferroptosis Pathway Genes

SLC7A11 (xCT)	Inhibitor	Imports cystine for glutathione synthesis; supports GPX4 function (Lee et al., 2022).
GCLC, GCLM, GSS	Inhibitor	Genes involved in glutathione synthesis; indirectly inhibit ferroptosis (Tang et al., 2021).
FTH1 / FTL	Inhibitor	Ferritin chains; sequester iron, reducing ROS formation (Tang and Kang, 2024).
NFE2L2 (NRF2)	Inhibitor	Antioxidant transcription factor; activates GPX4, FTH1, SLC7A11(Tang and Kang, 2024).
MT1G	Inhibitor	Binds metal ions; antioxidant activity, suppresses ferroptosis (Tang and Kang, 2024).
CDKN1A (p21)	Inhibitor	P53 target; protects cells by suppressing ferroptosis (Tang and Kang, 2024).
LOX	Effector	Catalyses lipid peroxidation (Jiang et al., 2021).
POR	Effector	Promotes lipid peroxidation through electron transfer (Jiang et al., 2021).

The genes associated with ferroptosis, as identified in the Table 2.3, are categorized as follows: activators include ALOX15, ACSL4, LPCAT3, TFRC, NOX1/NOX4, and SAT1; inhibitors consist of GPX4, SLC7A11 (xCT), GCLC, GCLM, GSS, FTH1/FTL, NFE2L2 (NRF2), MT1G, and CDKN1A (p21); and effectors include LOX and POR. The p53 protein plays a complex and context-dependent role in ferroptosis. Research has shown that p53 can function both as an activator and an inhibitor of ferroptosis, depending on the cellular context. For instance, a study by (**Tang et al., 2021**) demonstrated that p53 can induce ferroptosis by suppressing the expression of SLC7A11, a cystine/glutamate antiporter, thereby weakening the cell's antioxidant defence mechanisms. This highlights p53's role as a pro-ferroptotic activator. On the other hand, p53 can also act protectively under certain conditions by enhancing the expression of GPX4 or activating pathways that limit lipid peroxidation, thus inhibiting ferroptosis. Therefore, p53's influence on ferroptosis varies based on factors such as cell type, stress levels, and interactions with other molecules. These findings underscore p53's multifaceted regulatory role in cell death mechanisms, extending beyond its traditional function as a tumor suppressor. Therefore, p53 has

been included in both the activator and inhibitor gene sets within the ferroptosis pathway in analyses due to its dual regulatory role.

Figure 2.6 shows the enrichment output for the gene set identified as associated with ferroptosis, and the results demonstrate that these genes have a strong functional association with the ferroptosis mechanism.

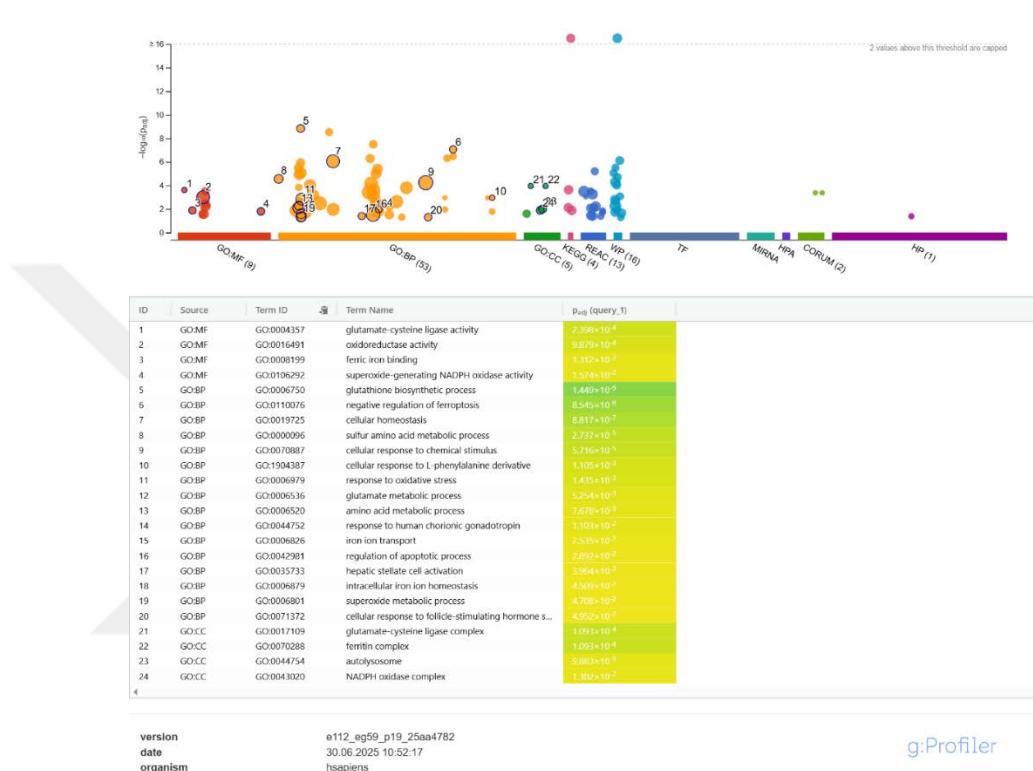


Figure 2.6: Functional Enrichment Analysis of Ferroptosis-Related Genes

As a result of the analysis, the term “negative regulation of ferroptosis” was found to be statistically significant, indicating that the analyzed genes are directly involved in the regulation of the ferroptosis process. In addition, the significant enrichment of terms such as “glutathione biosynthetic process”, “cellular response to oxidative stress”, “superoxide-generating NADPH oxidase activity”, and “ferritin complex” demonstrates a strong association between these genes and key components of ferroptosis, including iron metabolism, the glutathione system, and reactive oxygen species (ROS) generation. Moreover, molecular functions such as “glutamate-cysteine ligase activity”, “intracellular iron ion homeostasis”, and “oxidoreductase activity” are consistent with processes that either trigger or suppress ferroptosis. These findings

strongly support that the analyzed gene set is not only theoretically but also functionally and biologically connected to the core components of the ferroptosis mechanism.

2.4 Parthanatos and Related Genes

Parthanatos is a caspase-independent, poly (ADP-ribose) polymerase 1 (PARP-1)-dependent programmed cell death pathway. This process is distinct from apoptosis, necrosis, or other known forms of cell death, and is typically triggered by excessive activation of PARP-1 in response to DNA damage. Parthanatos is characterized by a cell death mechanism involving accumulation of PAR (poly (ADP-ribose)) and translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus. This pathway is characterized by large-scale DNA fragmentation, chromatin condensation, and is associated with energy depletion (loss of NAD⁺ and ATP) (Huang et al., 2022). The mechanism of Parthanatos primarily involves excessive PARP-1 activation, PAR accumulation, binding of PAR to AIF, release of AIF from mitochondria, nuclear translocation of the AIF/MIF complex, and large-scale DNA fragmentation mediated by MIF (Liu et al., 2022). Its name is derived from "Thanatos," the Greek god of death, and plays a significant role especially in conditions such as cancer, neurodegenerative diseases like Alzheimer's and Parkinson's, and ischemic injury (Zhang et al., 2025).

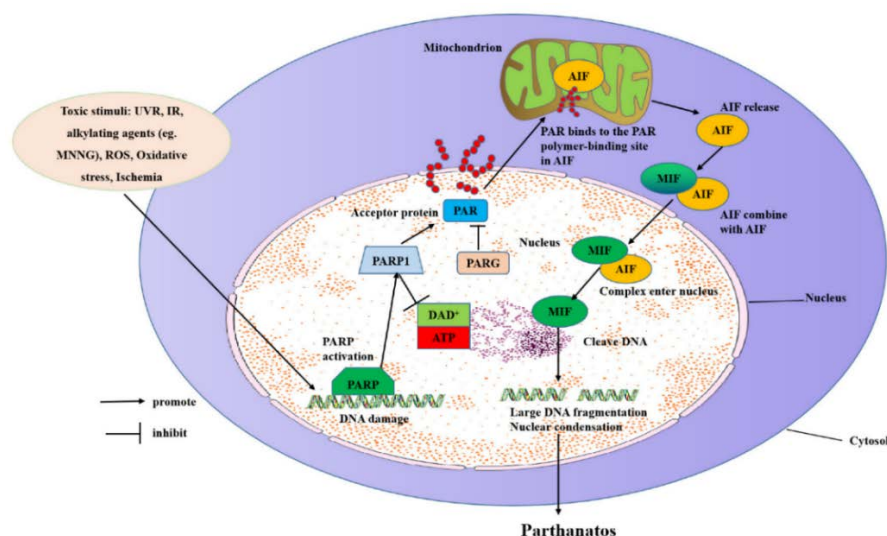


Figure 2.7: Parthanatos with Pathway Genes (Huang et al., 2022)

The genes associated with parthanatos, including activators, inhibitors, and parallel effectors, along with their respective roles, were identified through a literature review and are presented in Table 2.4.

Table 2.4: Parthanatos Pathway Genes

Gene	Role	Description
PARP1	Activator	Activated by DNA damage; causes energy depletion and AIF release (Song et al., 2024).
AIFM1 (AIF)	Activator	Released from mitochondria after PARP1 activation; initiates chromatin condensation and DNA fragmentation (Song et al., 2024).
MIF	Activator	Interacts with AIF; amplifies parthanatos (Song et al., 2024).
MACROD1 / MACROD2	Inhibitor	Hydrolyze PAR polymers; inhibit mitochondrial AIF release (Moura et al., 2024).
NUDT16	Inhibitor	Clears PAR fragments; reduces cytotoxicity (Martínez-Morcillo et al., 2021; Arroyo et al., 2023).
ARH3	Inhibitor	Degrades PAR chains; suppresses parthanatos (Mashimo et al., 2019).
SIRT1	Inhibitor	Inhibits PARP1 via deacetylation; involved in energy balance (Zhang et al., 2025).
PARG	Effector	Cleaves PAR polymers; imbalance can either protect or trigger parthanatos (Moura et al., 2024).
XRCC1	Effector	DNA repair factor: deficiency increases susceptibility to parthanatos (Zhang et al., 2025).
H2AX (γ H2AX)	Effector	Phosphorylated in DNA damage; used as DNA damage marker (Zhang et al., 2025).

The genes associated with parthanatos, as identified in the Table 2.4, are categorized as follows: activators include PARP1, AIFM1 (AIF), and MIF; inhibitors consist of MACROD1/MACROD2, NUDT16, ARH3, and SIRT1; and effectors include PARG, XRCC1, and H2AX (γ H2AX).

Figure 2.8 shows the enrichment analysis output for the gene set associated with parthanatos, confirming that these genes are significantly linked to the parthanatos cell death mechanism at both the biological and molecular levels.



Figure 2.8: Functional Enrichment Analysis of Parthanatos-Related Genes

The significant enrichment of terms such as “poly-ADP-D-ribose binding”, “damaged DNA binding”, and “DNA damage response” indicates a direct association between the analyzed gene set and the core components of the parthanatos process, including DNA damage, PARP1 activation, and PAR polymer formation. Additionally, molecular functions such as “ADP-ribosyl/glutamate hydrolase activity” and “deacetylase activity” support the roles of regulatory genes like PARG, MACROD1/2, and SIRT1 in this cell death mechanism. The enrichment of cellular components such as “site of DNA damage” and “chromosome, telomeric region” further suggests that these genes are active in damaged genomic regions and participate in AIF-mediated DNA degradation. Collectively, these findings confirm that the analyzed genes are not only supported by existing literature but are also statistically and functionally integrated into the parthanatos pathway.

2.5 Autophagic Cell Death and Related Genes

Autophagic Cell Death (ACD) is a type of programmed cell death occurring through autophagy, characterized by the breakdown of cellular components via lysosomes (**Galluzzi et al., 2018**). Although autophagy generally functions as a survival mechanism, under specific conditions, its excessive activation can lead to cell death (**Denton & Kumar, 2019**). ACD involves key autophagic steps, including autophagosome formation, fusion with lysosomes, and subsequent degradation of contents (**Levine & Kroemer, 2019**). This process begins with the inhibition of the mTOR (mechanistic target of rapamycin) complex; for example, in nutrient-deprivation conditions, decreased mTOR activity activates the ULK1 complex, initiating autophagosome formation (**Kim & Guan, 2019**). Subsequently, autophagy-related (Atg) proteins, particularly Atg5 and Atg7, facilitate the elongation and closure of the autophagosomal membrane (**Mizushima et al., 2020**). Once autophagosomes fuse with lysosomes, the enclosed materials are degraded by hydrolytic enzymes, and this excessive degradation can result in ACD (**Galluzzi et al., 2018**).

Autophagic cell death is suggested to play roles in both physiological and pathological processes, with reduced autophagic activity contributing significantly to cancer development. Deficiencies in autophagy-related proteins such as Beclin-1, Atg5, LC3, and Fip200 have been associated with various cancer types, and impaired autophagy is thought to promote tumor progression through mechanisms like NF- κ B activation, oxidative stress, and increased mutation rates. Additionally, decreased JNK activity can prevent autophagic cell death, resulting in uncontrolled cellular proliferation and acquisition of a malignant phenotype. Since current cancer therapies mainly target apoptosis, developing novel anticancer agents aimed at autophagic cell death has gained importance. Compounds capable of stimulating autophagy with potent anticancer effects have been identified, and optimizing these into pharmaceutical forms could provide effective new therapeutic options for resistant cancer types (**Shimizu, 2015**). In the past 15-20 years, studies elucidating autophagy's role in cancer and its potential as a therapeutic target have significantly accelerated (**Debnath et al., 2023**).

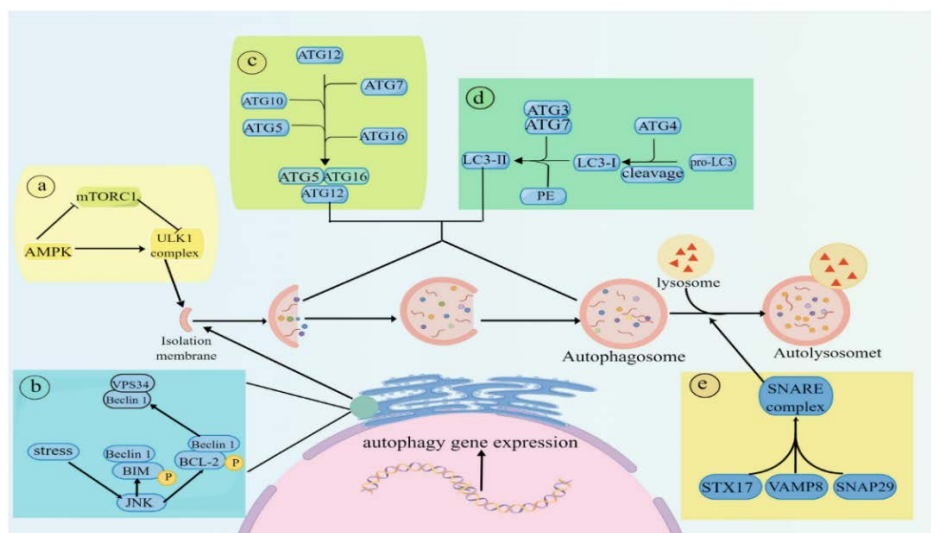


Figure 2.9: Autophagic Cell Death with Pathway Genes (Liu et al., 2023)

The genes associated with ACD, including activators, inhibitors, and parallel effectors, along with their respective roles, were identified through a literature review and are presented in Table 2.5.

Table 2.5: ACD Pathway Genes

Gene	Role	Description
BECN1 (Beclin-1)	Activator	Forms complex with Vps34 to initiate autophagy; may trigger cell death (Linder & Kögel, 2019).
ATG5	Activator	Key regulator of autophagosome formation (Changotra et al., 2022).
ATG7	Activator	Critical for LC3 and ATG12 conjugation; essential for autophagic flux (Collier et al., 2021).
ULK1	Activator	Initiates autophagy when mTOR is inhibited (Liu et al., 2023).
VPS34	Activator	Forms lipid kinase complex with Beclin-1; initiates autophagy (Liu et al., 2023).
FOXO3	Activator	Enhances transcription of autophagy-related genes (Fitzwalter et al., 2018).
BNIP3 / BNIP3L	Activator	Induces mitophagy, playing a key role in cell death (Liu et al., 2023).
MTOR	Inhibitor	Suppresses autophagy when active (Liu et al., 2023).
AKT1	Inhibitor	Activates mTOR; inhibits autophagy (Zang et al., 2022).

Table 2.5 (Continued): ACD Pathway Genes

BCL2	Inhibitor	Interacts with Beclin-1, inhibiting autophagy (Dong et al., 2022).
MAP1LC3B (LC3B)	Effector	Binds autophagosome membrane; LC3-II form elevated during cell death (Chen et al., 2025).

The genes associated with autophagy-dependent cell death (ACD), as identified in the Table 2.5, are categorized as follows: activators include BECN1 (Beclin-1), ATG5, ATG7, ULK1, VPS34, FOXO3 and BNIP3/BNIP3L; inhibitors consist of MTOR, AKT1, and BCL2; and the effector is MAP1LC3B (LC3B).

Figure 2.10 presents the results of the functional enrichment analysis performed for the gene set associated with autophagic cell death, revealing that these genes are significantly and biologically associated with the autophagy process and related cellular components.

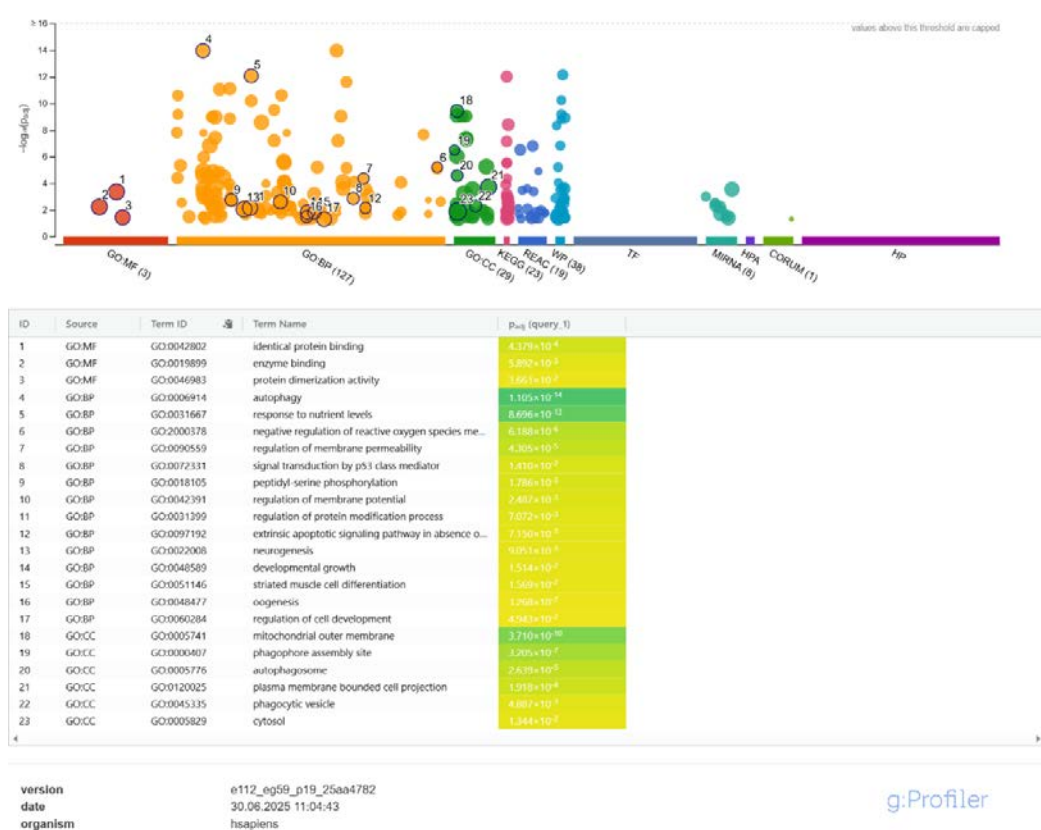


Figure 2.10: Functional Enrichment Analysis of ACD-Related Genes

The significant enrichment of terms such as “autophagy”, “autophagosome”, and “mitophagy” indicates that the analyzed gene set is actively involved in the formation and regulation of autophagic structures and directly contributes to autophagy activity that may trigger cell death. Furthermore, the enrichment of cellular components such as “phagophore assembly site”, “mitochondrial outer membrane”, and “phagocytic vesicle” demonstrates that these genes are integrated into the autophagic cell death process at a subcellular level. Biological processes such as “regulation of membrane permeability”, “regulation of membrane potential”, and “response to nutrient levels” also align with autophagy’s role in maintaining energy balance and responding to cellular stress. Overall, this analysis confirms that the selected gene set is not only related to autophagy in general but also contributes to the pathological forms of autophagy that can lead to cell death, demonstrating a strong functional and structural association with this mechanism.

2.6 Cuproptosis and Related Genes

Cuproptosis is a newly identified programmed cell death mechanism triggered by the excessive accumulation of copper ions, first described by Tsvetkov and colleagues in 2022 (**Tsvetkov et al., 2022**). Unlike other types of cell death, this process occurs through the direct interaction of copper with lipoylated proteins (notably DLAT and PDH) in the mitochondrial respiratory chain (**Chen et al., 2022**). When copper ions bind to these proteins, they induce protein aggregation and mitochondrial dysfunction, disrupting the cell’s energy metabolism and leading to its death (**Tang et al., 2023**). This process causes an interruption of the TCA cycle, a reduction in ATP production, and an increase in reactive oxygen species (ROS) (**Wang et al., 2023**). Cuproptosis is considered a potential target in cancer therapy, as copper ionophores like elesclomol can selectively activate this death mechanism in cancer cells (**Chen et al., 2023**). Conversely, copper chelators may inhibit this process, offering therapeutic benefits in conditions involving copper accumulation, such as neurodegenerative diseases (**Tang et al., 2023**). One of the key advantages of cuproptosis is its potential to overcome drug resistance in tumors. Cancer cells exhibiting metabolic alterations, such as increased mitochondrial metabolism and glycolysis, may become particularly vulnerable to cuproptosis. Furthermore, activation of this mechanism could alter the

tumor microenvironment, enhancing immune cell infiltration and improving responses to immunotherapy (Zhang et al., 2024).

However, the precise molecular mechanisms of cuproptosis and its full role in physiological and pathological processes remain incompletely understood, necessitating further research (Tsvetkov et al., 2022; Wang et al., 2024). Unravelling the mechanism of cuproptosis is still an emerging field. The complex dynamics of copper, which can play both tumor-promoting and tumor-suppressing roles, present challenges that must be addressed before clinical applications can be realized. Therefore, additional studies are required to fully elucidate the mechanism and develop safe therapeutic strategies (Zhang et al., 2024).

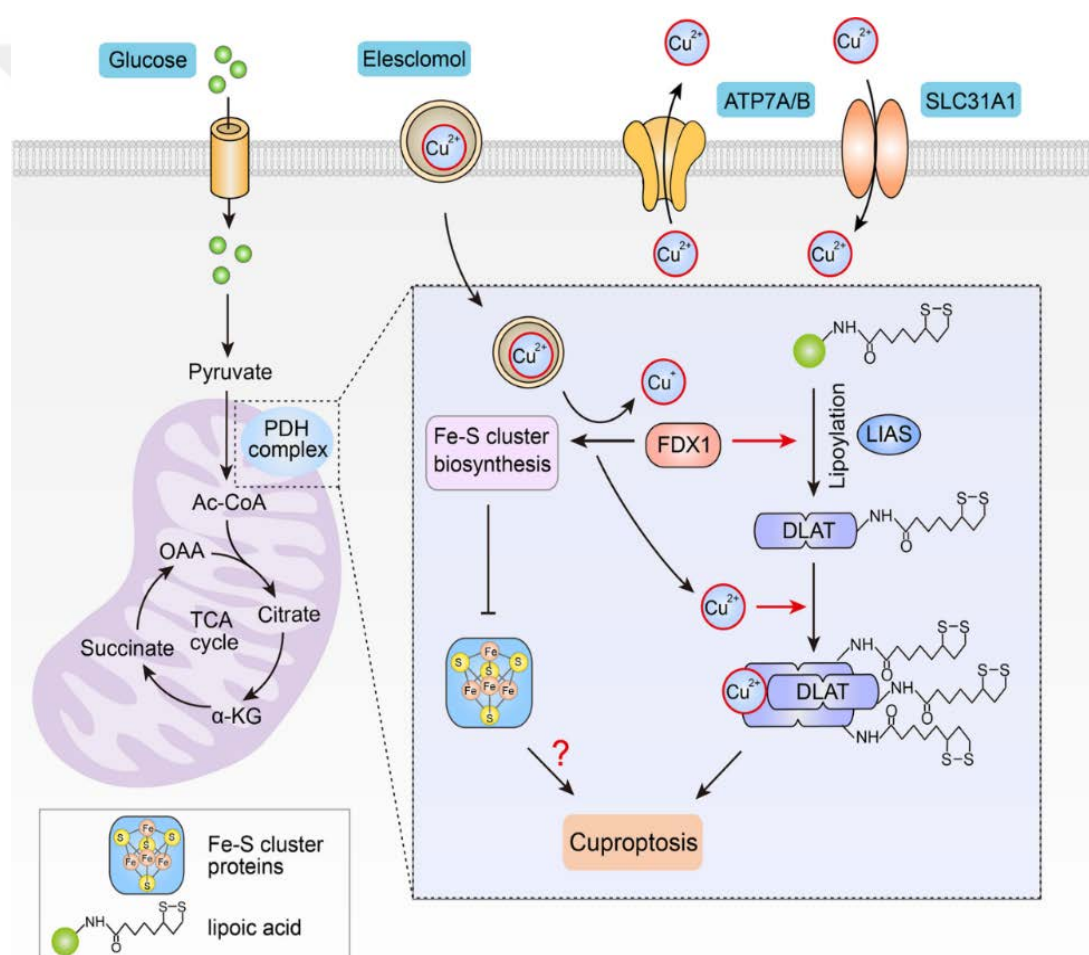


Figure 2.11: Cuproptosis with Pathway Genes (Wang et al., 2022)

The genes associated with cuproptosis including activators, inhibitors, and parallel effectors, along with their respective roles, were identified through a literature review and are presented in Table 2.6

Table 2.6: Cuproptosis Pathway Genes

Gene	Role	Description
FDX1	Activator	Reduces Cu^{2+} to Cu^{+} ; stabilizes lipoylated proteins, triggering cuproptosis (Tsvetkov et al., Science, 2022).
LIAS	Activator	Synthesizes lipoic acid for lipoylated proteins (Tsvetkov et al., Science, 2022).
LIPT1	Activator	Transports lipoic acid into mitochondria (Tsvetkov et al., Science, 2022).
DLD	Activator	Part of lipoylated proteins; triggers cuproptosis (Tsvetkov et al., Science, 2022).
DLAT	Activator	Component of PDH complex; forms toxic complexes with Cu^{2+} (Tsvetkov et al., Science, 2022).
PDHA1	Activator	Subunit of pyruvate dehydrogenase; interacts with copper (Tsvetkov et al., Science, 2022).
PDHB	Activator	Another subunit of pyruvate dehydrogenase; contributes to cuproptosis (Tsvetkov et al., Science, 2022).
MTF1	Inhibitor	Regulates copper homeostasis; suppresses cuproptosis (Tsvetkov et al., Science, 2022).
GLS	Inhibitor	Maintains redox balance; protective against copper stress (Zhang et al., 2024).
CDKN2A	Inhibitor	Cell cycle regulator; protects against copper stress (Fan et al., 2023).
SLC31A1 (CTR1)	Parallel	Copper transporter; indirectly promotes cuproptosis (Xie et al., 2023).
ATP7A / ATP7B	Parallel	Export excess copper; protect cells from cuproptosis (Xie et al., 2023).

The genes associated with cuproptosis, as identified in the Table 2.6, are categorized as follows: activators include FDX1, LIAS, LIPT1, DLD, DLAT, PDHA1, and PDHB; inhibitors consist of MTF1, GLS, and CDKN2A; and parallel effectors include SLC31A1 (CTR1), ATP7A, and ATP7B.

Figure 2.12 presents the results of the functional enrichment analysis conducted for the gene set associated with cuproptosis. The analysis outputs reveal that the gene set is statistically and biologically significantly associated with the cuproptosis

mechanism, highlighting its relevance to key processes involved in copper-induced cell death.



Figure 2.12: Functional Enrichment Analysis of Cuproptosis Genes

The significant enrichment of terms such as “pyruvate dehydrogenase activity”, “pyruvate decarboxylation to acetyl-CoA”, and “pyruvate dehydrogenase complex” indicates a direct association between the analyzed gene set and the core components of cuproptosis—namely, lipoylated proteins and the pyruvate dehydrogenase complex (Lin et al., 2024). In addition, the enrichment of copper homeostasis-related terms such as “copper ion binding”, “copper ion transmembrane transporter activity”, and “intracellular copper ion homeostasis” confirms the involvement of these genes in regulating intracellular copper balance and toxicity. Furthermore, the prominence of cellular components such as the “mitochondrial matrix” supports the notion that cuproptosis is a mitochondria-specific form of cell death and shows that the gene set is well-aligned with the subcellular context of the process.

3. OBJECTIVES AND METHODS

The study is structured to encompass several key steps. In the initial phase, activator and inhibitor genes associated with cuproptosis, ferroptosis, and pyroptosis were compiled from literature, followed by the retrieval of bulk RNA sequencing data for pan-cancer analysis from The Cancer Genome Atlas (TCGA) database. Subsequently, the activity levels of gene sets related to non-apoptotic programmed cell death mechanisms (including activator and inhibitor gene sets) were examined in matched tumor and normal tissue samples obtained from the TCGA database. This analysis covered 15 distinct cancer types: BLCA, BRCA, COAD, ESCA, HNSC, KICH, KIRC, KIRP, LIHC, LUAD, LUSC, PRAD, STAD, THCA, and UCEC. Gene set activity was quantified using the Gene Set Variation Analysis (GSVA) method, and differences in GSVA scores between tumor and normal tissues were evaluated using the Wilcoxon signed-rank test, a non-parametric statistical method for analysis of paired data. In the subsequent phase, feature engineering was performed to extract various features from the data for use in machine learning models. Multi-feature visualization was conducted using UMAP and t-SNE to differentiate cancer types based on non-apoptotic cell death mechanisms through gene expression profiles. The study investigated whether features derived from the expression profiles of genes associated with non-apoptotic programmed cell death mechanisms could reveal distinct patterns, particularly among cancer types identified as active in the initial GSVA-based analysis. Gene expression data from both normal and tumor samples were analyzed to assess the roles of six different non-apoptotic cell death pathways potentially associated with cancer progression. Analyses on normal tissues were conducted to determine whether observed patterns were due to tissue specificity or malignant transformation. Figure 3.1 illustrates the overall workflow of the study.

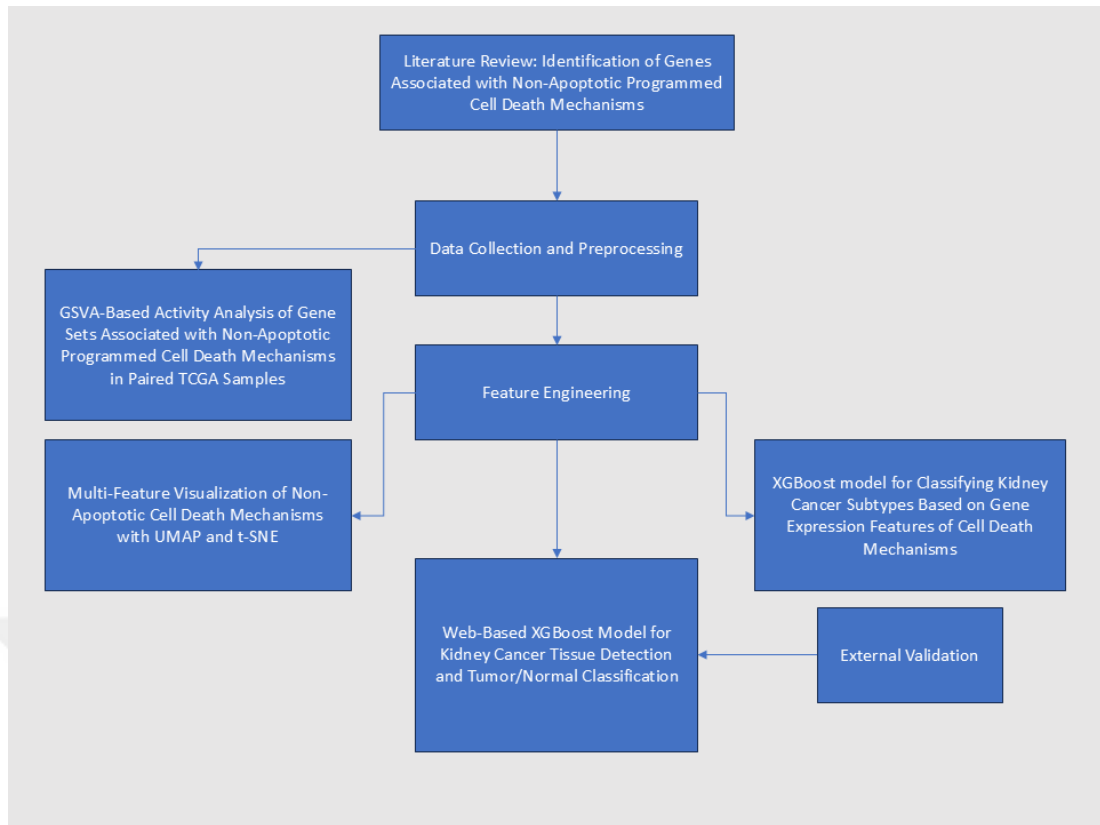


Figure 3.1: Overall workflow of the study

The study found that activator and inhibitor gene sets associated with non-apoptotic cell death mechanisms effectively distinguished kidney cancer subtypes. The subsequent phase focused on developing a multi-class machine learning model to differentiate the three primary kidney cancer subtypes—KIRC, KIRP, and KICH. Various models, including XGBoost, RandomForest, Support Vector Machine, and K-Nearest Neighbors, were tested, with the XGBoost model selected based on its accuracy and average cross-validation scores. In the final phase, XGBoost-based models were developed using features derived from non-apoptotic programmed cell death genes, establishing a highly accurate machine learning system for identifying kidney tissue and distinguishing between tumor and normal samples. These models were deployed through a Flask-based web application, enabling automatic tissue type and tumor/normal classification results from uploaded external data (GEO datasets). This has facilitated the creation of a modular and scalable diagnostic platform to enhance the rapid and reliable diagnosis of kidney cancer in research and clinical settings.

3.1 Data Collection and Preprocessing

In this study, gene expression data were retrieved from The Cancer Genome Atlas (TCGA) database and processed using the TCGAplot R package. The TCGA Pan-Cancer Atlas is a comprehensive repository that provides integrative analyses of genomic, transcriptomic, epigenomic, and proteomic data across 33 distinct cancer types, as part of The Cancer Genome Atlas (TCGA) project (**National Cancer Institute [NCI], 2023**). The study utilized both paired data, encompassing matched tumor and normal tissue samples from 15 cancer types, and unpaired datasets comprising tumor and normal tissue samples from all 33 cancer types.

The data preprocessing pipeline was implemented using the `get_all_paired_tpm()` and `get_all_tpm()` functions within the TCGAplot package (**Liao & Wang, 2023**). These functions facilitate the retrieval of RNA-Seq data from the TCGA data portal and perform preprocessing to transform the data into a format suitable for analysis. Consequently, paired samples from tumor and normal tissues were automatically aligned, and redundant (duplicated) samples were excluded from the dataset.

Gene expression levels were normalized using the Transcripts Per Million (TPM) metric. TPM is a widely adopted normalization method in RNA sequencing data analysis, enabling robust comparisons across genes and samples. This approach adjusts the expression level of each gene based on both its length and the total transcript abundance within the sample. Throughout this study, the abbreviation TPM represents the normalized values of gene expression. Genes with a TPM value of zero across all samples were systematically removed from the dataset. Only protein-coding genes were retained, and their expression values were subjected to a $\log_2(\text{TPM} + 1)$ transformation to achieve further normalization. Each sample was annotated with metadata specifying cancer type and group classification (tumor or normal). This structured annotation yielded a refined data matrix, readily applicable to comparative analyses and the development of classification models.

Paired data, within the context of the TCGA project, refers to the comparative analysis of tumor tissue and normal tissue (typically healthy or adjacent to the tumor) samples obtained from the same patient. In this study, paired gene expression data from the TCGA project were employed, enabling comparative analyses between tumor and

normal tissue samples from the same patient. Unpaired data, conversely, were utilized in advanced analyses, particularly for the development of machine learning models.

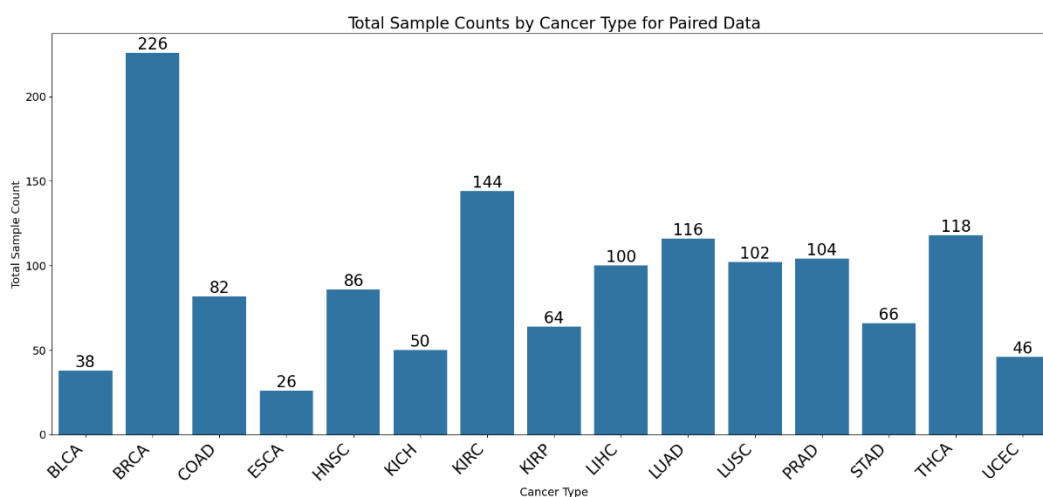


Figure 3.2: Distribution of paired tumor and normal samples across 15 cancer types

The TCGA paired dataset includes 15 cancer types. Among them, breast cancer (BRCA) has the highest number of matched samples ($n = 226$), followed by clear cell renal carcinoma (KIRC, $n = 144$), lung adenocarcinoma (LUAD, $n = 116$), and thyroid carcinoma (THCA, $n = 118$). The cancer types with the lowest number of samples are esophageal carcinoma (ESCA, $n = 26$) and uterine corpus endometrial carcinoma (UCEC, $n = 46$). Use of paired data minimizes inter-individual biological variability, allowing for a more reliable assessment of the differences between tumor and normal tissues.

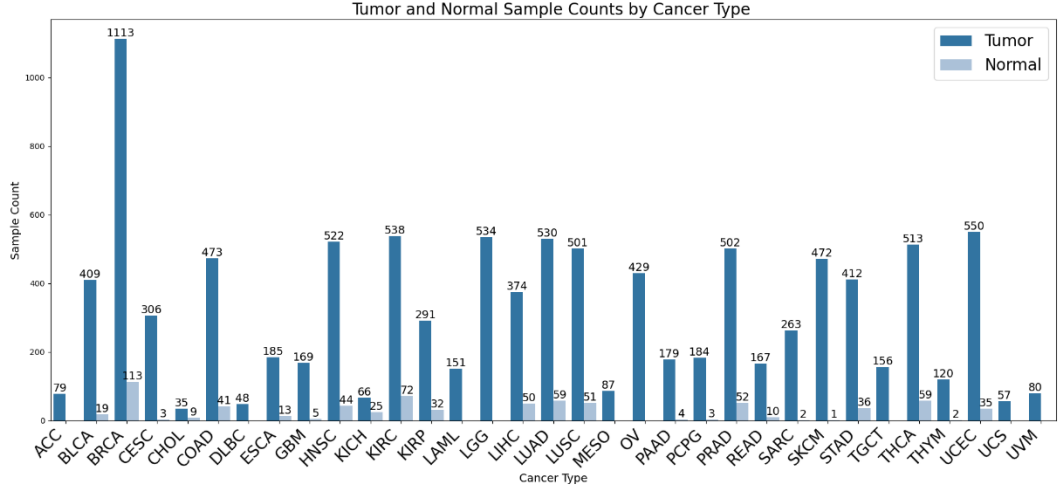


Figure 3.3: Distribution of tumor and normal samples across 33 cancer types

Figure 3.3 compares the number of tumor and normal samples across 33 different cancer types, with tumor samples generally far outnumbering normal ones; for instance, BRCA has the highest count with 1113 tumor samples and 113 normal samples, while cancers like BRCA, LUAD, and LUSC also have over 400 tumor samples, though in some cancer types such as ACC, DLBC, and SARC, normal samples are either zero or very low. In the graph, it is evident that the number of tumor samples is markedly higher than that of normal samples across various cancer types; for example, in some cancer types, tumor sample counts approximate 1,200 while normal samples are around 250, whereas in other groups, tumor counts are approximately 500 with normal samples ranging from 0 to 50.

For model testing, external microarray-based datasets obtained from the GEO (Gene Expression Omnibus) database were used. These datasets were directly provided as input to the model. However, to ensure compatibility with the genes used during training, only the overlapping genes between the training and test sets were selected. To reduce discrepancies arising from cross-platform differences, the microarray data were also subjected to z-score normalization using the mean and standard deviation values derived from the RNA-seq training data. This process aligned the distribution of the test data with that of the training data, thereby preserving the generalization capability of the model.

Through these preprocessing steps, data obtained from different biological measurement platforms were made suitable for integrated analysis by the model, and the impact of external datasets on classification performance was evaluated.

In the final phase of the study, data from the GEO database, specifically GSE11151 and GSE105288-GPL10558, were utilized to validate the web application developed for kidney tissue and tumor detection. The GSE11151 dataset, generated using the Affymetrix Human Genome U133 Plus 2.0 microarray platform, comprises gene expression profiles from human kidney tissues and includes 67 samples, encompassing fetal and adult normal kidney tissues as well as various tumor subtypes such as Wilms tumor, renal cell carcinoma (KIRC), and mesoblastic nephroma (Peterfi et al., 2023). This dataset aims to comparatively analyze kidney tumors. The GSE105288 series, specifically the GPL10558 subset (Illumina HumanHT-12 v4.0 expression beadchip), consists of a 44-sample transcriptome array created by researchers at the University of Alabama at Birmingham (UAB) and made publicly available on October 16, 2018. It includes 9 healthy kidney tissue samples, 9 primary clear cell renal cell carcinoma (ccRCC) samples, and 26 metastatic ccRCC samples (Nam et al., 2019).

Additionally, the microarray dataset with GEO series number GSE44861, published under the title “Affymetrix expression data from colon cancer patient tissues,” containing 111 colon tissue samples, was used in the experiment (Ryan et al., 2014).

3.2 GSVA of Non-Apoptotic Programmed Cell Death Mechanisms Across 15 Cancer Types with Paired Samples

Cancer types with paired samples were used in this stage of the study, the activity levels of gene sets (including activators and inhibitors) associated with non-apoptotic programmed cell death mechanisms were investigated in paired tumor and normal tissue samples obtained from The Cancer Genome Atlas (TCGA) database. This analysis encompassed 15 distinct cancer types: BLCA, BRCA, COAD, ESCA, HNSC, KICH, KIRC, KIRP, LIHC, LUAD, LUSC, PRAD, STAD, THCA, and UCEC. The activity of these gene sets was quantified using the Gene Set Variation Analysis (GSVA) method, and differences in GSVA scores between tumor and normal tissues were evaluated using the Wilcoxon signed-rank test, a non-parametric statistical

method for comparison of means in paired data. The results were visualized through paired box plots.

GSVA is an unsupervised and non-parametric approach that calculates enrichment scores by capturing expression variability of predefined gene sets across different samples. By employing Gaussian kernel density estimation, GSVA reduces multidimensional expression data into gene set scores, thereby achieving dimensionality reduction. This process minimizes noise while amplifying biological signals **(Hänzelmann et al., 2007)**. In this study, TPM data were transformed into a matrix, and gene set scores were computed, with each sample represented by a single numerical value. This approach facilitated the examination of tumor-normal differences across the 15 cancer types by converting individual gene variations into collective pathway activity, thus enhancing interpretability. The primary function utilized for GSVA in this study was the `gsva()` method from the R package **(Hänzelmann et al., 2007)**.

To assess whether the gene set activity scores exhibited statistically significant differences, the Wilcoxon signed-rank test was subsequently applied. This test was chosen due to the potential non-normal distribution of GSVA scores; as a non-parametric method, it does not require the assumption of normality and yields reliable results for such data **(Schober et al., 2020)**. Furthermore, given that the study utilized paired samples (tumor and normal tissues from the same individual), a paired test was essential to control for inter-individual variation. The Wilcoxon signed-rank test accounts for this pairing by analyzing the tumor-normal differences expression values of each gene in each individual. Specifically, the test calculates the difference between tumor and normal scores for each individual, ranks the absolute values of these differences, and then considers their direction (positive or negative) to generate a test statistic. This statistic evaluates whether the observed differences are random, producing a corresponding p-value. The significance symbols in the resulting plots denote the degree of statistical significance of the gene set activity differences between tumor and normal tissues, providing a clear representation of the findings.

In this study, particular attention was paid to cases where both activator and inhibitor genes were significantly upregulated or downregulated together specifically in tumor tissues. Such patterns may indicate a disruption in the regulation of the corresponding cell death mechanism within that tumor type. Therefore, activators and inhibitors that

were co-upregulated or co-downregulated with high statistical significance compared to normal tissues were examined in detail.

This approach has enabled us to understand in which cancer types the regulation of non-apoptotic programmed cell death mechanisms is disrupted.

3.3 Feature Engineering and Dimensionally Reduction

The features derived from gene expression profiles of non-apoptotic programmed cell death mechanisms have been constructed through a detailed feature engineering process to capture biological diversity and complexity. The feature set comprises two main components: summary scores (mean values, PCA scores) and Shannon entropy values. These features aim to highlight differences between samples while preserving the biological structures of gene sets and elucidating molecular distinctions among cancer types.

For the mechanism-specific means, the mean expression values of the predefined activator and inhibitor genes were calculated separately for each mechanism, providing a concise representation of the overall activity level of these gene groups.

The mean values were computed using the classical arithmetic mean formula:

$$\text{Mean Activity} = \frac{1}{n} \sum_{i=0}^n x_i \quad (3.1)$$

where x_i denotes the TPM (Transcripts Per Million) expression value of the corresponding gene.

Principal Component Analysis aims to reduce high-dimensional data into a lower-dimensional space while preserving the directions of maximum variance (i.e., the most informative components). In the context of high-dimensional gene expression data, PCA identifies the principal axes along which the data vary most. The transformation is mathematically defined as follows (Ma & Dai, 2011):

$$Z = XW \text{ where } W = \text{eigenvectors of } \text{Cov}(X) \quad (3.2)$$

The data matrix X is composed of rows representing samples (individuals) and columns representing variables (genes), with a dimensionality of $n \times p$ where n denotes the number of samples and p denotes the number of genes. The weight (loading) matrix W , obtained through PCA, consists of the eigenvectors of the covariance matrix and has dimensions $p \times k$ where k indicates the number of selected principal components (e.g., $k = 1$ if only PC1 is used). The score matrix $Z = XW$ is computed by projecting each sample onto the principal components, and its dimensions are $n \times k$, representing the new coordinates of each sample in the reduced-dimensional space.

PCA was applied to the gene sets associated with each mechanism, and the first principal component (PC1) scores were extracted as features. Since PC1 captures the direction of maximum variance within a gene set, these scores provide valuable information summarizing the internal structural diversity of each mechanism. Additionally, the PCA loadings indicate which genes contribute most to the observed variation, offering further insight into the dominant regulatory patterns within each pathway.

Finally, for each cell death mechanism, Shannon entropy was calculated based on the combined expression values of activator and inhibitor genes. Entropy was used to quantify the degree of irregularity or information content within the gene expression distribution of each sample. During the calculation, all genes within the gene set—regardless of whether they were categorized as activators or inhibitors—were considered collectively. Shannon entropy is a well-established metric for evaluating the complexity or information content of biological networks (**Carels, 2024**).

Entropy is computed based on the probability distribution of a signal, taking into account the number and probability of transitions between different states within the system. In this context, the distribution of expression values among genes serves as a proxy for state variability. Shannon entropy enables the quantitative assessment of information content based on the probability that a given node (gene) exhibits a particular expression level within the network. It is calculated using the following equation, originally proposed by **Shannon (1948)**:

Let p_i denote the probability of each outcome X_i within the signal X for $i = 1, \dots, N - 1$

$$Entropy = \sum_{i=1}^{N-1} p_i \log\left(\frac{1}{p_i}\right) \quad (3.3)$$

The more regular or predictable the values of the genes within a gene set are, the lower the resulting entropy will be. Conversely, the more irregular or chaotic the signal is, the higher its entropy will be. When gene expression values within a gene set are similar to each other, entropy tends to be low; when they exhibit greater variation, entropy increases. Biologically, this characteristic reflects whether the underlying mechanism operates in a more ordered or chaotic manner, thereby contributing to the understanding of pathophysiological differences across cancer types. Derived measures such as entropy account not only for the magnitude of gene expression levels but also for their distribution patterns, offering a multidimensional and enriched feature set for analysis.

Integrating features that represent different levels of information—tpm data, summary statistics, and entropy—provided a multi-layered perspective for the machine learning analysis and enhanced the biological interpretability of the resulting classes. Through this holistic approach, the study aimed to more clearly elucidate the molecular differences underlying distinct cancer types.

3.4 Visualization with UMAP and t-SNE

In this phase of the study, it was investigated whether features derived from the expression profiles of genes associated with non-apoptotic programmed cell death mechanisms could reveal distinct patterns across cancer types, particularly those identified as active in the initial GSVA-based analysis. Gene expression data (TPM) from both normal and tumor samples were analyzed to assess the roles of six distinct non-apoptotic cell death pathways potentially associated with cancer progression. Analyses on normal tissues were performed to determine whether the observed patterns were driven by tissue specificity or malignant transformation.

For each cell death mechanism, summary features were constructed by calculating the mean expression levels of activator and inhibitor genes, the loadings of the first principal component obtained through Principal Component Analysis (PCA) and Shannon entropy scores, which reflect the biological complexity and variability within the gene set. These features were combined with TPM to form a comprehensive feature

set aimed at preserving high-dimensional biological structures and identifying distinct patterns across samples. To visualize these patterns in two-dimensional space, dimensionality reduction techniques such as t-distributed Stochastic Neighbour Embedding (t-SNE) and Uniform Manifold Approximation and Projection (UMAP) were applied.

The visualization outcomes were presented using scatter plots to demonstrate separation by cancer type. Analyses using t-SNE and UMAP allowed for the assessment of whether the observed patterns were primarily influenced by cancer type or tissue origin. The primary research question was whether gene expression signatures of non-apoptotic cell death mechanisms could effectively differentiate between cancer types. The findings revealed that these gene sets, particularly those associated with kidney cancers (KIRC, KIRP, KICH), demonstrated distinct separation patterns, suggesting that the dysregulation of non-apoptotic cell death pathways may serve as a biologically relevant marker for distinguishing certain cancer types.

3.5 Multi-Class Machine Learning Models for Classifying Kidney Cancer Subtypes

The classification model section of this study focuses on developing a multi-class machine learning model to distinguish the three major subtypes of kidney cancer: KIRC, KIRP, and KICH. As stated in Sections 3.2 and 3.4, an important finding is that these subtypes may be associated with the expression levels of activator and inhibitor genes related to non-apoptotic cell death mechanisms such as necroptosis, pyroptosis, ferroptosis, parthanatos, autophagic cell death, and cuproptosis. In this context, Section 3.5 describes the creation of an effective classification model for KIRC, KIRP, and KICH using features derived from the gene expression profiles of these mechanisms. In the study, various models such as XGBoost, RandomForest, Support Vector Machine, and K Nearest Neighbours were tested, and based on accuracy rates and average cross-validation scores, the XGBoost model was selected.

In this study, unmatched tumor tissue samples from The Cancer Genome Atlas (TCGA) database were selected to increase the dataset size. As detailed in Section 3.3, feature engineering was a critical step in transforming gene expression data into biologically meaningful predictors. The classification task was framed as a multi-class problem, with KIRC, KIRP, and KICH being encoded as 0, 1, and 2, respectively. The

dataset was split into 70% training and 30% testing, and the features were standardized using StandardScaler. The XGBoost model was configured with a learning rate of 0.1, a maximum depth of 3, and the mlogloss evaluation metric, with a fixed random seed (42) to ensure reproducibility. Model performance was evaluated via 5-fold cross-validation and accuracy, precision, recall, F1-scores, and confusion matrices of classification results. The results demonstrated strong discriminative power between subtypes with high accuracy and minimal misclassifications, and the cross-validation scores further validated the model's consistency and generalizability.

To enhance the interpretability of the model, advanced analyses were conducted. The feature importance scores of XGBoost were used to identify the 30 most influential features, with the reliability of these scores strengthened by standard deviations calculated in cross-validation layers. However, the feature importance ranking based on metrics such as gain, coverage, or split frequency, while effective in measuring the contribution of features to the decision trees of the model, does not provide detailed insights into how individual features influence specific predictions or capture interactions between features.

To address this limitation, SHAP (SHapley Additive exPlanations) was employed to deepen the interpretability of the model. Based on game theory, SHAP calculates the contribution of each feature to individual predictions using Shapley values, offering a fair and comprehensive decomposition of feature effects (**Lundberg & Lee, 2017**). Unlike XGBoost's feature importance ranking, which highlights the general effect of features, SHAP explains the magnitude and direction (positive or negative) of each feature's contribution both at the individual and collective levels. For instance, while a high importance score in XGBoost shows the overall effect of a feature, SHAP reveals which subtypes (e.g., KIRC or KIRP) the feature predominantly impacts and whether it increases or decreases predictions for a specific class. Additionally, SHAP provides a more holistic interpretation by considering feature interactions.

In this study, SHAP analysis was applied to explain the predictions of the XGBoost model on the test set. SHAP values visualized the contributions of each mechanism (e.g., necroptosis, pyroptosis, ferroptosis) and derived features (e.g., activator/inhibitor mean scores, PCA components, entropy) to the classification of KIRC, KIRP, and KICH. SHAP summary plots displayed the most influential features for each class and

their directional effects on predictions. By aggregating SHAP values per mechanism, the most discriminative cell death pathways for each subtype were clarified.

To test whether the model is biologically meaningful, the true mechanism genes were replaced with other random genes in the dataset, and the same feature extraction steps were reapplied. Predictions were made on the random genes using the same trained model. Subsequently, the model's accuracy score and confusion matrix were calculated based on this random gene set, and it was evaluated that the model is more meaningful with the mechanism genes.

In conclusion, this study developed an XGBoost-based multi-class classification model to differentiate KIRC, KIRP, and KICH using gene expression features derived from non-apoptotic cell death mechanisms. The model's high accuracy, combined with feature importance analysis and SHAP values, highlights its value not only as a diagnostic tool but also as a platform for biological discovery. SHAP analysis complements XGBoost's feature importance ranking, making predictions more transparent and biologically interpretable. By highlighting key genes and pathways, this study lays the foundation for precision oncology in kidney cancer.

3.6 Platform for Kidney Cancer Diagnosis Using Genes Linked to Non-Apoptotic Programmed Cell Death

The final phase of the study encompasses two core components of a bioinformatics system that develops machine learning models using genetic data and provides these models to users via a web application. In the first part, XGBoost-based models were trained to classify tissue types and distinguish tumor from normal kidney tissue. In the second part, these models were made accessible through a Flask-based web service. Flask is an open-source web application framework written in the Python programming language. It is designed for the development of web applications and application programming interfaces (APIs). As will be seen in Results section below, Section 4.2 presents that the gene sets associated with non-apoptotic programmed cell death mechanisms exhibited irregular regulation in kidney cancer. In Section 4.3, UMAP and t-SNE projections created using gene expression values from non-apoptotic cell death-related genes obtained from normal tissue samples demonstrate that liver, lung, and kidney tissues form distinct clusters. In contrast, the analysis of tumor samples showed that the subtypes of kidney cancer differentiated from each

other (Figure 4.8). In Section 4.4, kidney cancer subtypes were classified with high accuracy using XGBoost, based on the features derived from the non-apoptotic cell death-related genes described in Section 3.3. These findings led to the development of a system capable of successfully predicting kidney tissue and distinguishing between tumor and normal samples. The system was designed in two stages. In the first stage, data preprocessing, feature engineering, and model training steps were followed. A feature matrix for the non-apoptotic cell death gene sets, as discussed in Section 3.3, was created. The dataset containing all tissue types was split into 70% for training and 30% for testing. A multi-class model for kidney tissue prediction was trained using the XGBoost algorithm. The model's performance was evaluated using accuracy, precision, recall, and F1-score metric. In the second stage, only kidney tissue samples were filtered, and "Normal" and "Tumor" labels were encoded as 0 and 1, respectively. The dataset was split into 60% for training and 40% for testing, ensuring class balance with stratification. After optimizing the hyperparameters of XGBoost for binary classification through manual tuning, selecting parameters such as `max_depth=1`, `learning_rate=0.02`, `n_estimators=100`, `subsample=0.5`, `colsample_bytree=0.6`, `min_child_weight=5`, `gamma=0.3`, `reg_lambda=2.0`, and `reg_alpha=0.5` to address class imbalance and prevent overfitting, the model was trained and evaluated with test data, achieving good performance as indicated by cross-validation and test accuracy metrics. The trained models were made available through a Flask-based web API for user access. To test the system, external data obtained from the GEO database (GSE111151, GSE105288) were downloaded using the R GEOquery library. Sample IDs and gene expression data associated with non-apoptotic cell death mechanisms were log2-transformed and saved as CSV files. The CSV files are uploaded via the GUI, the tool applies the same feature extraction procedures (mean, PCA, entropy, ratio), and generates two predictions: one for tissue type and the other indicating whether kidney tissue is tumor or normal. For missing genes in the input data, neutral values are added to ensure compatibility with the models. The model expects that only pre-specified genes (non-apoptotic cell death mechanisms related genes) from external datasets are provided as input. Under this condition, the model's predictive performance will be at its highest. However, if there are missing genes in the input data, neutral values (zeros) are assigned to the expression values of these missing genes to ensure compatibility with the model. In such cases, the model informs the user about the presence of missing genes in the dataset and indicates that the accuracy of

predictions may be reduced due to these missing genes. Predictions, along with sample IDs, are returned to the user via the web application. Error handling, such as incorrect file formats or missing model files, is also addressed. The purpose of this web application is to make the trained models practically usable in laboratory or clinical settings. For instance, a researcher can quickly determine whether samples derived from tissues associated with cell death mechanisms are from kidney tissue and if the samples are from kidney, whether if the samples are cancerous. This system aims to automate manual analysis processes and expedite diagnostic procedures. The binary classification model has the potential to contribute to the early detection of kidney cancer, improving patient outcomes. By transforming the complexity of genetic data into understandable results, the system supports biomedical research and clinical decision-making processes. Furthermore, due to its modular structure, the system can be adapted to other genetic datasets or different biological questions. This phase of the study presents an automated analysis and prediction system for kidney cancer diagnosis based on genetic data. Biological meaningful features were extracted, models were developed, and these models were made accessible through a web application.

4. RESULTS AND DISCUSSION

Based on the literature review, genes associated with non-apoptotic programmed cell death mechanisms were classified as activators, inhibitors, and effectors through an extensive literature survey. Table 4.1 shows the genes associated with these mechanisms.

Table 4.1: The Genes Associated with Necroptosis, Pyroptosis, Ferroptosis, Parthanatos, ACD and Cuproptosis

	Necroptosis	Pyroptosis	Ferroptosis	Parthanatos	ACD	Cuproptosis
Activators	RIPK1, RIPK3, ZBP1, TNF, CYLD, TICAM1	CASP1, CASP4, CASP5, NLRP3, AIM2, NLRC4, GZMA, GZMB, PYRIN, ASC (PYCARD), NLRP1	ALOX15, ACSL4, LPCAT3, TFRC, NOX1/ NOX4, SAT1 P53	PARP1, AIFM1 (AIF), MIF	BECN1 (Beclin-1), ATG5, ATG7, ULK1, VPS34, FOXO3 BNIP3/ BNIP3L	FDX1, LIAS, LIPT1, DLD, DLAT, PDHA1, PDHB
Inhibitors	CASP8, FADD, cFLIP	CARD16 (COP), CARD18 (ICEBERG), NLRX1, POP1/POP2, CASP8, XIAP, BCL2, IL10	GPX4, SLC7A11 (xCT), GCLC, GCLM, GSS, FTH1/FTL, NFE2L2 (NRF2), MT1G, CDKN1A (p21) P53	MACROD1, MACROD2, NUDT16, ARH3, SIRT1	MTOR, AKT1, BCL2	MTF1, GLS, CDKN2A

Table 4.1 (Continued): The Genes Associated with Necroptosis, Pyroptosis, Ferroptosis, Parthanatos, ACD and Cuproptosis

Effectors	MLKL	TNF, CASP3, GSDMD, GSDME, GSDMB, GSDMC, IL1B, IL18, P2X7R, ESCRT-III.	LOX, POR	PARG, XRCC1, H2AX	MAP1LC3B (LC3B).	SLC31A1 (CTR1), ATP7A, ATP7B.
-----------	------	---	-------------	-------------------------	----------------------------	--

After the genes associated with cell death mechanisms were obtained from the literature, a functional enrichment analysis was conducted using the g:Profiler platform to further evaluate whether the analyzed gene sets are biologically associated with the corresponding cell death mechanisms. As a result of this analysis, the selected genes were found to be significantly associated with biological processes related to the defined cell death mechanisms across various Gene Ontology (GO) categories.

Subsequently, the activity levels of non-apoptotic programmed cell death (PCD) mechanisms were examined across 15 different cancer types using data from The Cancer Genome Atlas (TCGA) database. The activities of activator and inhibitor gene sets were quantified using the Gene Set Variation Analysis (GSVA) method, and differences in gene expression levels between tumor and normal tissues were evaluated with the Wilcoxon test. The findings revealed heterogeneity in PCD mechanisms across cancer types, with a general trend of upregulation in kidney cancers (KIRC, KIRP) and downregulation in cancers such as LUSC and COAD. This indicates that PCD may exert tumor-suppressive or tumor-promoting roles depending on the cancer type.

In machine learning analyses, t-SNE and UMAP visualizations showed organ-specific clustering of normal tissues, with tumor tissues exhibiting distinct separation of KIRC, KIRP, and KICH. For kidney cancer subtype classification, XGBoost, Random Forest, SVM, and KNN models were developed, with XGBoost achieving the highest performance at 94% accuracy. High accuracy was observed for KIRC and KIRP (98% and 87%, respectively), while KICH showed lower performance due to a limited sample size. Feature importance rankings highlighted pyroptosis and autophagy-

related genes, with SHAP analyses confirming their influence on subtype classification.

In the final stage, an XGBoost-based web application (kidneycancerscope.com) was developed for kidney cancer tissue detection and tumor/normal classification, utilizing data from all 33 cancer types in the TCGA database. Kidney tissue classification achieved a 98% F1-score, while tumor/normal classification yielded a 97% F1-score for tumor detection. Validation with external datasets demonstrated high performance in both tissue and tumor detection.

4.1 Regulation of Non-Apoptotic Cell Death Mechanisms in Cancer: Distinct Patterns Concentrated in Renal Malignancies

The activity levels of gene sets (activators and inhibitors) associated with non-apoptotic programmed cell death mechanisms were investigated in paired tumor and normal tissue samples obtained from The Cancer Genome Atlas (TCGA) database across 15 different cancer types (BLCA, BRCA, COAD, ESCA, HNSC, KICH, KIRC, KIRP, LIHC, LUAD, LUSC, PRAD, STAD, THCA, and UCEC). The Gene Set Variation Analysis (GSVA) method was employed to quantify the activity of these gene sets. Figure 4.1 illustrates the expression differences and statistical significance of necroptosis-related activator and inhibitor gene sets, as calculated by GSVA scores, between tumor and normal tissues. Differences in GSVA scores between tumor and normal tissues were assessed using the Wilcoxon signed-rank test for paired data with p-values corrected using the Bonferroni method to account for multiple comparisons.

The results were visualized using paired box plots, with statistical significance denoted by the following symbols: *** ($p \leq 0.001$), ** ($p \leq 0.01$), * ($p \leq 0.05$), and ns ($p > 0.05$). For each Figure 4.1 to 4.6, panels (a) and (b) present the differences in GSVA scores for activator (a) and inhibitor (b) gene sets, respectively, between normal and tumor samples across 13 different cancer types. Each box plot illustrates the distribution of GSVA scores for the respective gene set in tumor and normal groups.

Individual lines represent the changes between paired samples. Panel (c) summarizes the direction of change (Up/Down) for activator and inhibitor scores in each cancer type, along with the corresponding levels of statistical significance. Red shades

indicate high significance, yellow shades denote moderate significance, and gray shades represent statistically non-significant differences.

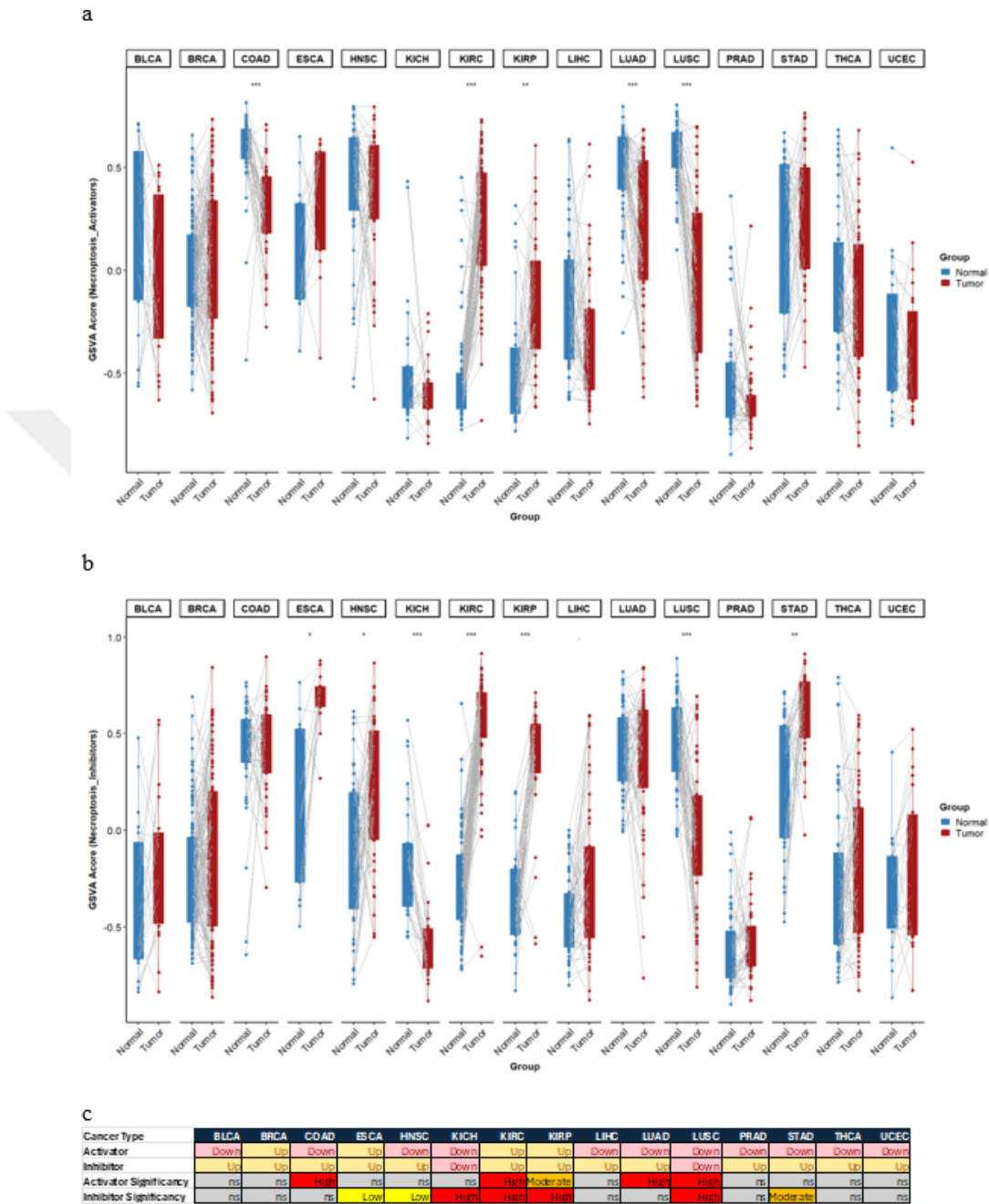


Figure 4.1: GSVA Score Differences and Statistical Significance Analysis of Necroptosis-Related Gene Sets Between Tumor and Normal Tissues

GSVA scores for gene sets comprising activator and inhibitor genes associated with necroptosis were significantly upregulated or downregulated in various tumor tissues compared to normal tissues (Figure 4.1). Notably, in KIRC, both activators and

inhibitors exhibited highly significant upregulation ($p \leq 0.001$). Another kidney cancer subtype, KIRP, also displayed upregulation, with high significance for activators ($p \leq 0.01$) and inhibitors ($p \leq 0.001$). In contrast, LUSC showed very high and high significance for downregulation of activators and inhibitors, respectively. **(Yan et al., 2022)** demonstrated that the relationship between necroptosis and cancer is bidirectional, exhibiting both tumor-suppressive and tumor-promoting effects. These findings align with **(Yan et al., 2022)**, emphasizing that the dual role of necroptosis depends on cancer type, tumor microenvironment, and the regulation of cell death pathways.

Figure 4.2 illustrates the expression differences and statistical significance of GSVA scores for pyroptosis-associated activator and inhibitor gene sets between tumor and normal tissues. The activator and inhibitor gene sets associated with pyroptosis are significantly upregulated with very high statistical significance in KIRC and KIRP. In COAD, activators are downregulated with low significance, while inhibitors are downregulated with high significance. In THCA, activators and inhibitors are observed to be significantly upregulated and downregulated, respectively, with moderate statistical significance.

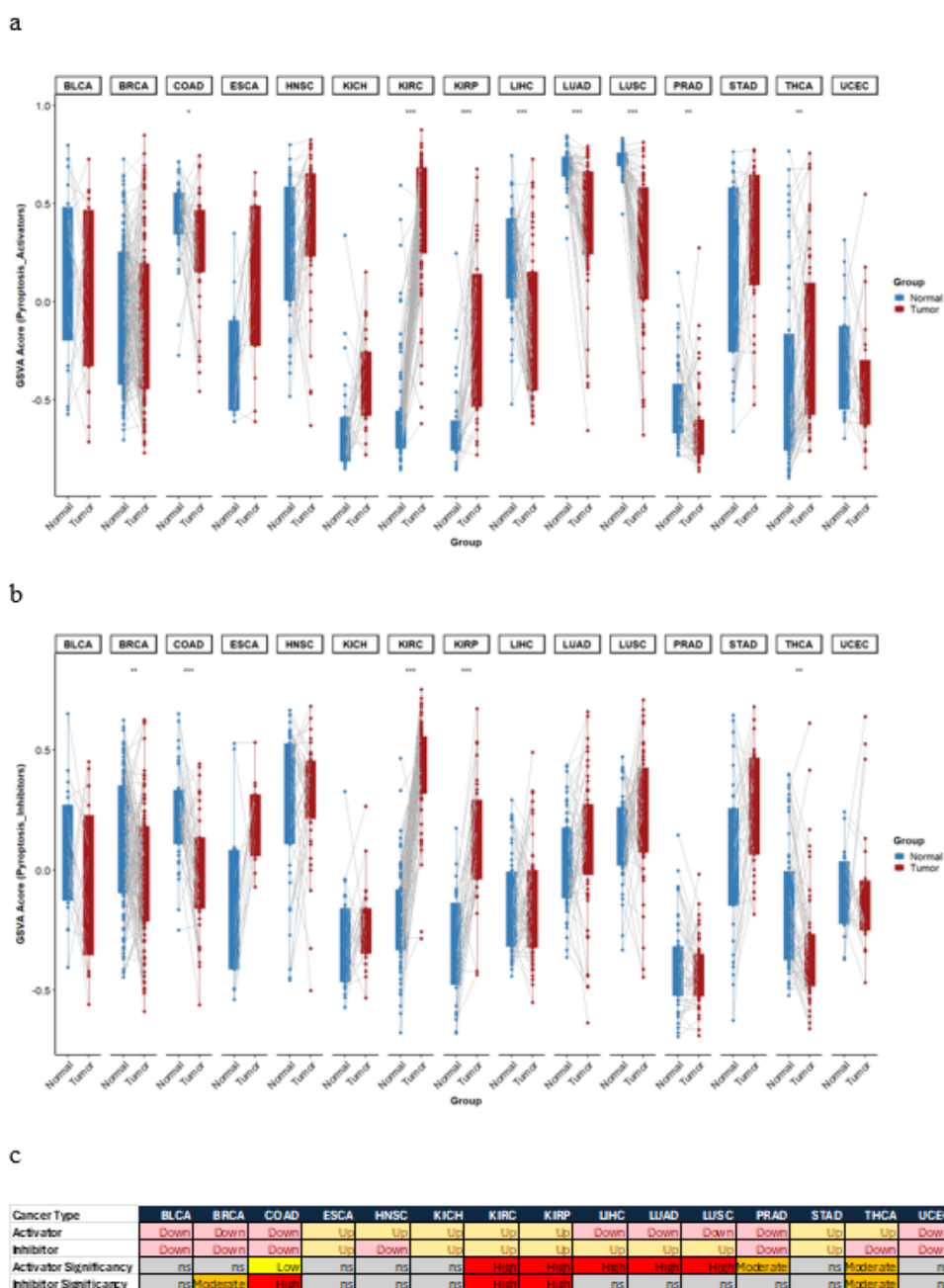
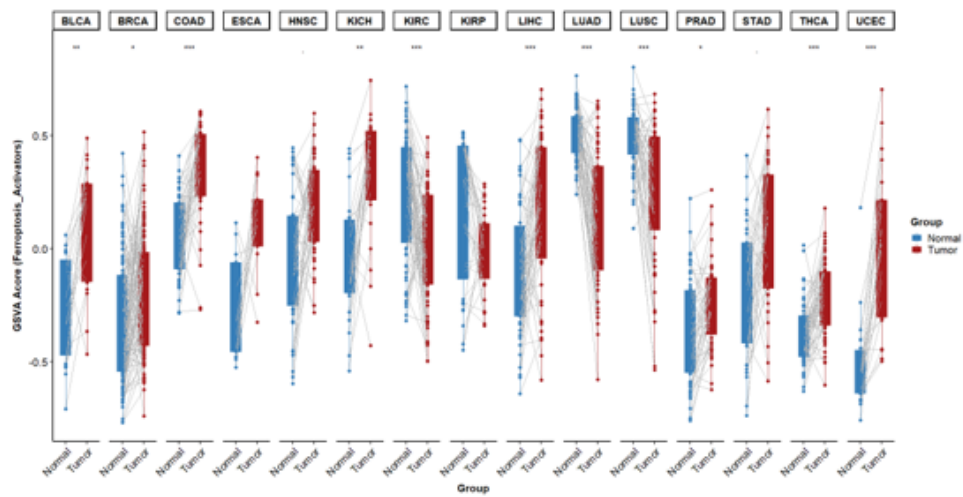


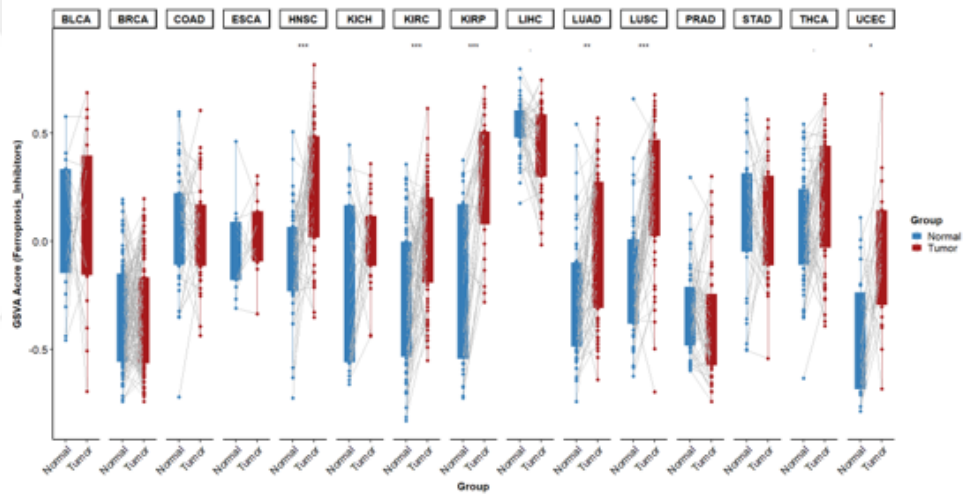
Figure 4.2: GSVA Score Differences and Statistical Significance Analysis of Pyroptosis-Related Gene Sets Between Tumor and Normal Tissues

The result for GSVA of ferroptosis related genes is shown in Figure 4.3 In KIRC, activators are downregulated with high significance and inhibitors are upregulated with high significance. In LUAD, activators are downregulated with high significance, while inhibitors are upregulated with moderate significance. In UCEC both activators and inhibitors are upregulated with high and low significance respectively.

a



b



c

Cancer Type	BLCA	BRCA	COAD	ESCA	HNSC	KICH	KIRC	KIRP	LIHC	LUAD	LUSC	PRAD	STAD	THCA	UCEC
Activator	Up	Up	Up	Up	Up	Up	Down	Down	Up	Down	Down	Up	Up	Up	Up
Inhibitor	Up	Down	Down	Down	Up	Down	Up	Up	Down	Up	Up	Down	Down	Up	Up
Activator Significance	Moderate	Low	High	ns	ns	Moderate	High	ns	High	High	High	Low	ns	High	High
Inhibitor Significance	ns	ns	ns	ns	High	ns	High	High	ns	Moderate	High	ns	ns	ns	Low

Figure 4.3: GSVA Score Differences and Statistical Significance Analysis of Ferroptosis-Related Gene Sets Between Tumor and Normal Tissues

Figure 4.4 shows the expression differences and statistical significance of parthanatos-related activator and inhibitor gene sets, as calculated by GSVA scores, between tumor and normal tissues.

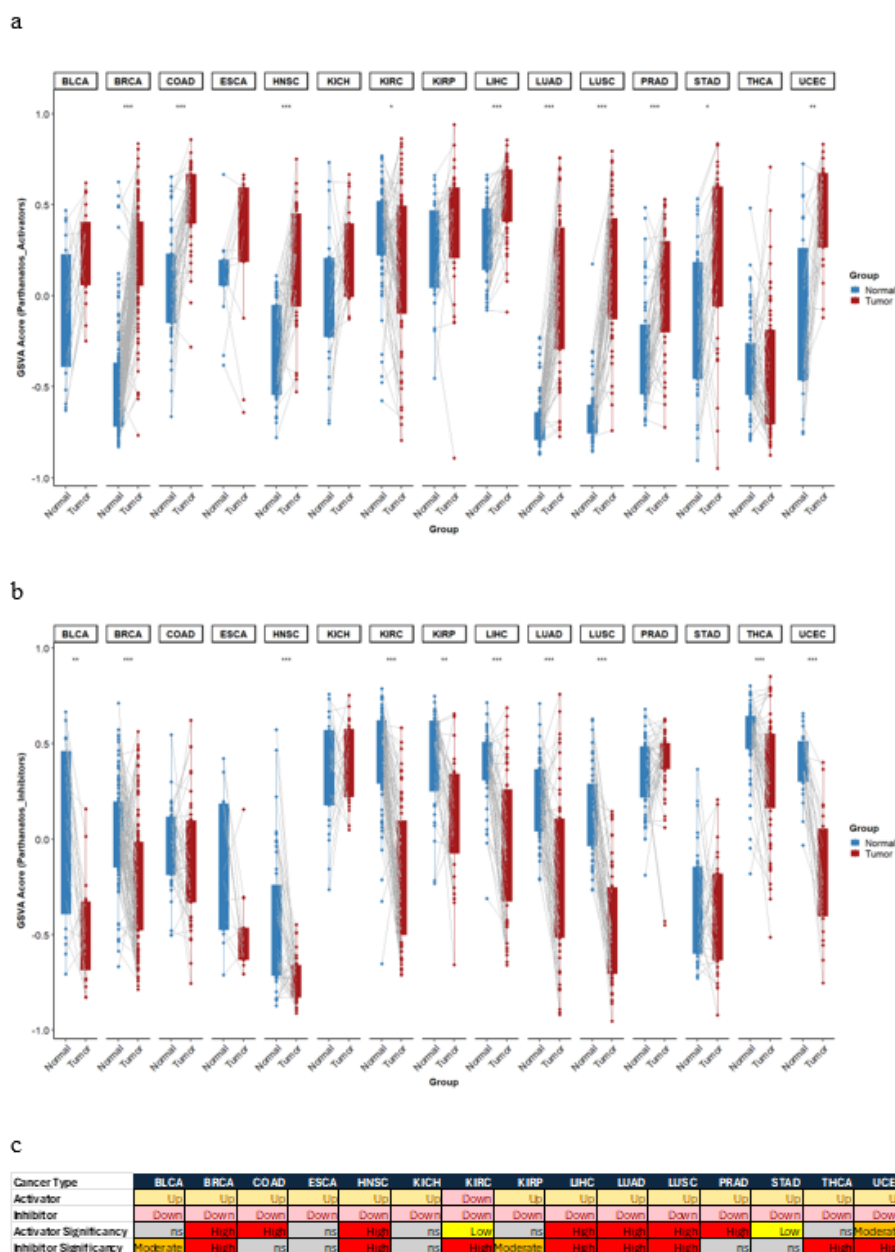


Figure 4.4: GSVA Score Differences and Statistical Significance Analysis of Parthanatos-Related Gene Sets Between Tumor and Normal Tissues

The activator gene sets associated with parthanatos are upregulated with low significance in STAD and with high significance in BRCA, COAD, HNSC, LIHC, LUAD, LUSC and with moderate significance in UCEC. The inhibitor gene sets are downregulated with moderate significance in BLCA and with high significance in BRCA, HNSC, LIHC, LUAD, LUSC, THCA and UCEC. KIRC is the only cancer type where both activator and inhibitor gene sets are concurrently downregulated with moderate and high significance, respectively.

Figure 4.5 illustrates the expression differences and statistical significance of ACD-related activator and inhibitor gene sets, as calculated by GSVA scores, between tumor and normal tissues.

The activator and inhibitor gene sets associated with autophagic cell death (ACD) are downregulated in THCA with moderate and high statistical significance, respectively. In KICH, activators are downregulated with high significance. In KIRC, activators are upregulated with high significance.,

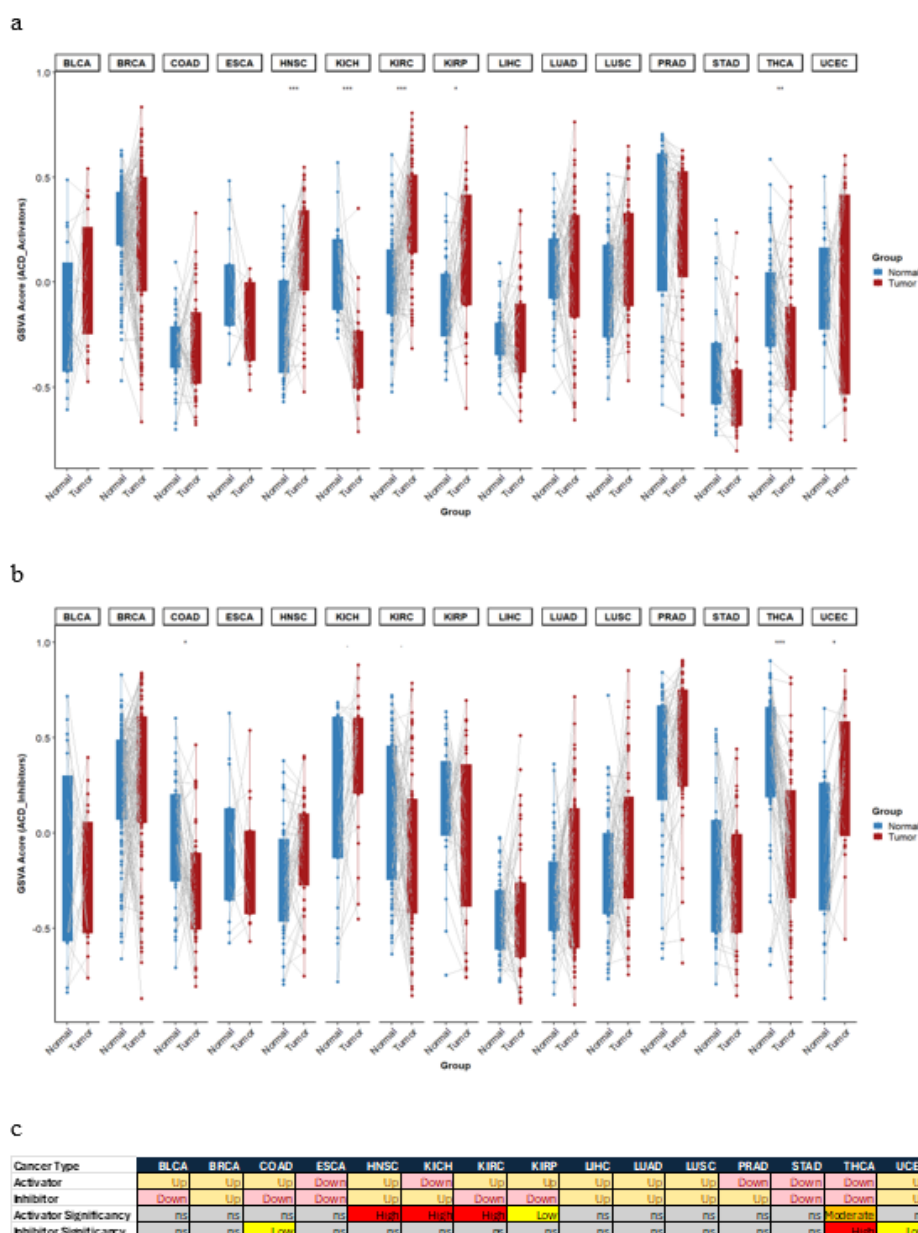


Figure 4.5: GSVA Score Differences and Statistical Significance Analysis of ACD-Related Sets Between Tumor and Normal Tissues

Figure 4.6 illustrates the expression differences and statistical significance of cuproptosis-related activator and inhibitor gene sets, as calculated by GSVA scores, between tumor and normal tissues.

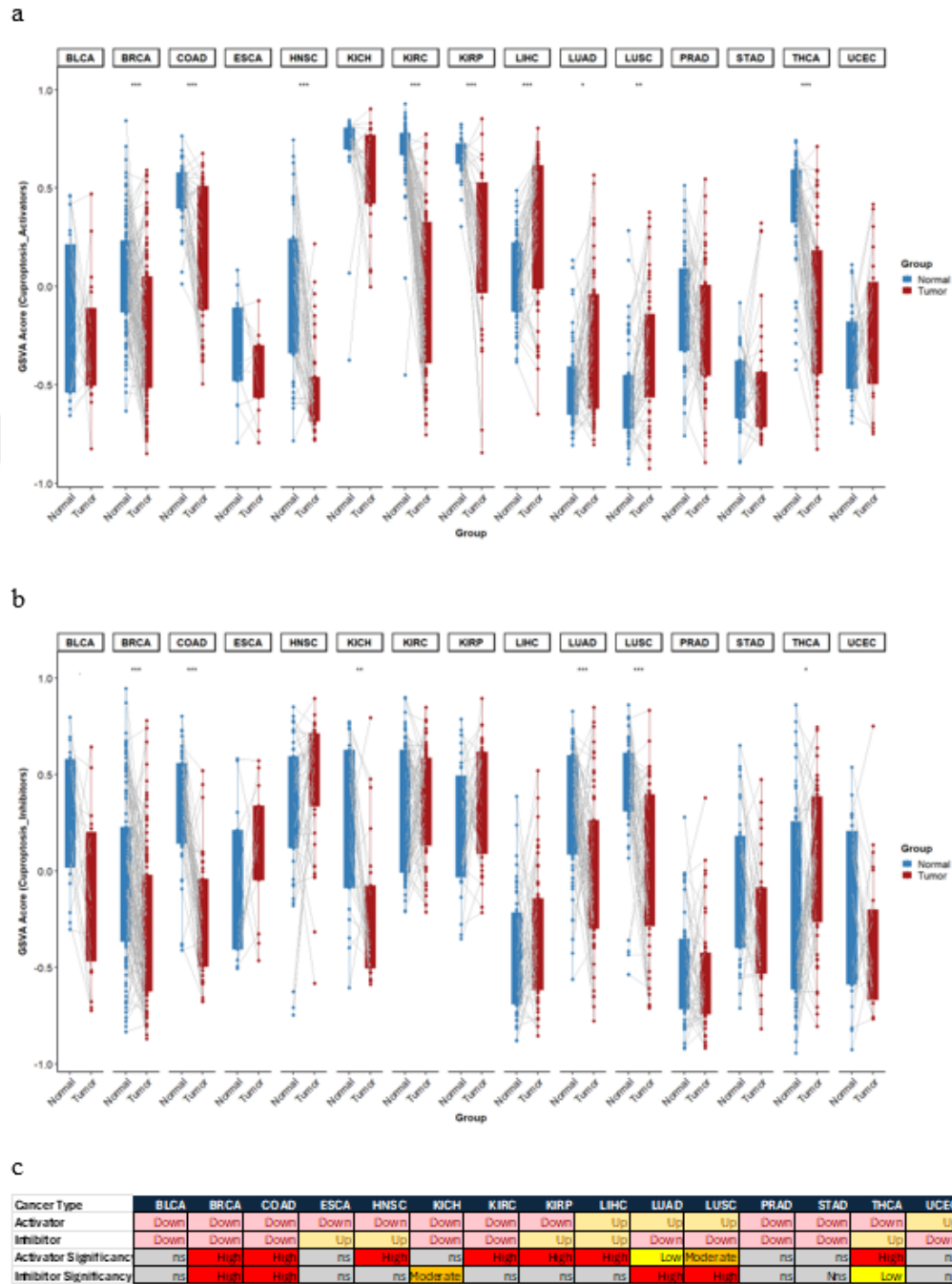


Figure 4.6: GSVA Score Differences and Statistical Significance Analysis of Cuproptosis-Related Gene Sets Between Tumor and Normal Tissues

The activator and inhibitor gene sets associated with cuproptosis are concurrently downregulated with high significance in BRCA and COAD. In KICH, inhibitors are

downregulated with moderate significance. In LUAD and LUSC, activators are upregulated with low and moderate significance, while inhibitors are downregulated with high significance. In THCA, activators are downregulated with high significance, while inhibitors are upregulated with low significance.

This analysis reveals that non-apoptotic programmed cell death (PCD) mechanisms exhibit significant heterogeneity across cancer types, with their roles in cancer biology varying depending on cancer type, tumor microenvironment, and genetic and epigenetic regulation. The bidirectional nature of PCD mechanisms, capable of exerting both tumor-suppressive and tumor-promoting effects, reflects a complex balance in cancer development and progression. For instance, the general upregulation of PCD pathways in renal cancers such as KIRC and KIRP suggests that cell death mechanisms may be active in a tumor-suppressive capacity in these cancers, whereas downregulation in cancers like LUSC and COAD may indicate tumor evasion strategies. These findings underscore the critical importance of understanding PCD pathways in a cancer-type-specific context for the development of targeted therapies. In particular, therapeutic approaches modulating the activity of these pathways should be designed by considering cancer-type-specific molecular profiles. The study highlights the potential of PCD mechanisms as novel therapeutic targets in cancer treatment while indicating the need for further functional and clinical research in this field. These results provide a significant foundation for understanding the complexity of cancer biology and advancing personalized medicine approaches.

4.2 Machine Learning Clustering with Mechanism-Derived Features: Investigating Cancer Heterogeneity

The data were filtered to include only "Normal" samples. Features for activator and inhibitor genes, specific to each mechanism, were generated as described in the Methods section. These features were subsequently integrated with gene expression data and analyzed using UMAP or t-SNE dimensionality reduction techniques. The results were visualized through scatter plots to illustrate the separation of tissue types, and the outputs were recorded for further analysis.

In Figures 4.7- 4.8, panels (a) t-SNE plot illustrate the clustering of tissue samples from normal samples of 15 cancer types based on the expression of genes associated with cell death mechanisms. Panel (b) UMAP plot depicts the same tissue samples as

in panel (a), with clustering patterns reinforcing the tissue-specific separation observed in the t-SNE analysis. Each point represents a sample, with colors indicating different cancer types as per the legend.

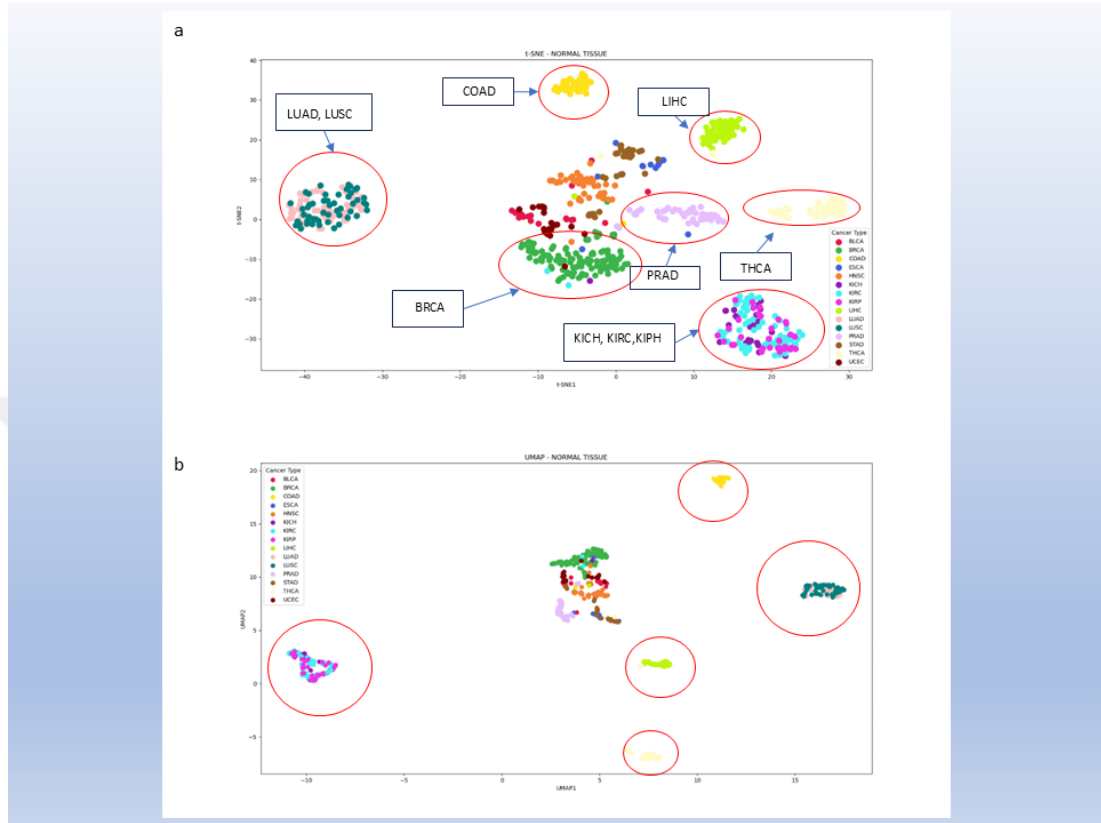


Figure 4.7: Normal Tissue-Specific Clustering of Normal Samples Across Cancer Types Using t-SNE and UMAP Dimensionality Reduction

Preliminary analyses of normal samples from 15 cancer types obtained from The Cancer Genome Atlas (TCGA) database revealed that normal tissues exhibit a tendency to cluster based on tissue type. A central research question of this study was to determine whether genes associated with cell death mechanisms could effectively distinguish between cancer types. However, preliminary findings indicated that the expression levels of these genes are primarily tissue-dependent, with tissue-specific expression patterns exerting a dominant influence on the analyses. Hence it can be concluded that the expression of genes related to non-apoptotic programmed cell death cell death mechanisms is organ-specific in normal tissues, cell death mechanisms are not only associated with disease states but also with tissue-specific physiological roles. For instance, normal samples of cancers originating from the same organ, such as KIRP and KICH or LUSC and LUAD, clustered together in the analyses (Figure 4.7).

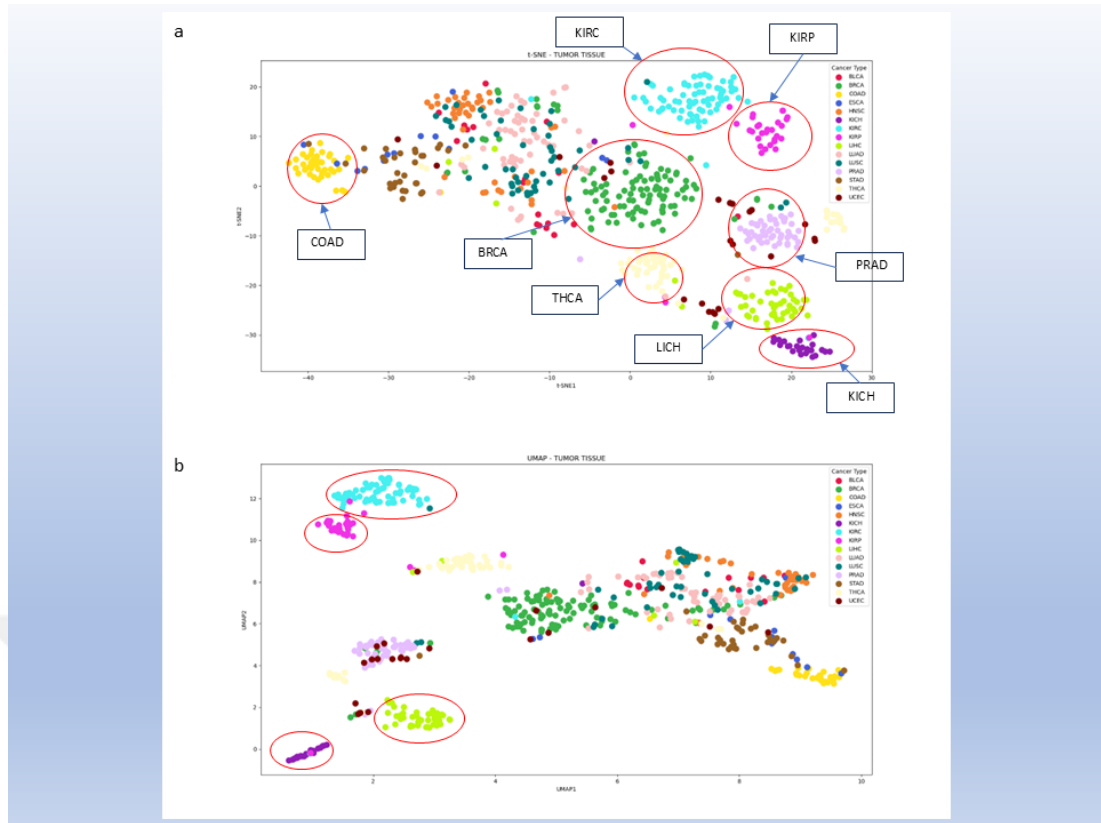


Figure 4.8: Tumor Tissue-Specific Clustering of Normal Samples Across Cancer Types Using t-SNE and UMAP Dimensionality Reduction

The dataset was subsequently filtered to include only "Tumor" samples. We observed that the targeted cancers, specifically KIRC, KICH, and KIRP, exhibited robust clustering independent of tissue type and were distinctly separated from one another (Figure 4.8). These findings confirm that KIRP, KIRC, and KICH are closely associated with the expression levels of activator and inhibitor genes involved in non-apoptotic cell death mechanisms, and that these cancers can be effectively classified using features derived from these mechanisms. Furthermore, for other cancer types, the clustering separation between tumor tissues and normal tissues became less pronounced. This finding indicates that non-apoptotic programmed cell death activities exhibit more divergent behavior in tumor tissues compared to normal tissues.

One of the primary objectives of the initial phase of our study was to investigate the differential expression of specific gene sets or pathways between tumor and normal tissues. The goal was to elucidate the distinct responses of non-apoptotic cell death mechanisms in specific cancer types, thereby enabling us to focus our subsequent analyses on these cancers. Consistent with this aim, our preliminary analysis identified

kidney cancers as the primary target cancers for further investigation as the primary target cancers for further investigation, as their subtypes were well distinguished among tumor groups. Moreover, in the GSVA analysis, kidney cancer subtypes showed significant differences across tumor tissues for most cell death mechanisms.

4.3 Classification of Kidney Cancer Subtypes Using Non-Apoptotic Cell Death Mechanisms and XGBoost

In this phase of the study, multi-class machine learning models, including XGBoost, Random Forest, Support Vector Machine, and K-Nearest Neighbours, were developed to classify three subtypes of kidney cancer (KICH, KIRC, and KIRP) based on gene expression profiles associated with non-apoptotic programmed cell death mechanisms. The accuracy and mean CV score of these models were compared (Figure 4.9). The Mean CV (Cross-Validation) score is a metric used to evaluate the generalization performance of a machine learning model. In the k-fold cross-validation method, the dataset is divided into k equal parts, and the model is trained and evaluated k times, with each part serving as the test set once. The performance score (e.g., accuracy or F1 score) is calculated for each fold, and the average of these scores yields the mean CV score. This approach ensures the model's consistency across different data subsets and reduces the risk of overfitting, providing a more reliable performance estimate. In this study, 5-fold cross-validation was performed, and the scores from each fold were averaged to report the mean CV score.

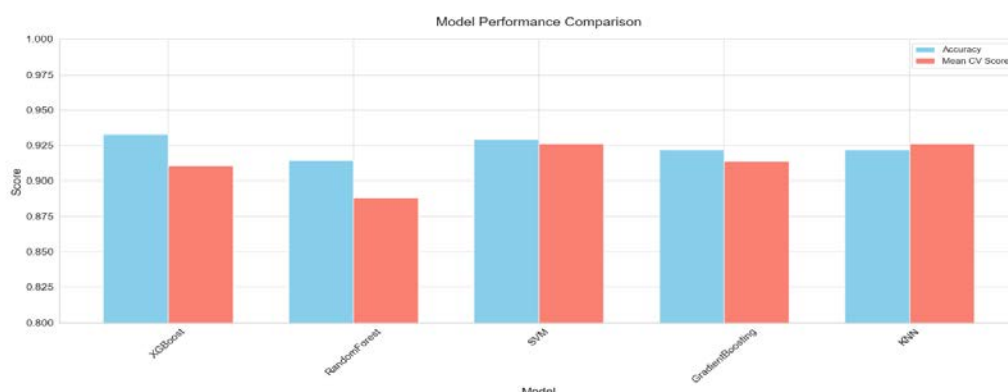


Figure 4.9: Performance comparison between XGBoost, Random Forest, SVM, Gradient Boosting and KNN

The XGBoost model achieved an overall accuracy of approximately 94% on an independent test set. Owing to its superior performance, an XGBoost-based machine learning model was further developed. The choice of XGBoost is based on several factors: its ability to balance low variance and low bias, ensuring high accuracy and generalizability; its robustness in handling missing or noisy biological data; its native support for multi-class classification tasks; its ability to efficiently process high-dimensional datasets with GPU-accelerated training; and its feature importance ranking, which facilitates biological interpretation (Lin et al., 2024). Beyond being a classification tool, the model provides valuable insights for biomarker discovery by identifying the most critical cell death mechanisms that differentiate kidney cancer subtypes.

Table 4.2 shows the classification report of the model. The highest performance was observed for the KIRC subtype, where the model demonstrated superior results with an accuracy of 0.98, a recall of 0.95, and an F1-score of 0.96. Represented by 173 samples, the KIRC subtype exhibited low false positive and false negative rates, indicating the model's high capacity to distinguish this subtype. Similarly, strong performance was achieved for the KIRP subtype, with an accuracy of 0.87, a recall of 0.95, and an F1-score of 0.91. The notably high recall value indicates that 95% of true KIRP samples were correctly classified, with only a limited number of samples missed (78 samples). In contrast, the KICH subtype, represented by only 18 samples, was classified with more limited success (accuracy of 0.88, recall of 0.83, and F1-score of 0.86), likely due to the small sample size. This suggests that limited sample sizes may adversely affect the model's generalization capacity. Figure 4.10 presents the confusion matrix of the multi-class XGBoost model for kidney cancer subtype classification.

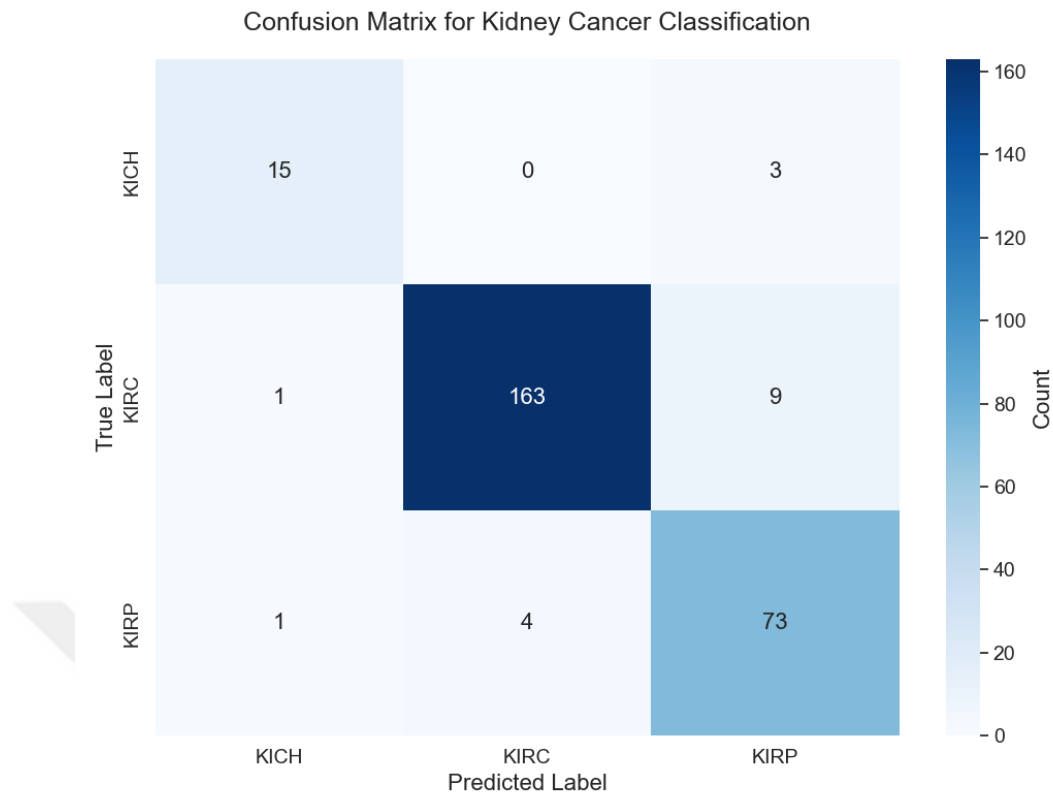


Figure 4.10: Confusion Matrix of Multi-Class XGBoost Model for Kidney Cancer Subtype Classification

The generalizability of the model was further assessed using 5-fold cross-validation, yielding accuracy scores of 0.881, 0.928, 0.880, 0.968, and 0.880, respectively. The average cross-validation score of 0.91 (approximately 90.7%) demonstrates that the model consistently achieves high performance across different subsets of the dataset. The highest accuracy score of 0.968 indicates that the model correctly classified nearly all samples in certain folds, while the lowest scores (0.880) may be attributed to the limited number of samples in the KICH class or variations in data distribution. The narrow range of accuracy scores (approximately 8.8%) suggests that the model avoids overfitting and effectively adapts to different data subsets.

Table 4.2: Classification Report of Multi-Class XGBoost Model for Kidney Cancer Subtype Classification

Class	precision	recall	f1-score	support
KICH	0.88	0.83	0.86	18
KIRC	0.98	0.94	0.96	173
KIRP	0.86	0.94	0.90	78

Table 4.2 (Continued): Classification Report of Multi-Class XGBoost Model for Kidney Cancer Subtype Classification

accuracy			0.93	269
macro avg	0.91	0.90	0.90	269
weighted avg	0.94	0.93	0.93	269

To assess the biological significance of the model, true mechanism genes were replaced with random genes from the dataset, and the same feature extraction steps were reapplied. Predictions were made on the random gene set using the trained model, and the resulting accuracy score and confusion matrix confirmed that the model is more meaningful when using mechanism genes. Figure 4.11 presents the confusion matrix of the multi-class XGBoost model for kidney cancer subtype classification with random genes.

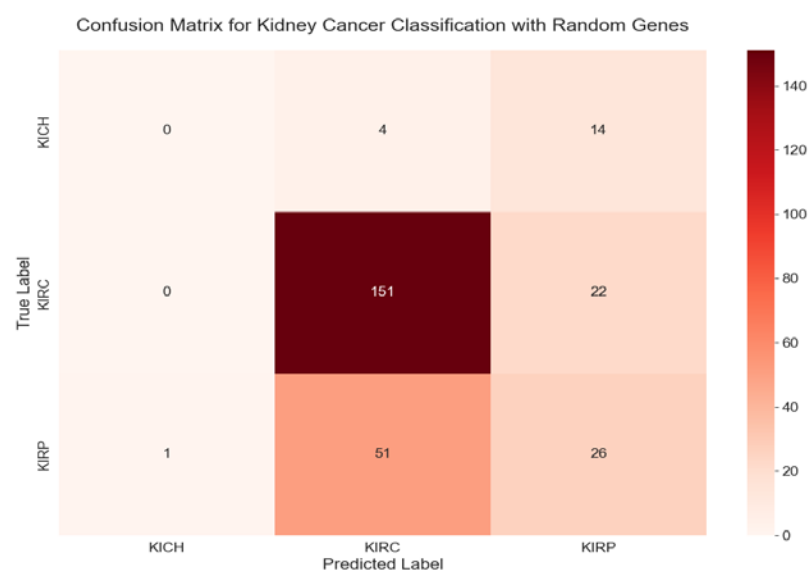


Figure 4.11: Confusion Matrix of Multi-Class XGBoost Model for Kidney Cancer Subtype Classification with Random Genes

Based on the results presented in Figure 4.11 and Table 4.3, the classification experiments conducted using artificially constructed features from randomly selected genes show a significant decrease in model accuracy (accuracy: 0.66). In particular, the KICH subtype was completely misclassified, and the macro-average F1-score was observed to be only 0.39. These findings indicate that the features derived from

mechanism-based gene sets contribute biologically meaningful information to the classification performance.

Table 4.3: Classification Report of Multi-Class XGBoost Model for Kidney Cancer Subtype Classification with Random Genes

Class	precision	recall	f1-score	support
KICH	0.00	0.00	0.00	18
KIRC	0.73	0.87	0.80	173
KIRP	0.42	0.33	0.37	78
accuracy			0.66	269
macro avg	0.38	0.40	0.39	269
weighted avg	0.59	0.66	0.62	269

The validation method with the random gene set was repeated 5 times, and the following accuracy rates were obtained. Table 4.4 shows the accuracy rates of the 5 runs conducted with random genes.

Table 4.4: Accuracy Rates of the 5 Runs Conducted with Random Genes for Kidney Cancer Subtype Classification

Run	Accuracy
1	0.7
2	0.68
3	0.72
4	0.65
5	0.69

These findings align with the classification report's overall accuracy of 94%, underscoring the model's potential as a reliable diagnostic tool for kidney cancer subtypes. In contrast, when the same model was tested using artificially generated features based on randomly selected genes, the average classification accuracy dropped significantly across five independent runs (Table 4.4). This supports its value in contributing to biomarker discovery and precision oncology applications.

Figure 4.12 illustrates the feature importance ranking of the XGBoost model, while Figure 4.13 depicts the mechanism importance ranking. Feature importance was calculated based on their contributions to the model's decision trees (e.g., using metrics such as gain, coverage, or split frequency). The top 30 features were ranked,

with their importance scores displayed on the horizontal axis. The feature with the highest importance score was identified as the first principal component of pyroptosis activator genes, followed by the first principal component of autophagic cell death inhibitor genes and the combined entropy of autophagic cell death. The presence of features related to all cell death mechanisms among the top 30 features indicates that all identified non-apoptotic cell death mechanisms contribute to distinguishing kidney cancer subtypes.

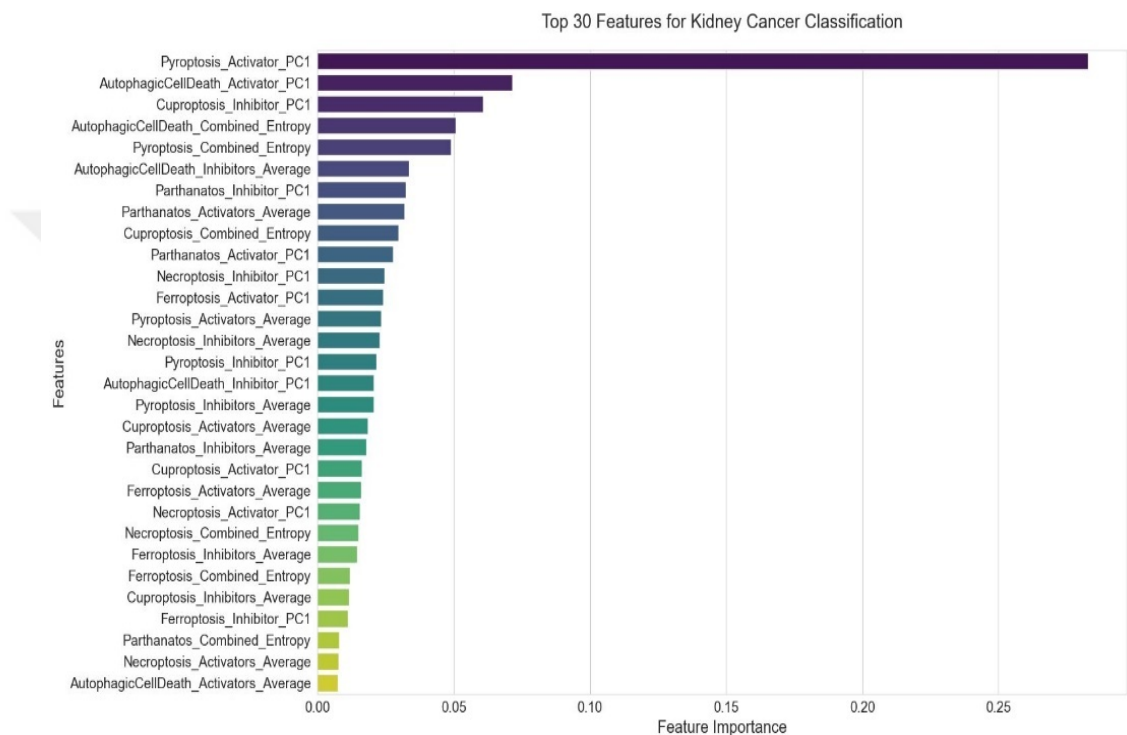


Figure 4.12: Feature Importance Ranking Determined by XGBoost Feature Importance

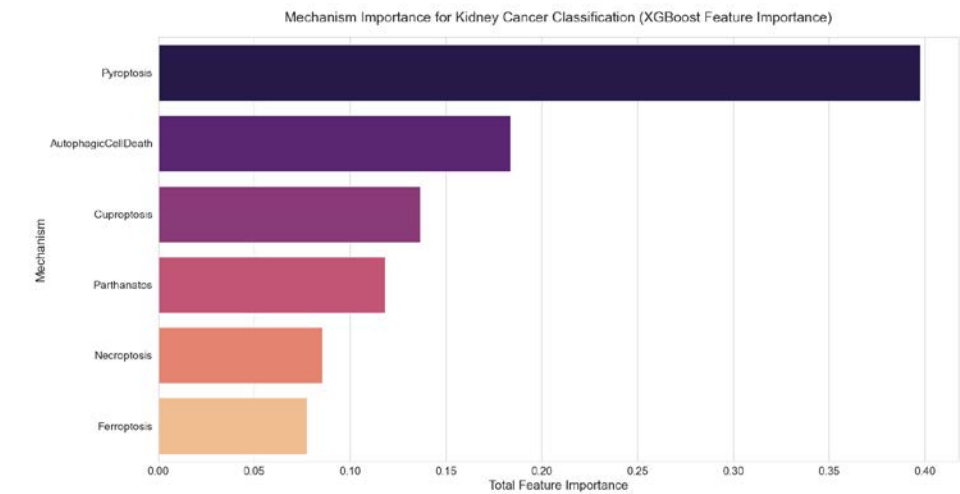


Figure 4.13: Mechanism Importance Ranking of the XGBoost Model

Figure 4.14 illustrates the total contribution of features from each cell death mechanism to the model’s predictions using SHAP (SHapley Additive exPlanations) values. SHAP values fairly quantify the impact of each mechanism on the model’s predictions and visually represent these effects collectively. Figure 4.15 shows the total contribution of each cell death mechanism to the model’s predictions using SHAP values.

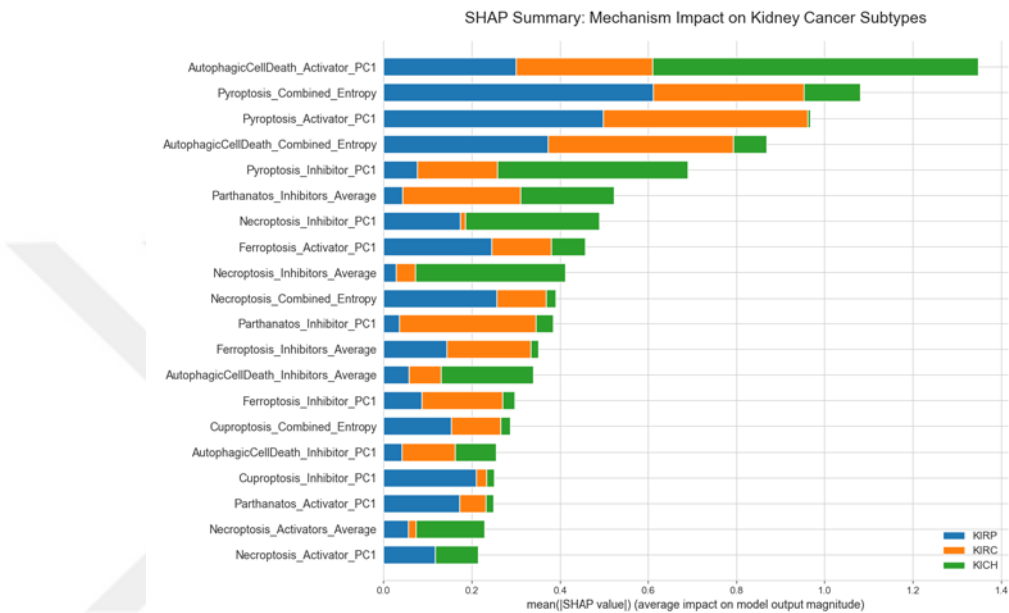


Figure 4.14: Feature Importance Ranking Determined by SHAP

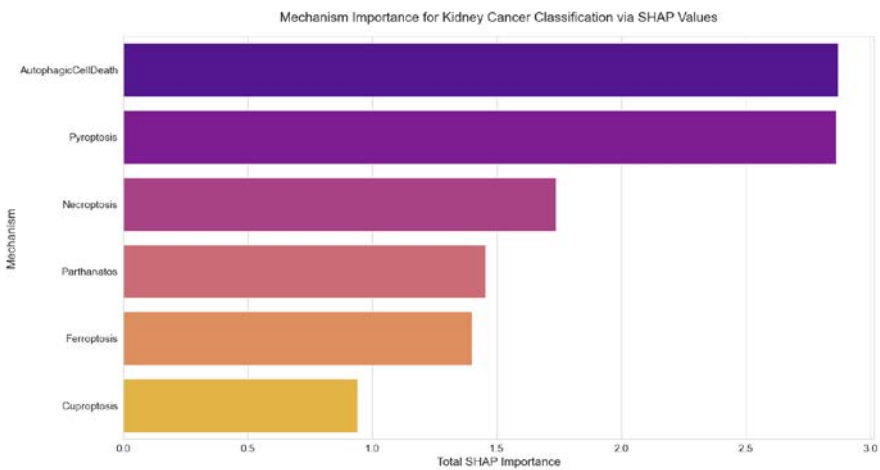


Figure 4.15: Mechanism Importance Ranking Determined by SHAP

Two graphs used in kidney cancer classification evaluate the impact of mechanisms and features through XGBoost feature importance and SHAP values. Figure 4.12 ranks the 30 most important features according to the XGBoost model. Pyroptosis Activator PC1 (0.25) and Cuproptosis Inhibitor PC1 (approximately 0.20) have the highest importance, while features like AutophagicCellDeath Combined Entropy, Pyroptosis Combined Entropy, and Parthanatos Activator PC1 also stand out. Features related to Pyroptosis, AutophagicCellDeath, and Cuproptosis (PC1, entropy, and mean scores) rank highly, indicating frequent use in the model's decision tree splits and significant contributions to classification performance.

Figure 4.14 analyzes the effect of features on KIRP, KIRC, and KICH subtypes using SHAP values. AutophagicCellDeath Activator PC1 emerges as the most impactful feature with an average SHAP value of approximately 1.4, followed by Pyroptosis Combined Entropy and Pyroptosis Activator PC1 (each around 1.2). The graph distinguishes the impact on each subtype with colors (KIRP: blue, KIRC: orange, KICH: green).

Both analyses highlight the dominance of Pyroptosis, AutophagicCellDeath and Necroptosis mechanisms. SHAP values not only reveal the features' contributions to the model's overall performance but also their specific effects on individual subtype classification, confirming that all mechanisms (including Cuproptosis, Parthanatos, Necroptosis, and Ferroptosis) contribute to classification to varying degrees and provide deeper biological insights into subtype-specific effects.

Autophagy is a process that breaks down and recycles unnecessary organelles or damaged cellular contents. It has been found to have dual functions in renal cell carcinoma (RCC). Many autophagy-related proteins are considered prognostic markers for RCC. Researchers have explored synthetic and phytochemical drugs targeting autophagy for RCC treatment (**He et al., 2021**). Targeting the pyroptosis pathway could lead to new therapeutic options, offering significant opportunities, particularly in controlling inflammation and directing cell death (**Zhou & Li, 2023, Wang et al., 2021**). Necroptosis-related genes can predict ccRCC prognosis: Risk models based on necroptosis-related genes (e.g., NRGscore, NPRGs, N-Score) show strong correlations with clinical variables such as patient survival, immune cell infiltration, and immunotherapy response (**Sheng et al., 2022, Cai et al., 2023, Chen et al., 2022**). The prognostic and therapeutic roles of autophagy, pyroptosis, and

necroptosis mechanisms in kidney cancer, as discussed in the literature, have been supported by XGBoost and SHAP analyses, confirming their critical importance in the classification of kidney cancer subtypes.

4.4 Validation of a Web-Based XGBoost Model for Kidney Cancer Tissue Detection and Tumor/Normal Classification Using Non-Apoptotic Cell Death Mechanisms

In the initial part of the final phase of the study, kidney cancer subtypes KIRP, KICH, and KIRC were combined and mapped as the Kidney class, and the XGBoost model was employed to predict the Kidney class among all cancers using all data in the TCGA. The Kidney class achieved precision, sensitivity, and F1-score of 0.98, making it one of the best-performing classes in the model. This indicates that the model can detect kidney tissue with high accuracy and has a very low likelihood of confusing it with other tissues.

Supported by 317 samples, this high performance confirms the model's reliability in classifying the Kidney class. Compared to the overall test accuracy (87.5%) and cross-validation average (0.857), the Kidney class performance (0.98 F1-score) demonstrates that the model achieves significantly above-average success in this class (Figure 4.16). The model also accurately classified other well-clustered cancers, such as LIHC, THCA, and PRAD, as shown in t-SNE and UMAP projections generated with TCGA paired data in Section 4.2 of the project (Figure 4.8).

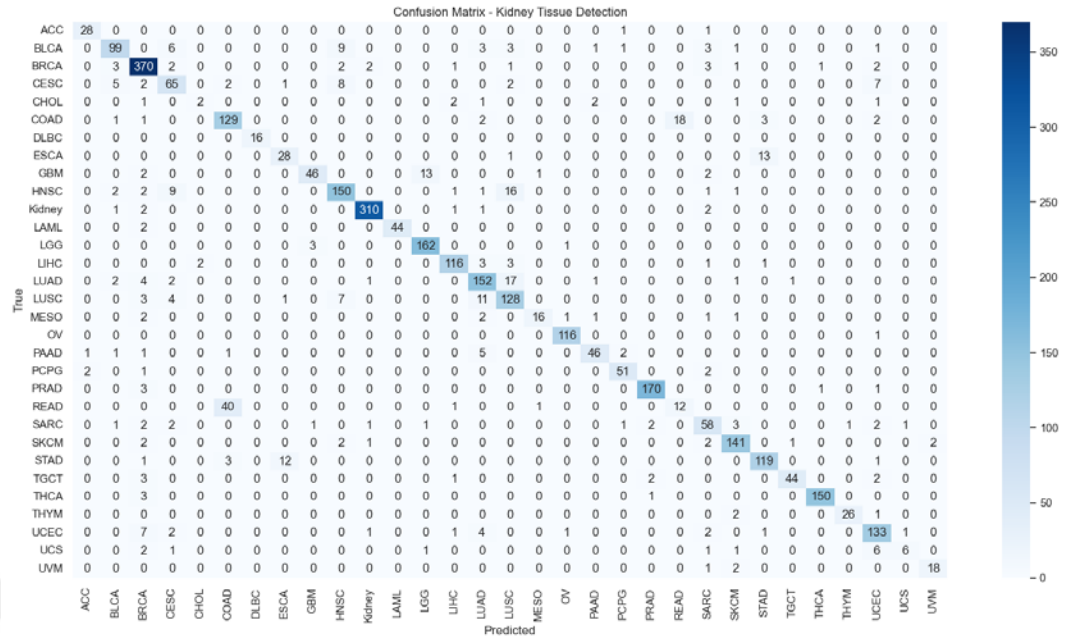


Figure 4.16: Confusion Matrix for Multi-Class Tissue Detection - Kidney-Focused Analysis

The observed confusion in the classification of COAD and READ by the model likely stems from their shared molecular mechanisms. Both cancer types, as part of the colorectal cancer spectrum, exhibit highly similar genetic and molecular profiles, including frequent APC, KRAS, and TP53 mutations, as well as microsatellite instability (MSI) (Cancer Genome Atlas Network, 2012).

In the second phase of the study, tumor/normal classification was performed using the XGBoost model, trained exclusively on data from kidney normal and tumor samples. The model classifies the Tumor class with near-perfect performance (F1-score 0.97, sensitivity 1.00), indicating high success in detecting cancerous cases. However, due to low sensitivity (0.58) in the Normal class, some healthy samples are mistakenly classified as Tumor. This is attributed to the imbalance in the dataset (Tumor: 358, Normal: 52). The overall accuracy (94.6%) and cross-validation average (0.946 ± 0.027) demonstrate the model's generally robust performance (Figure 4.17).

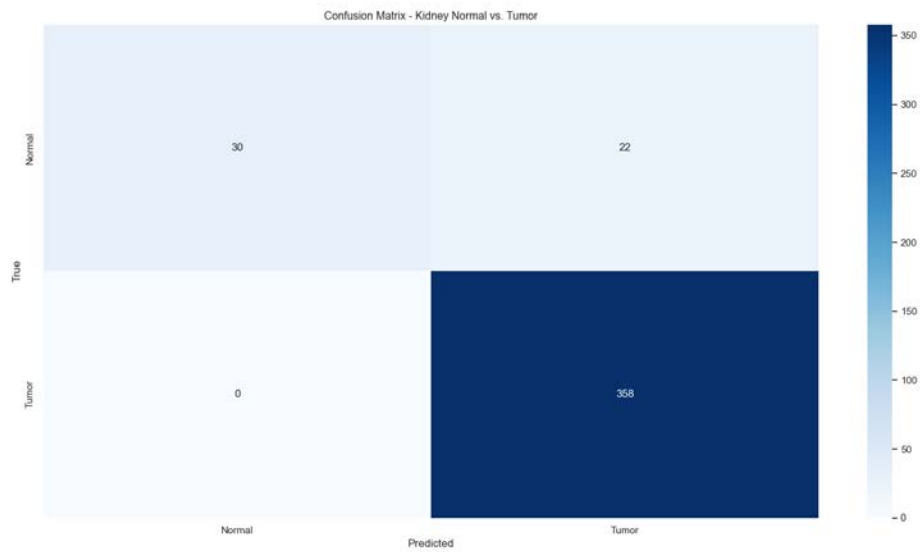


Figure 4.17: Confusion Matrix for Kidney Normal vs. Tumor Classification

In all data obtained from TCGA, the proportion of normal samples is significantly lower than that of tumor samples, as indicated in Figure 3.3. This is the reason for the low precision in detecting normal classes. Table 4.5 presents the classification report resulting from training with all TCGA data. The dataset contains 77 normal and 537 tumor samples.

Table 4.5: Classification Report of Multi-Class XGBoost Model for Kidney Cancer Normal/Tumor Classification using all Data in TCGA

Class	precision	recall	f1-score	support
Normal	1.00	0.58	0.73	52
Tumor	0.94	1.00	0.97	358
accuracy			0.95	410
macro avg	0.97	0.79	0.85	410
weighted avg	0.95	0.95	0.94	410

When training was performed with paired data balanced between normal and tumor classes, the recall value for the normal class was 0.98, as shown in Table 4.6. The paired dataset consists of 77 normal and 77 tumor samples.

Table 4.6: Classification Report of Multi-Class XGBoost Model for Kidney Cancer Normal/Tumor Classification using Paired Data in TCGA

Class	precision	recall	f1-score	support
Normal	0.93	0.98	0.95	52
Tumor	0.98	0.92	0.95	52
accuracy			0.95	104
macro avg	0.95	0.95	0.95	104
weighted avg	0.95	0.95	0.95	104

In the final part of the study, a Flask-based web API was developed for kidney cancer tissue detection and tumor/normal classification, and a web application accessible at kidneycancerscope.com was created. Compatible with Python 3.6+, this tool system analyzes gene expression data to identify kidney tissue and classify the detected kidney tissues as tumor or normal. The API processes gene expression data, calculating features (mean expression, PCA, Shannon entropy, activator/inhibitor ratio) derived from gene sets associated with necroptosis, pyroptosis, ferroptosis, parthanatos, autophagic cell death, and cuproptosis cell death mechanisms. Using XGBoost models, it performs tumor/normal classification and kidney tissue prediction. The /predict endpoint, which accepts data in CSV format, returns sample names, tissue type, tumor/normal predictions, and tumor probabilities in JSON format (Figure 4.18).

Kidney Tumor Prediction

Upload CSV File

Dosya Seç GSE11151.csv

Predict Now

CSV File Format

Your CSV file should contain non-apoptotic programmed cell death mechanism-related gene expression data with the following structure:

- Gene Names:** RIPK1, RIPK3, ZBP1, CYLD, TICAM1, TNF, MLKL, CASP8, FADD, CFLAR, CASP1, CASP4, CASP5, NLRP3, AIM2, NLRC4, GZMA, GZMB, PYCARD, NLRP1, CARD16, CARD18, NLRX1, POP1, BCL2, IL10, ALOX15, ACSL4, LPCAT3, TFRC, NOX1, NOX4, SAT1, GPX4, SLC7A11, GCLC, GCLM, GSS, FTH1, FTL, NFE2L2, MT1G, CDKN1A, PARP1, AIFM1, MIF, MACROD1, MACROD2, NUDT16, SIRT1, BECN1, ATG5, ATG7, ULK1, FOXO3, BNIP3, BNIP3L, AKT1, MTOR, FDX1, UAS, LIPT1, DLD, DLAT, PDHA1, PDHB, MTF1, GLS, CDKN1A.
- Row Index:** Sample names (e.g., Sample1).
- Columns:** Gene names (e.g., RIPK1, RIPK3, CASP8).
- Values:** Numeric values (float).
- Optional:** sample_title column for metadata.

Example:

```
,RIPK1,RIPK3,CASP8,...,sample_title
Sample1,5.2,3.1,2.4,...,SampleTitle1
Sample2,4.8,2.9,2.1,...,SampleTitle2
```

Prediction Results

Sample: GSM261278 Predicted Tissue: Kidney Prediction: Tumor Tumor Probability: 57.9%	Sample: GSM261279 Predicted Tissue: Kidney Prediction: Tumor Tumor Probability: 51.3%	Sample: GSM261280 Predicted Tissue: Kidney Prediction: Tumor Tumor Probability: 56.4%
Sample: GSM261281 Predicted Tissue: Kidney Prediction: Tumor Tumor Probability: 51.3%	Sample: GSM261282 Predicted Tissue: Kidney Prediction: Tumor Tumor Probability: 51.3%	Sample: GSM261283 Predicted Tissue: Kidney Prediction: Tumor Tumor Probability: 57.1%

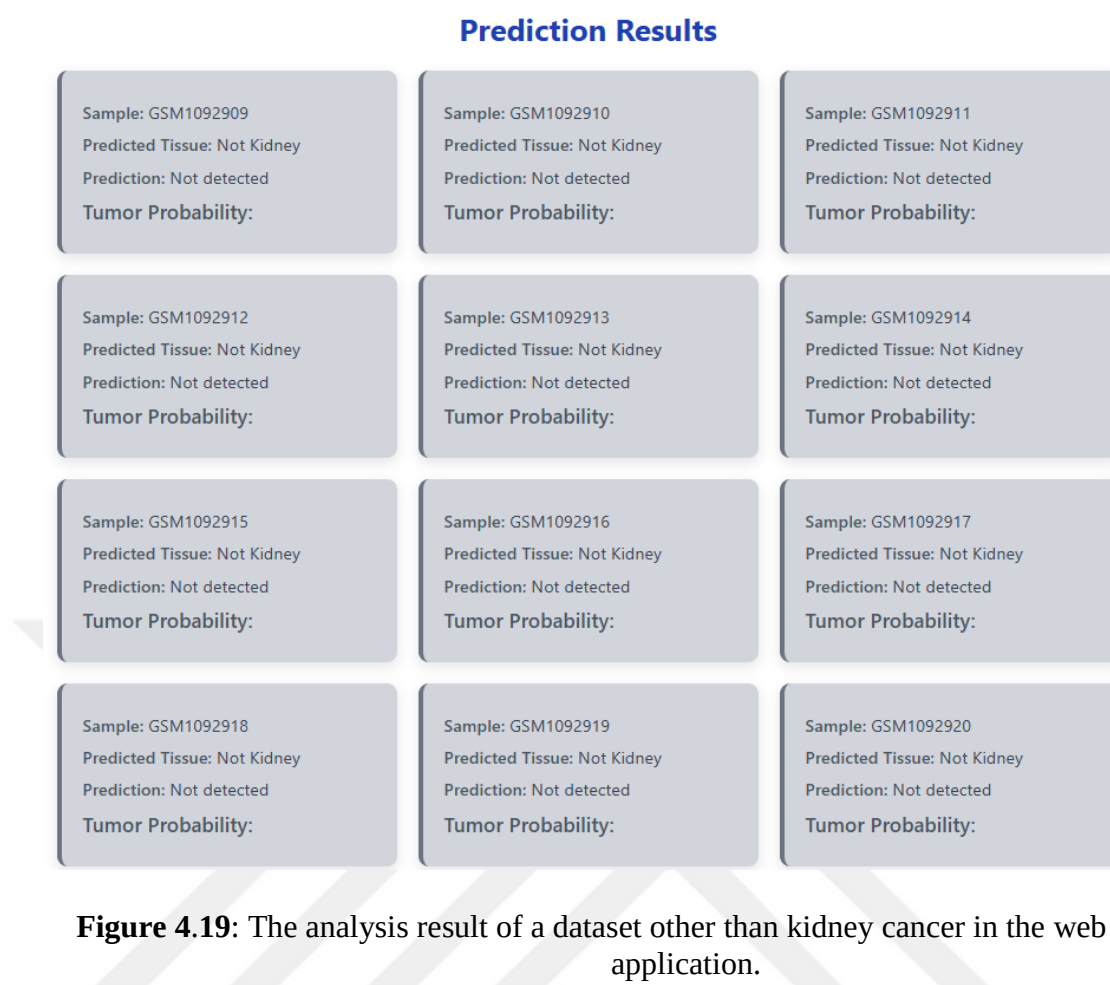
Figure 4.18: Web Application for Kidney Tissue and Tumor Prediction Using an XGBoost Model with Features Extracted from Non-Apoptotic Programmed Cell Death Mechanisms (kidneycancerscope.com)

The web application analyzed the GSE11151 dataset, which includes 67 kidney tissue samples: 5 normal (2 fetal, 3 adult) and 62 tumor samples. Tissue prediction achieved 100% accuracy, correctly identifying all samples as kidney tissue. For sample classification, the 3 adult normal samples were accurately classified as normal, but the 2 fetal normal samples were misclassified as tumors. Additionally, 1 tumor sample (Wilms tumor) was incorrectly predicted as normal. Thus, of the 5 normal samples, only the 3 adult normal samples were correctly classified. For tumor samples, 61 out of 62 were accurately classified, yielding a high success rate. As shown in Figure 4, the absence of fetal data in the TCGA dataset likely caused the misclassification of fetal samples, particularly due to the model's lack of training on fetal kidney tissue.

The GSE105288-GPL10558 dataset, comprising 44 kidney tissue samples, includes 9 normal and 35 tumor samples. Tissue prediction again achieved 100% accuracy, correctly identifying all samples as kidney tissue. In sample classification, 5 samples were predicted as normal. However, 1 tumor sample) was incorrectly classified as normal, and 5 normal samples were misclassified as tumors. All other tumor predictions were accurate.

Experiments on these datasets demonstrated excellent tissue identification performance, with 100% accuracy in both cases. Tumor detection showed strong performance, despite some misclassifications. Adult normal samples were classified with high accuracy, but the misclassification of fetal normal samples and certain normal samples as tumors indicates a need for improved model discriminability for these classes. Overall, tumor detection achieved a high accuracy rate with few exceptions.

The web application operates as follows: If the detected tissue is not kidney tissue, it returns a “Not Kidney” response and does not perform tumor-normal classification. In this context, the model was also tested with datasets containing non-kidney tissue data. All samples in the GSE44861 dataset, obtained from GEO and containing 111 colon tissue samples, were classified as “Not Kidney.” Some of the results are shown in Figure 4.19.



Experiments on these two external datasets demonstrated excellent performance in tissue identification, with 100% accuracy achieved in both cases. Tumor detection showed relatively good performance, despite some misclassifications. Notably, adult normal tissues were correctly classified with high accuracy, but the misclassification of fetal normal tissues and certain normal samples as tumors highlights the need for improved model discriminability in these classes. Tumor detection, with few exceptions, achieved a high accuracy rate.

This application has achieved significant success as an automated and rapid analysis tool for classifying kidney tissue and kidney cancer samples. The 100% accuracy in tissue type identification underscores the application's robust capability to reliably detect kidney tissue. The results in tumor and normal tissue classification, particularly the high accuracy in tumor detection, suggest that the application holds potential as a supportive tool in clinical and research settings. However, the misclassification of fetal

normal tissues and some normal samples indicates areas for improvement, which could be addressed through training with larger datasets or enhanced feature engineering.

This web application serves as a valuable tool in bioinformatics and oncology research, particularly for understanding serious health conditions such as kidney cancer at the molecular level. Its validation of the impact of non-apoptotic cell death mechanisms on kidney tissue and cancer using external datasets confirms its reliability in exploring biological mechanisms. In clinical settings, the application could function as a supportive diagnostic tool for the rapid and accurate differentiation of tumor and normal tissues. Furthermore, it enables researchers to analyze large datasets to identify disease-related biomarkers, thereby contributing to the development of personalized medicine approaches and the identification of novel therapeutic targets for kidney cancer treatment. In conclusion, this application significantly enhances and accelerates biomedical data analysis, offering substantial value in both academic research and clinical applications.

4.5 Discussion and Future Directions

This study examines the roles of non-apoptotic programmed cell death (PCD) mechanisms—necroptosis, pyroptosis, ferroptosis, parthanatos, autophagic cell death, and cuproptosis—in cancer biology, focusing on their regulatory dynamics across different cancer types. Analysis of paired tumor and normal tissue samples from The Cancer Genome Atlas (TCGA) identified notable gene expression differences in these mechanisms, particularly in kidney cancer subtypes (KIRC, KIRP, KICH). Using Gene Set Variation Analysis (GSVA) and Wilcoxon signed-rank tests, the study found that activator and inhibitor gene sets for these mechanisms exhibit varied regulation in tumor tissues compared to normal tissues. These observations suggest that non-apoptotic PCD pathways may influence cancer progression in both tumor-suppressive and tumor-promoting capacities (Yan et al., 2022; Chen et al., 2023).

The regulatory differences observed in kidney cancers indicate potential sensitivity to non-apoptotic PCD mechanisms. For example, in KIRC and KIRP, upregulation of necroptosis and pyroptosis gene sets, including both activators and inhibitors, may suggest a tumor-suppressive role. In contrast, downregulation of these gene sets in cancers such as LUSC and COAD could imply that tumor cells evade these death

pathways. These cancer type-specific patterns suggest the potential for tailored therapeutic strategies targeting non-apoptotic PCD mechanisms.

Dimensionality reduction techniques, including UMAP and t-SNE, showed clear separation of kidney cancer subtypes based on gene expression profiles, indicating that non-apoptotic PCD gene sets may provide biologically relevant features for distinguishing cancer subtypes. An XGBoost-based machine learning model achieved 94% accuracy in classifying KIRC, KIRP, and KICH subtypes, suggesting the clinical utility of these gene sets. SHAP analysis highlighted the contributions of pyroptosis and autophagic cell death to classification performance, pointing to their relevance in kidney cancer biology. The inflammatory characteristics of pyroptosis may offer opportunities for modulating immune responses in the tumor microenvironment, potentially informing immunotherapeutic approaches **(Wei et al., 2022)**.

The investigation of cuproptosis, a recently identified mechanism associated with mitochondrial metabolism and copper ion homeostasis, represents a novel aspect of this study. Cuproptosis may present opportunities for targeting metabolic vulnerabilities in cancer cells **(Tsvetkov et al., 2022)**. However, its roles across cancer types and clinical relevance require further exploration. Similarly, the cancer type-specific regulation of parthanatos and autophagic cell death highlights their complex functions in physiological and pathological contexts.

A Flask-based web application (kidneycancerscope.com), utilizing TCGA and GEO datasets (GSE11151, GSE105288), demonstrated high accuracy in tissue identification and tumor/normal classification. The effective classification of adult normal tissues suggests potential applications as a diagnostic tool in clinical and research settings. However, misclassification of fetal tissues may result from limited representation in the training dataset, indicating a need for more diverse datasets.

Limitations of the study include the limited availability of normal tissue samples for some cancer types and the small sample size for subtypes such as KICH. Additionally, *in vivo* and *in vitro* functional studies are needed to better understand the roles of non-apoptotic PCD mechanisms in cancer progression. Future research could further investigate their interactions with the tumor microenvironment and immune system and assess their therapeutic potential in clinical trials.

Renal cell carcinoma, a common malignant tumor, often shows limited response to radiotherapy and chemotherapy (Qin et al., 2022). Continued research on non-apoptotic PCD mechanisms in these cancers may contribute to the development of new therapeutic strategies. Overall, this study provides insights into the cancer type-specific roles of non-apoptotic PCD mechanisms, highlighting their biological and potential clinical relevance. The bioinformatics tool developed for kidney cancer diagnosis offers a step toward precision oncology, supporting further exploration of non-apoptotic PCD pathways as potential therapeutic targets. These findings contribute to ongoing efforts in cancer research and the advancement of personalized medicine.

Although the current performance of the developed machine learning model yields promising results for renal cancers and tissues, further improvements are required for the model to be widely adopted in clinical applications. The first aspect that needs enhancement is the generalization of the model to encompass all tissue types. For this, the model must be retrained using a larger and more diverse gene expression dataset. At present, the model relies solely on gene expression data. However, integrating heterogeneous data type such as epigenetic data (e.g., methylation profiles), proteomic data, and clinical information (e.g., patient age, gender, lifestyle factors) can significantly enhance the model's accuracy and clinical applicability. To achieve this, multi-modal machine learning approaches that combine multiple data types should be developed. Additionally, attention mechanisms can be employed to model correlations between different data modalities. For the model to be applicable in clinical settings, it must undergo extensive validation using real-world data (e.g., prospectively collected data from hospitals).

The second aspect that needs to be addressed for the model to gain widespread clinical use is its ability to produce results using data obtained from body fluids—particularly blood—instead of tissue samples. This is crucial because if a biopsy has already been taken from an organ, it likely indicates a pre-existing suspicion of cancer in that organ. Utilizing blood- and fluid-based biomarkers for cancer diagnosis would represent a significant innovation for both patients and clinicians. Unlike invasive methods such as biopsies, which are only performed on pre-diagnosed organs, blood samples can be collected non-invasively and used in broad screening programs.

REFERENCES

- Arroyo, A. B., Bernal-Carrión, M., Cantón-Sandoval, J., Cabas, I., Corbalán-Vélez, R., Martínez-Menchón, T., Ferri, B., Cayuela, M. L., García-Moreno, D., Mulero, V., “NAMPT and PARylation are involved in the pathogenesis of atopic dermatitis”, *International Journal of Molecular Sciences*, 2023, 24(9), 7992.
- Bersuker, K., Hendricks, J. M., Li, Z., Magtanong, L., Ford, B., Tang, P. H., Roberts, M. A., Tong, B., Maimone, T. J., Zoncu, R., Bassik, M. C., Nomura, D. K., Dixon, S. J., Olzmann, J. A., “The CoQ oxidoreductase FSP1 acts parallel to GPX4 to inhibit ferroptosis”, *Nature*, 2019, 575(7784), 688–692.
- Broz, P., Dixit, V. M., “Inflammasomes: mechanism of assembly, regulation and signalling”, *Nature Reviews Immunology*, 2016, 16(7), 407–420.
- Cai, M., Yang, Q., He, J., Wu, H., Li, Z., Fang, Z., Li, J., “Identification and validation of a necroptosis-related prognostic signature for kidney renal clear cell carcinoma”, *Stem Cells International*, 2023, 8446765.
- Carels, N., “Assessing RNA-Seq workflow methodologies using Shannon entropy”, *Biology (Basel)*, 2024, 13(7), 482.
- Changotra, H., Kaur, S., Yadav, S. S., Gupta, G. L., Parkash, J., Duseja, A., “ATG5: A central autophagy regulator implicated in various human diseases”, *Cell Biochemistry and Function*, 2022, 40(8), 758–768.
- Chen, J. W., Chen, S., Chen, G. Q., “Recent advances in natural compounds inducing non-apoptotic cell death for anticancer drug resistance”, *Cancer Drug Resistance*, 2023, 6, 729–747.
- Chen, J., Tao, Q., Lang, Z., Jin, Y., Chen, G., Li, X., Yu, Z., Li, Y., “Development and validation of a novel necroptosis-related score to improve the outcomes of clear cell renal cell carcinoma”, *Frontiers in Genetics*, 2022, 13, 926815.
- Chen, L., Min, J., Wang, F., “Copper homeostasis and cuproptosis in health and disease”, *Signal Transduction and Targeted Therapy*, 2022, 7, 378.
- Chen, Y., Yi, H., Liao, S., He, J., Zhou, Y., Lei, Y., “LC3B: A microtubule-associated protein influences disease progression and prognosis”, *Cytokine & Growth Factor Reviews*, 2025, 81, 16–26.
- Collier, J. J., Suomi, F., Oláhová, M., McWilliams, T. G., Taylor, R. W., “Emerging roles of ATG7 in human health and disease”, *EMBO Molecular Medicine*, 2021, 13(12), e14824.
- Debnath, J., Gammoh, N., Ryan, K. M., “Autophagy and autophagy-related pathways in cancer”, *Nature Reviews Molecular Cell Biology*, 2023, 24, 560–575.

Denton, D., Kumar, S., “Autophagy-dependent cell death”, *Cell Death and Differentiation*, 2019, 26, 605–616.

Devi, S., Stehlik, C., Dorfleutner, A., “An update on CARD only proteins (COPs) and PYD only proteins (POPs) as inflammasome regulators”, *International Journal of Molecular Sciences*, 2020, 21(18), 6901.

Ding, J., Wang, K., Liu, W., She, Y., Sun, Q., Shi, J., Sun, H., Wang, D. C., Shao, F., “Pore-forming activity and structural autoinhibition of the gasdermin family”, *Nature*, 2016, 535(7610), 111–116.

Dixon, S. J., Lemberg, K. M., Lamprecht, M. R., Skouta, R., Zaitsev, E. M., Gleason, C. E., Patel, D. N., Bauer, A. J., Cantley, A. M., Yang, W. S., Morrison, B. III, Stockwell, B. R., “Ferroptosis: an iron-dependent form of nonapoptotic cell death”, *Cell*, 2012, 149

Dong, X., Liang, Q., Pan, Y.-Z., Wang, X., Kuo, Y.-C., Chiang, W.-C., Zhang, X., Williams, N. S., Rizo, J., Levine, B., De Brabander, J. K., “Novel Bcl-2 inhibitors selectively disrupt the autophagy-specific Bcl-2–Beclin 1 protein–protein interaction”, *ACS Medicinal Chemistry Letters*, 2022, 13(9), 1510–1516.

Eckhart, L., Fischer, H., “Caspase-5: Structure, pro-inflammatory activity and evolution”, *Biomolecules*, 2024, 14(5), 520.

Fan, K., Dong, Y., Li, T., Li, Y., “Cuproptosis-associated CDKN2A is targeted by plicamycin to regulate the microenvironment in patients with head and neck squamous cell carcinoma”, *Frontiers in Genetics*, 2023, 13, 1036408.

Fang, Y., Tian, S., Pan, Y., Li, W., Wang, Q., Tang, Y., Yu, T., Wu, X., Shi, Y., Ma, P., Shu, Y., “Pyroptosis: A new frontier in cancer”, *Biomedicine & Pharmacotherapy*, 2020, 121, 109595.

Fitzwalter, B. E., Thorburn, A., “FOXO3 links autophagy to apoptosis”, *Autophagy*, 2018, 14(8), 1467–1468.

Galluzzi, L., Kepp, O., Chan, F. K., Kroemer, G., “Necroptosis: Mechanisms and relevance to disease”, *Annual Review of Pathology: Mechanisms of Disease*, 2017, 12, 103–130.

Galluzzi, L., Vitale, I., Aaronson, S. A., et al., “Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018”, *Cell Death and Differentiation*, 2018, 25(3), 486–541.

Gong, Y., Fan, Z., Luo, G., et al., “The role of necroptosis in cancer biology and therapy”, *Molecular Cancer*, 2019, 18, 100.

Hadian, K., Stockwell, B. R., “The therapeutic potential of targeting regulated non-apoptotic cell death”, *Nature Reviews Drug Discovery*, 2023, 22, 723–742.

Hanahan, D., Weinberg, R. A., “Hallmarks of cancer: the next generation”, *Cell*, 2011, 144(5), 646–674.

He, Y. H., Tian, G., “Autophagy as a vital therapy target for renal cell carcinoma”, *Frontiers in Pharmacology*, 2021, 11, 518225.

Huang, P., Chen, G., Jin, W., Mao, K., Wan, H., He, Y., “Molecular mechanisms of parthanatos and its role in diverse diseases”, *International Journal of Molecular Sciences*, 2022, 23(13), 7292.

Hughes, S. A., Lin, M., Weir, A., Huang, B., Xiong, L., Chua, N. K., Pang, J., Santavanond, J. P., Tixeira, R., Doerflinger, M., Deng, Y., Yu, C. H., Silke, N., Conos, S. A., Frank, D., Simpson, D. S., Murphy, J. M., Lawlor, K. E., Pearson, J. S., Silke, J., Pellegrini, M., Herold, M. J., Poon, I. K. H., Masters, S. L., Li, M., Tang, Q., Zhang, Y., Rashidi, M., Geng, L., Vince, J. E., “Caspase-8-driven apoptotic and pyroptotic crosstalk causes cell death and IL-1 β release in X-linked inhibitor of apoptosis (XIAP) deficiency”, *EMBO Journal*, 2023, 42(5), e110468.

Hänzelmann S, Castelo R, Guinney J (2013). “GSVA: gene set variation analysis for microarray and RNA-Seq data.” *BMC Bioinformatics*, 14, 7

Jiang, X., Stockwell, B. R., Conrad, M., “Ferroptosis: mechanisms, biology and role in disease”, *Nature Reviews Molecular Cell Biology*, 2021, 22(4), 266–282.

Jin, X., Ma, Y., Liu, D., Huang, Y., “Role of pyroptosis in the pathogenesis and treatment of diseases”, *MedComm*, 2023, 4(3), e249.

Kerr, J. F., Wyllie, A. H., Currie, A. R., “Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics”, *British Journal of Cancer*, 1972, 26(4), 239–257.

Kim, J., Guan, K. L., “mTOR as a central hub of nutrient signalling and cell growth”, *Nature Cell Biology*, 2019, 21, 63–71.

Kong, H., Zhao, H., Chen, T., et al., “Targeted P2X7/NLRP3 signaling pathway against inflammation, apoptosis, and pyroptosis of retinal endothelial cells in diabetic retinopathy”, *Cell Death & Disease*, 2022, 13, 336.

Lee, J., Roh, J. L., “SLC7A11 as a gateway of metabolic perturbation and ferroptosis vulnerability in cancer”, *Antioxidants (Basel)*, 2022, 11(12), 2444.

Levine, B., Kroemer, G., “Biological functions of autophagy genes: A disease perspective”, *Cell*, 2019, 176(1–2), 11–42.

Li, D., Li, Y., “The interaction between ferroptosis and lipid metabolism in cancer”, *Signal Transduction and Targeted Therapy*, 2020, 5, 108.

Li, J., Cao, F., Yin, H. L., et al., “Ferroptosis: past, present and future”, *Cell Death & Disease*, 2020, 11, 88.

Li, X., Li, F., Zhang, X., et al., “Caspase-8 auto-cleavage regulates programmed cell death and collaborates with RIPK3/MLKL to prevent lymphopenia”, *Cell Death and Differentiation*, 2022, 29, 1500–1512.

Li, Z., Mo, F., Wang, Y., et al., “Enhancing Gasdermin-induced tumor pyroptosis through preventing ESCRT-dependent cell membrane repair augments antitumor immune response”, *Nature Communications*, 2022, 13, 6321.

Liao, C., Wang, X., “TCGApilot: an R package for integrative pan-cancer analysis and visualization of TCGA multi-omics data”, *BMC Bioinformatics*, 2023, 24, 483.

Lin, C.-H., Chin, Y., Zhou, M., Sobol, R. W., Hung, M.-C., & Tan, M. (2024). Protein lipoylation: Mitochondria, cuproptosis, and beyond. *Trends in Biochemical Sciences*, 49(8), 729–744.

Lin, L., Long, Y., Liu, J., Deng, D., Yuan, Y., Liu, L., Tan, B., Qi, H., “FRP-XGBoost: Identification of ferroptosis-related proteins based on multi-view features”, *International Journal of Biological Macromolecules*, 2024, 262(Part 2), 130180.

Linder, B., Kögel, D., “Autophagy in cancer cell death”, *Biology*, 2019, 8(4), 82.

Liu, L., Li, J., Ke, Y., et al., “The key players of parthanatos: opportunities for targeting multiple levels in the therapy of parthanatos-based pathogenesis”, *Cellular and Molecular Life Sciences*, 2022, 79, 60.

Liu, M., Liu, K., Cheng, D., Zheng, B., Li, S., Mo, Z., “The regulatory role of NLRX1 in innate immunity and human disease”, *Cytokine*, 2022, 160, 156055.

Liu, S., Yao, S., Yang, H., et al., “Autophagy: Regulator of cell death”, *Cell Death & Disease*, 2023, 14, 648.

Lundberg, S. M., Lee, S.-I., “A unified approach to interpreting model predictions”, *Advances in Neural Information Processing Systems (NeurIPS)*, 2017, 30.

Ma, S., Dai, Y., “Principal component analysis based methods in bioinformatics studies”, *Briefings in Bioinformatics*, 2011, 12(6), 714–722.

Man, S. M., Kanneganti, T. D., “Regulation of inflammasome activation”, *Immunological Reviews*, 2015, 265(1), 6–21.

Martínez-Morcillo, F. J., Cantón-Sandoval, J., Martínez-Navarro, F. J., Cabas, I., Martínez-Vicente, I., Armistead, J., Hatzold, J., López-Muñoz, A., Martínez-Menchón, T., Corbalán-Vélez, R., Lacal, J., Hammerschmidt, M., García-Borrón, J. C., García-Ayala, A., Cayuela, M. L., Pérez-Oliva, A. B., García-Moreno, D., Mulero, V., “NAMPT-derived NAD⁺ fuels PARP1 to promote skin inflammation through parthanatos cell death”, *PLoS Biology*, 2021, 19(11), e3001455

Mashimo, M., Bu, X., Aoyama, K., Kato, J., Ishiwata-Endo, H., Stevens, L. A., Kasamatsu, A., Wolfe, L. A., Toro, C., Adams, D., Markello, T., Gahl, W. A., Moss,

J., “PARP1 inhibition alleviates injury in ARH3-deficient mice and human cells”, JCI Insight, 2019, 4(4), e124519.

Mizushima, N., Levine, B., “Autophagy in human diseases”, The New England Journal of Medicine, 2020, 383(16), 1564–1576.

Morgan, M. J., Kim, Y. S., “RIPK3 in necroptosis and cancer”, Molecules and Cells, 2025, 48(5).

Morgan, M. J., Kim, Y. S., “Roles of RIPK3 in necroptosis, cell signaling, and disease”, Experimental & Molecular Medicine, 2022, 54, 1695–1704

Moura, R. D., Mattos, P. D., Valente, P. F., Hoch, N. C., “Molecular mechanisms of cell death by parthanatos: More questions than answers”, Genetics and Molecular Biology, 2024, 47 Suppl 1, e20230357.

Nafe, R., Hattingen, E., “Forms of Non-Apoptotic Cell Death and Their Role in Gliomas—Presentation of the Current State of Knowledge”, Biomedicines, 2024, 12(7), 1546.

Nam, H. Y., Chandrashekar, D. S., Kundu, A., Shelar, S., et al., “Integrative epigenetic and gene expression analysis of renal tumor progression to metastasis”, Molecular Cancer Research, 2019, 17(1), 84–96.

National Cancer Institute (NCI), “The Cancer Genome Atlas (TCGA) Program”, U.S. Department of Health and Human Services, 2023.

Pedreira, L., Prieto Clemente, L., Dahlhaus, A., et al., “Ferroptosis triggers mitochondrial fragmentation via Drp1 activation”, Cell Death & Disease, 2025, 16, 40.

Peterfi, L., Yusenko, M. V., Kovacs, G., Beothe, T., “Lack of VEGFA/KDR signaling in conventional renal cell carcinoma explains the low efficacy of target therapy and frequent adverse events”, International Journal of Molecular Sciences, 2024, 25(13), 7525.

Qin, S., Wang, Y., Wang, P., et al., “Molecular mechanism of circRNAs in drug resistance in renal cell carcinoma”, Cancer Cell International, 2022, 22, 369.

Ratsimandresy, R. A., Chu, L. H., Khare, S., de Almeida, L., Gangopadhyay, A., Indramohan, M., Misharin, A. V., Greaves, D. R., Perlman, H., Dorfleutner, A., Stehlik, C., “The PYRIN domain-only protein POP2 inhibits inflammasome priming and activation”, Nature Communications, 2017, 8, 15556.

Ryan, B. M., Zanetti, K. A., Robles, A. I., Schetter, A. J., et al., “Germline variation in NCF4, an innate immunity gene, is associated with an increased risk of colorectal cancer”, International Journal of Cancer, 2014, 134(6), 1399–1407.

Schober P, Vetter TR. Nonparametric Statistical Methods in Medical Research. Anesth Analg. 2020 Dec;131(6):1862-1863.

Shannon, C. E., “A mathematical theory of communication”, *Bell System Technical Journal*, 1948, 27(3), 379–423.

Sheng, X., Jiaquan, M., Chen, D., Jiaxin, W., Yuchao, L., Jun, Y., Jia, H., Xiaming, L., Wei, G., Tao, W., Shaogang, W., Jihong, L., Wen, S., Xiaodong, S., “Identification and quantification of necroptosis landscape on therapy and prognosis in kidney renal clear cell carcinoma”, *Frontiers in Genetics*, 2022, 13, 891929.

Shi, C. S., Kehrl, J. H., “Bcl-2 regulates pyroptosis and necroptosis by targeting BH3-like domains in GSDMD and MLKL”, *Cell Death Discovery*, 2019, 5, 151.

Shi, J., Gao, W., Shao, F., “Pyroptosis: Gasdermin-Mediated Programmed Necrotic Cell Death”, *Trends in Biochemical Sciences*, 2017, 42(4), 245–254.

Shimizu, S., “Autophagic cell death and cancer chemotherapeutics”, in: *Innovative Medicine*, pp. 219–226, Springer, Tokyo, 2015.

Song, Z., Gao, M., Li, T., Zhang, Y., Chen, Z., Hu, L., Liu, J., Li, Y., Wang, X., Liu, Y., Mo, R., Xiang, R., Hua, D., Chen, H., Zhao, M., Chen, X., Yao, X., Yang, Y., “TRPV3-activated PARP1/AIFM1/MIF axis through oxidative stress contributes to atopic dermatitis”, *Journal of Investigative Dermatology*, 2024, 144(12), 2695–2705.e8.

Stockwell, B. R., Angeli, J. P. F., Bayir, H., et al., “Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease”, *Cell*, 2017, 171(2), 273–285.

Stockwell, B. R., Friedmann Angeli, J. P., Bayir, H., Bush, A. I., Conrad, M., Dixon, S. J., Fulda, S., Gascón, S., Hatzios, S. K., Kagan, V. E., Noel, K., Jiang, X., Linkermann, A., Murphy, M. E., Overholtzer, M., Oyagi, A., Pagnussat, G. C., Park, J., Ran, Q., Rosenfeld, C. S., Salnikow, K., Tang, D., Torti, F. M., Torti, S. V., Toyokuni, S., Woerpel, K. A., Zhang, D. D., “Ferroptosis: A regulated cell death nexus linking metabolism, redox biology, and disease”, *Cell*, 2017, 171(2), 273–285.

Tang, D., Chen, X., Kang, R., et al., “Ferroptosis: molecular mechanisms and health implications”, *Cell Research*, 2021, 31, 107–125.

Tang, D., Chen, X., Kroemer, G., “Cuproptosis: a copper-triggered modality of mitochondrial cell death”, *Cell Research*, 2022, 32(5), 417–418.

Tang, D., Kang, R., Berghe, T. V., et al., “The molecular machinery of regulated cell death”, *Cell Research*, 2019, 29(5), 347–364.

Tang, D., Kang, R., “NFE2L2 and ferroptosis resistance in cancer therapy”, *Cancer Drug Resistance*, 2024, 7, 41.

The Cancer Genome Atlas Network, “Comprehensive molecular characterization of human colon and rectal cancer”, *Nature*, 2012, 487, 330–337.

Tsvetkov, P., Coy, S., Petrova, B., Dreishpoon, M., Verma, A., Abdusamad, M., Rossen, J., Joesch-Cohen, L., Humeidi, R., Spangler, R. D., Eaton, J. K., Frenkel, E., Kocak, M., Corsello, S. M., Lutsenko, S., Kanarek, N., Santagata, S., Golub, T. R., “Copper induces cell death by targeting lipoylated TCA cycle proteins”, *Science*, 2022, 375(6586), 1254–1261.

Tsvetkov, P., Coy, S., Petrova, B., et al., “Copper induces cell death by targeting lipoylated TCA cycle proteins”, *Science*, 2022, 375(6586), 1254–1261.

Wang, J., Ren, H., Yuan, X., Ma, H., Shi, X., Ding, Y., “Interleukin-10 secreted by mesenchymal stem cells attenuates acute liver failure through inhibiting pyroptosis”, *Hepatology Research*, 2018, 48(3), E194–E202.

Wang, X., Hua, P., He, C., Chen, M., “Non-apoptotic cell death-based cancer therapy: Molecular mechanism, pharmacological modulators, and nanomedicine”, *Acta Pharmaceutica Sinica B*, 2022, 12(9), 3567–3593.

Wang, Y., Chen, Y., Zhang, J., Yang, Y., Fleishman, J. S., Wang, Y., Wang, J., Chen, J., Li, Y., Wang, H., “Cuproptosis: A novel therapeutic target for overcoming cancer drug resistance”, *Drug Resistance Updates*, 2024, 72, 101018.

Wang, Y., Li, Y., Xu, Y., “Pyroptosis in kidney disease”, *Journal of Molecular Biology*, 2022, 434(4), 167290.

Wei, X., Xie, F., Zhou, X., et al., “Role of pyroptosis in inflammation and cancer”, *Cellular & Molecular Immunology*, 2022, 19, 971–992.

Xia, X., Wang, X., Cheng, Z., et al., “The role of pyroptosis in cancer: pro-cancer or pro-‘host’?”, *Cell Death & Disease*, 2019, 10, 650.

Xie, J., Yang, Y., Gao, Y., et al., “Cuproptosis: mechanisms and links with cancers”, *Molecular Cancer*, 2023, 22, 46.

Yan, H. F., Zou, T., Tuo, Q. Z., et al., “Ferroptosis: mechanisms and links with diseases”, *Signal Transduction and Targeted Therapy*, 2021, 6, 49.

Yan, J., Wan, P., Choksi, S., Liu, Z. G., “Necroptosis and tumor progression”, *Trends in Cancer*, 2022, 8(1), 21–27.

Yao, K., Shi, Z., Zhao, F., Tan, C., Zhang, Y., Fan, H., Wang, Y., Li, X., Kong, J., Wang, Q., Li, D., “RIPK1 in necroptosis and recent progress in related pharmaceuticals”, *Frontiers in Immunology*, 2025, 16.

Ye, K., Chen, Z., Xu, Y., “The double-edged functions of necroptosis”, *Cell Death & Disease*, 2023, 14, 163.

Yu, M., Zeng, M., Pan, Z., Wu, F., Guo, L., He, G., “Discovery of novel AKT1 inhibitor induces autophagy-associated death in hepatocellular carcinoma cells”, *European Journal of Medicinal Chemistry*, 2020, 189, 112076

Yu, P., Zhang, X., Liu, N., Tang, L., Peng, C., Chen, X., “Pyroptosis: mechanisms and diseases”, *Signal Transduction and Targeted Therapy*, 2021, 6, 128.

Zang, X., Song, J., Li, Y., Han, Y., “Targeting necroptosis as an alternative strategy in tumor treatment: From drugs to nanoparticles”, *Journal of Controlled Release*, 2022, 349, 213–226.

Zhang, C., Huang, T., Li, L., “Targeting cuproptosis for cancer therapy: mechanistic insights and clinical perspectives”, *Journal of Hematology & Oncology*, 2024, 17, 68.

Zhang, C., Liu, X., Jin, S., et al., “Ferroptosis in cancer therapy: a novel approach to reversing drug resistance”, *Molecular Cancer*, 2022, 21, 47.

Zhang, J., Hu, X., Geng, Y., Xiang, L., Wu, Y., Li, Y., Yang, L., Zhou, K., “Exploring the role of parthanatos in CNS injury: Molecular insights and therapeutic approaches”, *Journal of Advanced Research*, 2025, 70, 271–286.

Zheng, D., Liwinski, T., Elinav, E., “Inflammasome activation and regulation: toward a better understanding of complex mechanisms”, *Cell Discovery*, 2020, 6, 36.

Zhou, Q., Meng, Y., Li, D., et al., “Ferroptosis in cancer: from molecular mechanisms to therapeutic strategies”, *Signal Transduction and Targeted Therapy*, 2024, 9, 55.

Zhou, Z., Li, Q., “The role of pyroptosis in the pathogenesis of kidney diseases”, *Kidney Diseases*, 2023, 9(6), 443–458.

BIOGRAPHY

Hakan Kahraman graduated from the Department of Electrical and Electronics Engineering at Eskişehir Osmangazi University in 2015. In 2016, he began his professional career in the automotive industry as a Wiring Harness Design Engineer at Ford Otosan R&D Center, working under the company Bertrandt. In 2019, he continued his career as a Software Development Engineer in the Treasury and Investment Operations Unit of the Software Development Department at Halkbank Information Technologies.

Since 2020, he has been working as an Artificial Intelligence / R&D Engineer at Livewell, a subsidiary of Türkiye İş Bankası, where he focuses on wearable health technologies. At Livewell, he is responsible for developing software and algorithms for the analysis of biosignals collected by wearable technologies using artificial intelligence and machine learning, with the goal of detecting various diseases.

During his time at Livewell, he served as the principal investigator in two TÜBİTAK-funded projects: “Livewell Home Healthcare Platform” (Project Code: 7210038) and “Smart ECG T-shirt and Pediatric Respiratory-Cardiac Disease Analysis Platform” (Project Code: 7230111). He is currently the project lead for Livewell in the ongoing international consortium project “TREAT – Transforming Healthcare Through Semantic Interoperability and Patient Self-Efficacy,” supported by ITEA (Information Technology for European Advancement).

PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Kahraman H. and Pir P., (2024), “Pan-Cancer Analysis of Non-Apoptotic Cell Death Mechanisms Using Stacking Classifier for Cancer Type Prediction”, The 17th International Symposium on Health Informatics and Bioinformatics (HIBIT) 2024, Fatih, İstanbul, 18-20 December.

